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Original Research Article

Studies on Prevalence, Antimicrobial Resistance and Survival of Cronobacter Sakazakii

Fatimah Y. Abdel-Galil[1], Hemmat K. Abdel-Latif[1], Ahmed M. Ammar[2], Fathy M.E.Serry[1]

[1]Dept of Microbiology and Immunology, Faculty of Pharmacy, Zagazig University.
[2]Dept of Microbiology, Mycology and Immunology, Faculty of Veterinary Medicine, Zagazig University.

Corresponding Author: Fatimah Y. Abdel-Galil

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ABSTRACT

Aim of the work: The present study aimed to investigate the prevalence of Cronobacter sakazakii in commercial powdered infant formula milk and powdered infant foods available in an Egyptian food market. Also, the study aimed to determine factors that affect survival and growth of *C.* sakazakii in powdered infant formula milk in order to control the spread of the organism. Also, aimed to determine susceptibility of C. sakazakii to different antibiotics and detect virulence genes by using PCR.

Keywords: Cronobacter sakazakii, Powdered Infant Formula milk, Druggan Forsythe Iversen media (DFI), Thermal resistance.

INTRODUCTION

C. sakazakii is a Gram-negative, facultative anaerobic, straight rod-shaped bacterium. It belongs to the family Enterobacteriaceae, and it was considered among the genus Enterobacter. [1] Unlike other members of the Enterobacteriaceae, Cronobacter possess the enzyme α-glucosidase, and this is exploited as a diagnostic feature in chromogenic media. [2] Brilliance™ Enterobacter sakazakii Isolation Agar(Druggan Forsythe Iversen media, DFI)was the first medium to incorporate a substrate for this enzyme, 5-bromo-4- chloro-3-indolyl α-D-glucopyranoside (X-α-glu), Cronobacter hydrolyze this colorless chromogen to produce characteristic blue green colonies for presumptive identification on the plate. [3] C. sakazakii may cause infections in premature babies and infants hospitalized in intensive care units who that are at higher risk of infection. The reason is that they are usually fed with formulas, which are the most common vehicle of transmission of the microorganism. [4] Although the incidence rate of the infection is low, the mortality rate ranges from 40 to 80% among infected infants, and those who survive the infection usually develop irreversible neurological sequelae. [5]

A strong association has been found only with Powdered Infant Formula (PIF). Intrinsic and extrinsic contamination of powdered infant formula with *C.* sakazakii can occur. Intrinsic contamination results from the introduction of the organism to the powdered infant formula at some stage during the manufacturing process. In contrast, extrinsic contamination may result from the use of contaminated utensils, such as blenders and spoons in the preparation of powdered infant formula. [6]

C. sakazakii does not survive in the heat of pasteurization used in the production of powdered milk; therefore, the organism mostly originates from the processing environment or from heat-sensitive ingredients added after pasteurization despite rigorous hygienic practices. Therefore, an end-product control measure is necessary to prevent the presence of the organism in the formulas. [7] *C.* sakazakii probably colonizes plant material and produces a novel heteropolysaccharide. This capsular material could facilitate the organism's attachment to plant surfaces. Combined with a tolerance to desiccation, this gives the organism an armory to colonize plant material and survive harsh environmental conditions. [2]

MATERIALS AND METHODS

Media and chemicals

Brilliance Enterobacter sakazakii Isolation Agar medium (Druggan Forsythe Iversen formula, (DFI)) and Violet Red Bile Glucose Agar (VRBGA) were obtained as dehydrated form from Oxoid, Hamshire, England. Tryptic Soy Agar (TSA), Buffered peptone water (BPW) and Enterobacteriaceae enrichment broth were obtained from Difco, USA.API RapiD 20E test galleries kits were obtained from BioMerieux, France. All antibiotic discs were obtained from Oxoid, UK. DreamTaq ™ Green Master Mix and 50xTAE buffer were supplied by Fermentas Life Science, England. Agarose was supplied by Sisco Research Laboratories PVT.LTD, Mumbai, India. Primers that amplified gluA and OmpA genes were obtained from Sigma Aldrich Company, USA.

Collection of samples

A total of 173 different commercial powdered infant formulas milk (recommended for infants from birth to one year old), 61 powdered infant foods obtained from 22 manufacturers, 7 blood samples obtained from septicemic infants admitted to ICUs in Zagazig University Hospital and 3 environmental samples obtained from hospital environment were tested for the presence of C. sakazakii.

Isolation of C. sakazakii

C. sakazakii was isolated from infant formula milk powder and infant food according to the International Organization for Standards Technical Specification (ISO / TS 22964), with some modifications. [8] Samples were diluted 1:10(w/v) in buffered peptone water (BPW) and homogenized. With regard to dried milk products and powders, 10 g of product was added to 100ml of BPW. Following an overnight incubation at 37°C, 10 ml of the pre-enrichment culture was inoculated into 90 ml Enterobacteriaceae Enrichment (EE) broth and incubated overnight at 37°C. A ten µl volume of the selective enrichment culture was then streaked onto a chromogenic media, Druggan Forsythe Iversen media (DFI).

Isolation of *C.* sakazakii from herbal products, environmental samples and clinical samples:

C. sakazakii were isolated from herbal samples, environmental samples and clinical samples according to the FDA method with modifications. [9] Briefly, 100 g of each sample were mixed thoroughly with 900 ml of pre-warmed sterile distilled water at 45˚C, and incubated for 15-20 min in a water bath at the same temperature. Ten ml of each mixture were resuspended in 90 ml of Enterobacteriaceae enrichment broth and incubated overnight at 37˚C. A loopful of the culture broth was streaked and another 0.1 ml of the same culture was spread onto Violet Red Bile Glucose Agar (VRBGA), and incubated for 24hr at 37˚C.All colonies were streaked onto Tryptic Soy Agar (TSA) and incubated for 24-48hr at 37˚C to look for the characteristic yellow colonies of Cronobacter spp. The isolates were then further confirmed by streaking onto (Druggan Forsythe Iversen (DFI), chromogenic agar containing 5-bromo-4-chloro-3-indolyl-α, D-glucopyranoside which upon hydrolysis of the substrate gives blue green colonies typical for Cronobacter spp.

Identification of C. sakazakii

1. Biochemical identification

Positive isolates producing blue green colonies on Brilliance Enterobacter sakazakii Isolation Agar (DFI) was identified using the kit API RapiD 20E test galleries according to the manufacturer's instructions.

2. *Detection and confirmation of identity of Cronobacter sakazakii using PCR.*

Identity of *C.* sakazakii was confirmed by PCR amplification fragment of gluA gene that encodes α- glucosidase enzyme according to. [10]

2.1. *Preparation of crude cell lysate*

Two ml aliquots of C. sakazakii cultures with approximately 10^9cfu/ ml were pelleted by centrifugation at 16.000 xg for 10 minutes, and the pellets were resuspended in 1 ml of sterile distilled water. The pellets were then boiled in a heating block for 10 minutes, quickly placed on ice for 5 minutes and centrifuged at 1.500x g for 30s, and the supernatant containing DNA was collected and stored at 4 °C for further PCR. [11]

2.2. *PCR amplification and cycling protocol*

PCR constitution was done according to the manufacturer's instructions (Fermentas), briefly, primers were optimized in 50 µl reaction mixture consisting of PCR Mix (Dream Taq ™ Green Master Mix) 25µl, Forward primer 1µl, Reverse primer 1µl, Template DNA 5µl, Water, nuclease-free to 50 µl. Sequences of primers used for the detection of genes encoding *gluA* are given in table 1.

Table 1: Oligonucleotide primers used for detection of *gluA* for identification and *OmpA* genes for detection of virulence of *Cronobacter sakazakii*.

primer	Nucleotide sequence	Target site	Amplicon size	References
EsgluA f *EsgluA r*	5'-TGAAAGCAATCGACAAGAAG-3' 5'-ACTCATTACCCCTCCTGATG-3'	*gluA*	1680bp	[10]
ESSF *ESSR*	5`-GGATTTAACCGTGAACTTTTCC-3` 5`-CGCCAGCGATGTTAGAAGA-3`	*OmpA*	469bp	[11]

For *gluA* gene, running condition was as described by. [10] The hot start polymerase was activated by incubation for 15 min at 95 °C ; followed by 30 cycles of denaturation, 94°C for 30 s, annealing, 56°C (gluA) for 1 min., extension, 72°C for 1.5 min., final extension period of 5min at 72°C. PCR cycling program was performed using thermal cycler (Biometra, UK).

2.3. Detection of PCR products: PCR products were analyzed using1.5% (w/v) agarose gel electrophoresis in TAE buffer and a constant voltage of 90 V for 90 minutes to confirm the presence of amplified DNA.

Detection of outer membrane protein A gene (*Omp A*) as a virulence factor of *C. sakazakii* using PCR.

The PCR was performed according to the method described by [11] PCR was done for the detection of *OmpA* gene that has a role in the organism penetrating the blood brain barrier. Sequences of primers used for the detection of genes encoding *OmpA* are given in table 1.For *OmpA* gene, the running conditions were94 °C for 2 minutes, 30 cycles of: denaturation, 94°C for 15 seconds annealing, 60°C for 15 seconds, extension, 72°C for 30 seconds, final extension period of 5 min. at 72 °C. The PCR products were visualized by agarose gel electrophoresis.

Determination of the sensitivity of *C.* sakazakii isolates to antimicrobial agents by agar disk diffusion method

C. sakazakii isolates were tested for their susceptibility to a total of 16 antimicrobial agents by agar diffusion method according to [12] The antimicrobial agents discs used are; Streptomycin (S, 10 µg), Norfloxacin (NOR, 10 µg), Ciprofloxacin (CIP, 5 µg), Levofloxacin (LEV, 5 µg), Gentamicin (CN, 10 µg), Rifampicin (RD, 5 µg), Ofloxacin (OFX, 5 µg), Augmentin (Amoxicillin /Clavulanic acid 2:1) (AMC, 30µg), Cephalexin (CL, 30 µg), Nalidixic Acid (NA, 30µg), Sulfamethoxazole/ Trimethoprim (SXT, 25µg), Ampicillin (AMP, 10µg), Aztreonam (ATM, 30 µg), Imipenem (IPM,10µg), Cefotaxime (CTX,30µg), Ceftazidime (CAZ, 30µg).

Survival of *Cronobacter sakazakii* at different temperatures

Survival of C. sakazakii at different temperatures in reconstituted infant products e.g. Complete balanced formula, Lactose free formula and Soy protein formula was studied according to [13] Forty-five ml of reconstituted milk or feeding formula were prepared according to the manufacturer's instruction in sterile 100 ml capacity Duran bottles. Each of the reconstituted products was preheated to 55, 60, 65, 70, 75, 80, 85 and 90˚C in shaking water bath (Jeo Tech, Seoul, Korea). One ml of the cell suspension was mixed with the 45 ml of temperature-equilibrated reconstituted product at each temperature to obtain approximately 10^8cfu/ml. At timed intervals, depending on temperature; samples (1ml) were transferred to sterile tubes and cooled immediately in running tap water. The tubes were left at room temperature and analyzed for viable C. sakazakii numbers within 15 minutes. Cronobacter survivors from thermal inactivation experiments were enumerated by spread plating aliquots of the samples and their appropriate dilutions in duplicate on Tryptic Soy Agar (TSA).After incubation aerobically at 37˚C for 24 hr, surviving cells were enumerated.

Effect of water temperature in reconstitution of powdered product on survival of Cronobacter sakazakii

C. sakazakii was mixed with each of the powdered products as described by [14] Briefly, 100 gram of powdered product e.g. Complete balanced formula, Lactose free formula, Soy protein formula, whole milk, low fat milk and skim milk was spread on the bottom of a sterile stainless steel bowl and 0.5 ml of the cell suspension was inoculated. To ensure homogenous distribution of C. sakazakii cells, the treated powder was mixed by a sterile spatula and passed through a sterile screen with 0.5mm pores to break up clumps. The inoculated formulas were then stored at 25˚C in 500ml sterile, screw-capped bottles for 24 hr. The initial level of C. sakazakii in the powdered products was approximately 10^8cfu/gm. The inoculated powdered products were reconstituted with 45 ml sterile water at 25 (Control), 60, 70, 75, 80 and 90˚C. The bottles were gently agitated by hand for 10 minutes at room temperature and then samples were analyzed for viable count of C. sakazakii by spread plating aliquots of the samples on Tryptic Soy Agar. After incubation aerobically at 37˚C for 24 hr, growing colonies were enumerated.

RESULTS

Isolation of C. sakazakii from infant formula, milk powder and infant food

Cronobacter sakazakii was isolated from 9 out of 173 samples of powdered infant formula milk and one out of 61 powdered infant foods making a total of 10 out of 234 samples with a prevalence rate of (4.27%).Table (2)

Table 2: Frequency of *C. sakazakii* from different sample types

Sample type	Total number	No.(%) of contaminated samples
Powdered infant formula milk.	173	9 (5.2%)
Powdered infant food	61	1 (1.6%)
Total	234	10(4.27%)

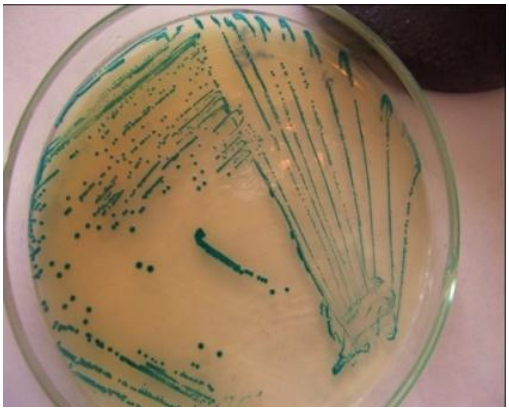
Among the 7 clinical specimens, only one Cronobacter sakazakii isolate was recovered, while no detection of organism was found in environmental samples. The result in table 2showed that powdered infant formula milk exhibited a higher frequency of isolation of the organism (5.2%) compared with powdered infant food (1.6%).

Identification of *Cronobacter sakazakii*

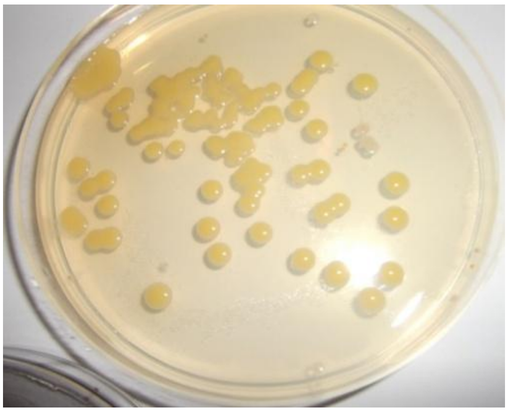
Colonial appearance

On Brilliance *Enterobacter sakazakii* Isolation Agar medium, *C. sakazakii* appeared as blue green colonies, while it gave characteristic yellow colonies on Tryptic Soy Agar medium. On violet red bile glucose agar, typical colonies of *C. sakazakii* appear as purple colonies surrounded by purple halo of precipitated bile acids (Figure1, a, b, c).

API RapiD 20E kit was carried out on the isolates of Cronobacter sakazakii. Results revealed seven digit profile numbers(5275772) which were identified through RapiD 20E analytical profile index (Ref. 20790)showing excellent *C. sakazakii* identification (99.9%:).



(a)



(b)

(c)

Genotypic identification of Cronobacter sakazakii using PCR

Identity of *Cronobacter sakazakii* was confirmed by PCR amplification of 1680 bp fragments of the *gluA* gene that encodes α- glucosidase enzyme (Figure 2).All isolates were found to have *gluA* gene.

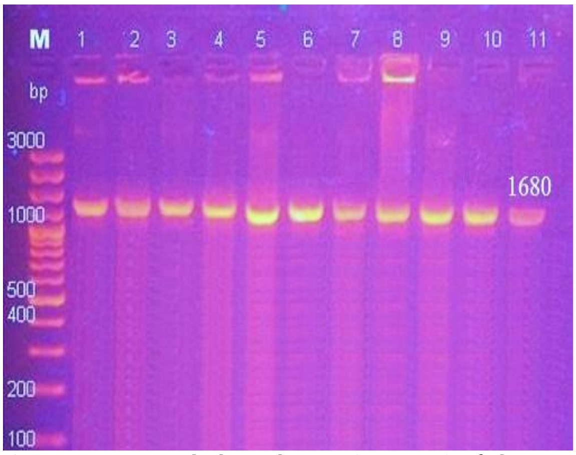


Figure2: Agarose gel electrophoresis (1.5% w/v) of the PCR products of *C. sakazakii* DNA isolated from powdered infant formula milk and food and blood of septicemic infant revealing that all isolates gave a characteristic band at 1680 bp which was specific for α-glucosidase gene. M: molecular weight marker, Lane 1: clinical isolate. Lane 2: isolate of powdered infant food, Lane 3, 4, 5, 6, 7, 8, 9, 10 and 11: isolates of powdered infant formula milk.

Detection of outer membrane protein A gene (*OmpA*) as a virulence factor of *C. sakazakii* using PCR

The presence of *Omp A* gene was examined in all eleven isolates by PCR amplification of 469 bp fragments for all isolates of *Cronobacter sakazakii*. All isolates were found to harbor *OmpA* (Figure 3).

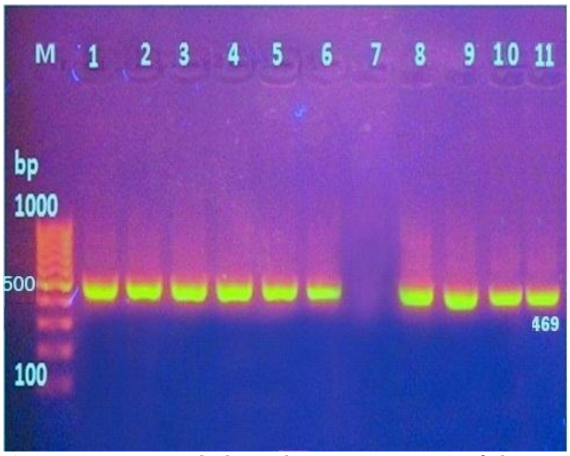


Figure 3: Agarose gel electrophoresis (1.5% w/v) of the PCR products of *C. sakazakii* DNA isolated from powdered infant formula milk, powdered infant food and blood of septicemic infant revealing that all isolates gave a characteristic band at 469 bp which was specific for *OmpA* gene. M: Molecular weight marker, Lane 1: Clinical isolate, Lane 2: Isolate from powdered infant food, Lane 3, 4, 5, 6, 8, 9, 10 and 11: Isolates from powdered infant formula milk.

Determination of the susceptibility of the isolates to antimicrobial agents by agar disc diffusion method

The results in table 3 revealed that all isolates demonstrated complete resistance to rifampicin (100%) and ampicillin (100%). All isolates were sensitive to levofloxacin (100%), norfloxacin (100%) and ofloxacin (100%).High susceptibility was observed to ciprofloxacin, nalidixic acid, gentamicin, imipenem, ceftazidime, sulfamethoxazole/ Trimethoprim (90.9% each), aztreonam (81.8%), and streptomycin (72.7 %).Intermediate sensitivity was observed to cefotaxime (54.5%) and low to amoxicillin /clavulanic acid (27.3 %) and cephalexin (9.09 %). The clinical isolate showed higher resistance to most of the tested antimicrobial chemotherapeutic agent compared to isolates from powdered infant products.

Table 3: Susceptibilities of *Cronobacter sakazakii* isolates to tested antibiotics

Isolate No.	LEV	NOR	OFX	CIP	NA	CN	IMP	CAZ	SXT	ATM	S	CTX	AMC	CL	RD	AMP
1	S	S	S	S	S	S	S	S	S	S	S	I	I	R	R	R
2	S	S	S	S	S	S	S	S	S	S	S	S	I	I	R	R
3	S	S	S	S	S	S	S	S	S	S	I	S	I	I	R	R
4	S	S	S	S	S	S	S	S	S	I	S	I	I	R	R	R
5	S	S	S	S	S	S	S	S	S	S	I	S	S	R	R	I
6	S	S	S	S	S	S	S	S	S	S	S	S	I	R	R	R
7	S	S	S	S	S	S	S	S	S	S	S	I	S	S	R	R
8	S	S	S	S	S	S	S	S	S	S	S	S	I	I	R	R
9	S	S	S	S	S	S	S	S	S	S	S	S	I	R	R	R
10	S	S	S	S	S	S	S	S	S	S	S	I	S	I	R	R
11	S	S	S	I	R	I	R	R	R	R	R	R	R	R	R	R

1-9: Isolates of *C. sakazakii* obtained from powdered infant formula milk. 10: isolate obtained from infant food. 11: clinical isolate. S, sensitive; R, resistant; I, intermediate; LEV, Levofloxacin; NOR, norfloxacin; OFX, ofloxacin; CIP, ciprofloxacin; NA, nalidixic acid; CN, gentamicin, IMP, imipenem; CAZ, ceftazidime; SXT, sulfamethoxazole/ trimethoprim; ATM, aztreonam; S , streptomycin; CTX, cefotaxime; AMC, amoxicillin/clavulanic acid (augmentin); CL, cephalexin; RD, rifampicin; AMP, ampicillin.

Survival of *C. sakazakii* at different temperatures in reconstituted products

For complete balanced and lactose free infant formula milk, the obtained results in figure 4 demonstrated that the numbers of the organism decreased with time at all temperatures used. At 70˚C, the reductions in log cfu of C. sakazakii were about 7 and 6 $\log_{10}$, respectively after 15 minutes with D-values of 2.5 minutes, while no visible organism was detected after 20 minutes. The increase in temperature from 55˚C to 70˚C reduced D- values by about three folds.

For soy protein formula, the thermal treatment at different temperatures for 30 minutes caused reductions in C. sakazakii numbers. Also, D- values for C. sakazakii at 55˚C and 70˚C were reduced from 6.87 minutes to 1.25 minute (more than 4 fold reduction).On the other hand, no viable C. sakazakii was found in the first sample taken after 5 minutes at treatment of temperatures of 75, 80, 85 and 90˚C.

Effect of water temperature in reconstitution of powdered product on survival of *C. sakazakii*

The results in (tables 4, 5) revealed that the reconstitution of infant milk formula with water at 70˚C decreased level of *C. sakazakii* by about 5.3 $\log_{10}$ in case of complete balanced powdered infant formula milk and lactose free infant formula, while in case of soy protein formula, the decrease was about 6.95 $\log_{10}$ at 70˚C.

In case of soy protein formula inoculated with *C. sakazakii,* heating with hot water at 60˚C for 10 minutes reduced numbers of the organism from about 7$\log_{10}$(at 25˚C) to 5.4 $\log_{10}$ with D-values 9.9 at 25˚C and 1.25 at 70˚C. The complete removal of the organism was at 70˚C for 10 minutes.

The thermal resistance of Cronobacter sakazakii in whole milk compared with low fat and skim milk formulae was studied. The results in table 5 revealed that the D- value was high in case of whole milk then followed by low fat formula and finally skim milk formula. On the other hand, no viable *C. sakazakii* was found in the first sample taken after 10 minutes at treatment of temperatures of 75, 80, 85 and 90˚C.

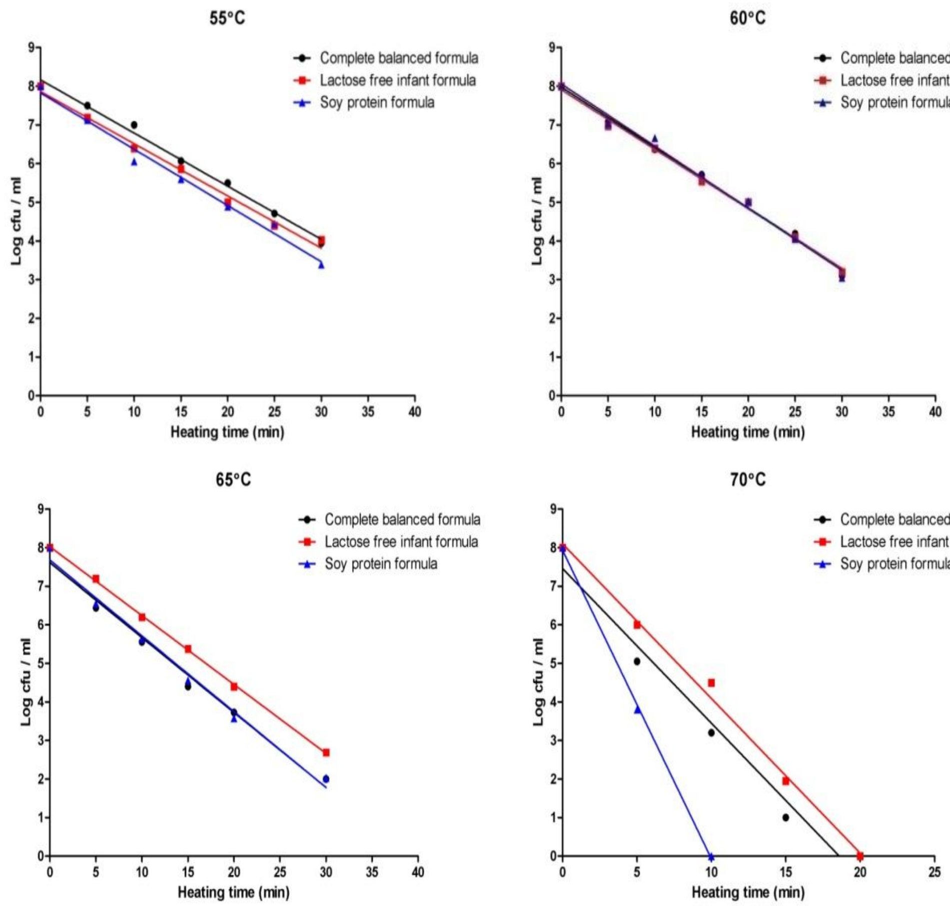


Figure4: Thermal inactivation of *C. sakazakii* at 55˚C, 60˚C, 65˚C and 70˚C in reconstituted lactose- free infant milk formula ( ), soy protein infant formula ( ) and complete balanced formula ( ). Results shown are the means of three replicate experiments

Table 4: Survivors of *C. sakazakii* in feeding formula reconstituted with hot water at different temperature

Product type	**Temp.**	**Time (min.)**	**cfu/ml***	**Log cfu/ml**	**D-value (min.)**
Complete balanced infant formula.	25˚C	10	$8.8x10^6$	6.94	9.43
	60˚C	10	$3.3x10^5$	5.51	4.01
	70˚C	10	$0.46x10^2$	1.66	1.57
	75,80,90˚C	10	0.00	0.00	1.25
Lactose free infant formula	25˚C	10	$9.2x10^6$	6.96	9.61
	60˚C	10	$3.9x10^5$	5.58	4.13
	70˚C	10	$0.9x10^2$	1.95	1.65
	75,80,90˚C	10	0.00	0.00	1.25
Soy protein formula.	25˚C	10	$9.8x10^6$	6.99	9.90
	60˚C	10	$2.7x10^5$	5.43	3.89
	70,75, 80,90˚C	10	0.00	0.00	1.25

Table5: survivors of C. sakazakii in milk powder reconstituted with hot water at different temperature

Product type	Temp.	Time (min.)	cfu/ml*	Log cfu/ml	D-value (min.)
Milk powder (Low fat).	25˚C	10	$5.03x10^6$	6.70	7.69
	60˚C	10	$6.7x10^5$	4.82	3.14
	70,75,80,90˚C	10	0.00	0.00	1.25
Skim milk powder	25˚C	10	$5.4 x10^6$	6.73	7.87
	60˚C	10	$5.9x10^4$	4.77	3.09
	70,75,80,90˚C	10	0.00	0.00	1.25
Whole milk powder	25˚C	10	$5.3 x10^6$	6.72	7.81
	60˚C	10	$5.1x10^5$	5.71	4.36
	70˚C	10	$2.4x10^2$	2.37	1.77
	75, 80, 90˚C	10	0.00	0.00	1.25

DISCUSSION

Cronobacter sakazakii is an emerging food borne pathogen that had been linked with infantile meningitis; septicemia and necrotizing enterocolitis transmitted through the consumption of contaminated powdered infant foods and other milk products. [15-17] In our study, the incidence of C. sakazakii in powdered infant formula milk and powdered infant foods available in Egyptian market (22 manufacturers) was 4.27%. These results are consistent with that obtained by [18-21] who reported a direct correlation between infant formula and C. sakazakii. The obtained percentage was less than that obtained by [18,22] and [23] who recorded 14.1%, 24%; and 27.1%, respectively. While our results were consistent with that reported by [20] who surveyed the presence of C. sakazakii in 120 dried infant milk samples (five manufactures) obtained from Canadian retail market and reported that the prevalence of this organism ranged between 0 and 12% of the samples.

Many studies have focused on the infant formula as the main source of Cronobacter sakazakii. [24,16,25] The infant milk and food formula are exposed to heat treatment during processing and the organism still isolated from these products. The presence of C. sakazakii may be due to post-processing contamination of infant formula from production environment. [26]

C. sakazakii can contaminate the powdered infant milk formula from the environment or from the addition of the ingredients which contain the organism at the powder stage especially the dry- mix process of the production. [27] and [26] Also, [28] reported that the presence of C. sakazakii in powdered infant milk formula depends on the process conditions and the nature of the products. Powdered infant formula has been known to be contaminated, on occasion with bacterial pathogens. [29] Therefore, hygienic measures and practices must be used during the manufacture of formula to minimize entry of contaminants into the process. [23]

In this study, the detection of Cronobacter sakazakii was carried out using Brilliance Enterobacter sakazakii Isolation agar media and subcultured onto Tryptic Soy Agar media (TSA). The complete identification of C. sakazakii was carried out by Violet Red Bile Glucose Agar (VRBGA). These cultures were sensitive for the detection of the organism than other culture media which used for bacteria from the family Enterobacteriaceae. These results agree with that reported by [30] and [31]who reported that Food and Drug Administration [32] method is not effective in detecting C. sakazakii as some ingredients used to prepare the particular selective and differential medium had prevented the recovery of injured cells. Hence, it is important to identify which enrichment and differential medium combination are more selective and specific for detection of C. sakazakii in powdered infant formula in order to lower the exposure risk of neonates and infants towards this organism that may lead to fatal infections such as meningitis, sepsis and necrotizing enterocolitis. [33]

In the present study, Identity of Cronobacter sakazakii was confirmed by PCR amplification of 1680 bp fragment of the gluA gene that encodes α- glucosidase enzyme. These results were consistent with that obtained by [34] and [10] The α-glucosidase based PCR, exclusively targets the gene responsible for the α-glucosidase activity in C. sakazakii. [10]

The presence of OmpA gene as a virulence factor was examined in all eleven isolates by PCR amplification of 469 bp fragment for all isolates of Cronobacter sakazakii. It was found that all isolates harbored OmpA. These results were consistent with that obtained by [11,35] and [36] The outer membrane protein A, encoded by the OmpA gene, is probably the best characterized virulence marker. [11] Outer membrane protein A is one of the determinants that contribute to C. sakazakii invasion of human brain microvascular endothelial cells (BMEC) in vitro, and may potentially play a role in the pathogenesis of neonatal meningitis caused by this organism. [37]

In our study, high sensitivity of C. sakazakii was found with levofloxacin, ofloxacin, norfloxacin, ciprofloxacin, gentamicin and sulfamethoxazole. These results are higher than that recorded by, [23] where they reported ofloxacin (92.1%), levofloxacin (79%) and gentamicin (65.8%).

In our study, sensitivity to streptomycin (72.7%) was less than that reported by [23] (94.7%). In the present study, the highest resistance was recorded for ampicillin and cephalexin. Also, complete resistance (100%) to rifampicin was found, which was consistent with that reported by [38] these results were compatible with that obtained by. [23]

Cronobacter sakazakii like other *Enterobacter* species have acquired resistance by inactivating beta-lactam antibiotics due to production of beta-lactamases. [39]

In our study, the reconstitution of infant milk formula with water at 70˚C decrease level of C. *sakazakii* by about 5.3 $\log_{10}$ in case of complete balanced powdered infant formula milk and lactose free infant formula, while in case of soy protein formula, the decrease was about 6.95 $\log_{10}$, these results are consistent with that obtained by. [40] In previous studies, D-values of Cronobacter sakazakii in reconstituted infant milk formula were with wide range. [41,3] and [26] reported D-values of 21.05- 0.07 minutes at 56-70˚C for clinical isolate and 16.4- 0.3 minutes at 54-62˚C, respectively. Also, [42] reported D-values of 54.79- 2.5 minutes at 52-60˚C.The obtained data revealed that the organism is sensitive to increase temperature. Differences in results can be explained by differences in products (milk formula) and bacterial strains. This hypothesis is consistent with [13,42] and [43]

CONCLUSION

Cronobacter sakazakii is an emerging pathogen, often transmitted through powdered milk and responsible for a series of infections, some of which with potential fatal outcomes, in a particular segment of the population (infants).

The heat resistance of C. sakazakii should not allow the survival of the pathogen during normal pasteurization treatment. The use of hot water (≥70˚C) during reconstitution appears to be an effective means to reduce the risk of Cronobacter sakazakii in milk and special feeding formula.

Recommendations

Breastfeeding should always be supported and encouraged since a mother's milk constitutes the preferred food for newborn infants especially in their early months. When this is not possible, a mother should be well informed and trained on the importance of hygiene while handling, preparing and storing powdered milk.

To reduce the probability of neonatal and infant infections caused by infant formulae, recommendations should be focused on controlling the initial populations of *C. sakazakii* in raw materials on receipt , reducing populations during heat treatment of raw milk and related ingredients, preventing an increase in population of *C. sakazakii* by avoiding post-processing contamination, applying microbiological criteria and providing appropriate information and preparation instructions, e.g. labeling and consumer education.

Reducing risks connected to *C. sakazakii* is mandatory for all people involved: producers, parents and health professionals. During production, raw materials should be monitored specifically ingredients which do not require further thermal treatment before mixing. The frequency of inspections on food production environments and on the end product should be increased.

At home, prepare only food enough for the meal avoiding the preparation of following meals; if necessary limit the number of meals prepared in advance to 1-2. Avoid leaving unused reconstituted milk at room temperature. The reconstituted product should be stored in a refrigerator. The lapse of time between the reconstitution of the formula and its use should be reduced as much as possible (shorter than 4 hours). The containers used for preparation should be cleaned and disinfected.

Appropriate control measures which can assess potential hazard should be enforced, critical control points (CCP) should be identified, non- conformities and necessary corrective actions should be monitored and results should be registered.

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REFERENCES

1. Farmer JJ, Asbury MA, Hickman FW, Brenner DJ. *Enterobacter sakazakii*: A new species of *Enterobacteriaceae* isolated from clinical specimens. Int. J. Sys. Bacteriol.1980; 30:569-584.
2. Forsythe S. *Cronobacter* species. Oxoid Culture. 2010; 31: 0965-0989.
3. Iversen C, Druggan P, Forsythe S.A selective differential medium for *Enterobacter sakazakii*, a preliminary study. International Journal of Food Microbiology.2004; 96:133-139.
4. Fiore A, Casale M, Aureli P. Enterobacter sakazakii: epidemiology, clinical presentation, prevention and control. ANN IST SUPER SANITA. 2008; 44 : 275-280.
5. Bowen AB, Braden CR. Invasive *Enterobacter sakazakii* disease in infants. Emerg. Infect Dis. 2006; 12:1185-1189.
6. Noriega FR, KotloffKL, MartinMA, Schwalbe RS.Nosocomial bacteremia caused by *Enterobacter sakazakii* and *Leuconostocmesenteroides* resulting from extrinsic contamination of infant formula. Pediatric Infectious Disease Journal.1990; 9:447-449.
7. Kandhai MC, Reij MW, Gorris LG, Guillaume-Gentile O, Vanscot-horst M.Occurrence of *Enterobactersakazakii* in food production environments and households. Lancet. 2004; 363: 39-40.
8. El-Sharoud W, O'Brien S, Negredo C, Iversen C, Fanning S, Healy B.Characterization of *Cronobacter* recovered from dried milk and related products. BMC Microbiol.2009; 9:24.
9. FDA.Isolation and enumeration of *Enterobacter sakazakii* from dehydrated powdered infant formula. 2002. http: // www. FDA. Gov / Food /ScienceResearch/ Laboratory Method/ucm114665.htm).(serial on line).
10. Lehner A, Nitzsche S, Breeuwer P, Diep B, Thelen K, Stephen R.Comparison of two chromogenic media and evaluation of two molecular based identification systems for *Enterobacter sakazakii* detection. BMC Microbiol.2006; 6:15.
11. Nair MKM and Venkitanarayanan KS.Cloning and Sequencing of the *OmpA* Gene of *Enterobacter sakazakii* and development of an *ompA*-targeted PCR for rapid detection of *Enterobacter sakazakii* in infant formula. Appl Environ Microbiol.2006; 72: 2539-2546.
12. Clinical and Laboratory Standards Institute Performance standards for Antimicrobial Susceptibility Testing. Twenty-Third Informational Supplement. 2013. CLSI document M100-S23.Wayne, Pennsylvania, USA.
13. Osaili TM, Shaker RR, Al-Haddaq MS, Al-Nabulsi S, Holley RA. Heat resistance of *Cronobacter* species (*Enterobacter sakazakii*) in milk and special feeding formula. J Appl. Microbiol.2009; 107:928-935.
14. Osaili TM, Shaker RR, Abu-Al-Hassan AS, Ayyash MM, Martin EM.Inactivation of *Enterobacter sakazakii in* infant milk formula by gamma irradiation: determination of D_{10}-value. J Food Sci.2007; 72: 85-88.
15. LaiK K. *Enterobacter sakazakii* infections among neonates, infants, children and adults: case reports and a review of the literature. Medicine. 2001; 80:113-122.
16. Van AckerJ, De Smet F, MuyldermansG, Bougatef A, Naessens A, Lauwers S.Outbreak of necrotizing enterocolitis associated with

Enterobactersakazakii in powdered milk formula. Journal of Clinical Microbiology. 2001; 39:293-297.

17. Bar-Oz B, Preminger A, Peleg O, Block C, Arad I.*Enterobacter sakazakii* infection inthenewborn. Acta Paedia.2001; 90:356-358.
18. Muytjens HL, Roelofs-Willemse H, Jasper GHJ. Quality of powdered substitutes for breast milk with regard to members of the family *Enterobacteriaceae*. Journal of Clinical Microbiology. 1988; 26:743-746.
19. Simmon BP, Gelfand MS, Haas M, Metts L, Ferguson J. *Enterobacter sakazakii* infections in neonates associated with intrinsic contamination of a powdered infant formula. Infect Control Hosp Epidemiol. 1989; 10: 398-401.
20. Nazarowec-White M and Farber JM. Incidence, survival and growth of *Enterobacter sakazakii* in infant formula. J. Food Protect.1997; 60:226-230.
21. Shaker R, Osaila T, Al-Omary W, Jaradat Z, Al-Zuby M.Isolation of *Enterobacter sakazakii* and other *Enterobacter* sp. from food and food production environments. Food Control. 2007; 18: 1241-1245.
22. Iversen C and Stephan F.Isolation of *Enterobacter sakazakii* and other *Enterobacteriaceae* from powdered formula milk and related products. Food Microbiology.2004; 21: 771-777.
23. Aigbekaen BO and Oshoma CE. Isolation of *Enterobacter sakazakii* from Powdered Foods locally consumed in Nigeria. Pakistan Journal of Nutrition. 2010; 9:659-663.
24. PostupaRand Aldova E. *Enterobacter sakazakii*: A Tween 80 esterase-positive representative of the genus *Enterobacter* isolated from powdered milk specimens. J. Hygi. Epidemiol. Microbiol. 1984; 28:435-440.
25. Block C, Peleg O, Minster N,Bar-OzB, SimhonA, Arad I, ShapiroM. Cluster of neonatal infections in Jerusalem due to unusual biochemical variant of *Enterobacter sakazakii*. Eur J Clin Microbiol Infect Dis.2002; 21: 613-616.
26. IversenC,Lane M,Forsythe SJ.The growth profile, thermo tolerance and biofilm formation of *Enterobactersakazakii* grown in infant formula milk. Lett. Appl. Microbiol.2004; 38: 378-382.
27. Nazarowec- White M and Farber JM. *Enterobacter sakazakii*: A review. International Journal of Food Microbiology.1997; 34:103-113.
28. Iversen C and Forsythe SJ. Risk profile of *Enterobacter sakazakii*, an emergent pathogen associated with infant milk formula. Trends Food Science Technology.2003; 14: 443-454.
29. ForsytheSJ. *Enterobactersakazakii* and other bacteria in powdered Infant Milk Formula. Maternal Child Nutr. 2005; 1:44-50.
30. Gurlter JB, Kornacki JL, Beuchat LR. *Enterobacter sakazakii:* A coli form of increased concern to infant health. International Journal of Food Microbiology. 2005; 104:1-34.
31. Al-Holy MA, Lin M, Al-Qadiri HM, Rasco, BAA.Comparative study between overlay method and selective-differential media for recovery of stressed *Enterobacter sakazakii* cells from infant formula. Food Microbiology.2008; 25: 22-28.
32. FDA/CFSAN. Health Professionals letters on *Enterobacter sakazakii* Infections associated with use of powdered (Dry) infant formulas in Neonatal Intensive Care Units. 2002. Available from http:www.cfsan.fda.gov/dms/inf-ltr3.html Accessed 6 March 2007.
33. Sani NA and Yi LY. *Enterobacteriaceae*, *Cronobacter* (*Enterobacter*) *sakazakii* and microbial population in infant formula products in the Malaysian market. Sain. Malays.2011; 40: 345-351.
34. Iversen C, Lehner A, Mullane N, Marugg J, Fanning S, Stephan R, Joosten H. Identification of "*Cronobacter"* spp. (*Enterobacter sakazakii*). Journal of Clinical Microbiology.2007; 45: 3814-3816.
35. Prasadarao NV, Wass CA, Weiser JN, Stins MF, Huang SH, Kim KS.Outer membrane protein A of *Escherichia coli* contributes to invasion of brain microvascular endothelial cells. Infect. Immun.1996; 64:146–153.

36. Kim KS.*E. Coli* invasion of brain microvascular endothelial cells as a pathogenetic basis of meningitis. Subcell. Biochem.2000;33: 47–59.
37. Nair MKM, Venkitanarayanan KS,Silbart LK, Kim KS.Outer Membrane Protein A (OmpA) of *Cronobacter sakazakii* Binds Fibronectin and Contributes to invasion of Human Brain Microvascular Endothelial Cells. Foodborne Path. Dis.2009;6: 495-501.
38. Stock I and Wiedemann B.Natural antibiotic susceptibility of *Enterobacter amnigenus*, *Enterobacter cancerogenus*, *Enterobacter gergoviae* and *Enterobacter sakazakii* strains. Clin. Microbiol. Infect.2002; 8:564-578.
39. Drudy D, Quinn NR, Wall PG, Fanning S. *Enterobacter sakazakii*: An emergent pathogen in powdered infant formula. Clin. Infect. Dis.2006; 42:996-1002.
40. Osaili TM, Shaker RR, Oliamat AN, Al-Nabulsi AA, Al-Holy MA, Forsythe SJ. Detergent and sanitizer stresses decrease the thermal resistance of *Enterobacter sakazakii* in infant milk formula. J Food Sci. 2008; 73: 154-157.
41. Edelson-Mammel SG and Buchanan RL. Thermal inactivation of *Enterobactersakazakii* in rehydrated infant formula. J food protec.2004; 67:60-63.
42. Nazarowec-WhiteM and FarberJM. Thermal resistance of *Enterobacter sakazakii* in reconstituted dried infant formula. Lett. Appl. Microbiol.1997; 24:9-13.
43. Kim SH and Park JH.Thermal resistance and inactivation of *Enterobacter sakazakii* isolates during rehydration of powdered infant formula. J Microbiol Biotechnol.2007; 17: 364-368.

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