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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]Department of Microbiology and Immunology, Faculty of Pharmacy, Zagazig University

[2]Department of Microbiology, Mycology and Immunology, Faculty of Veterinary Medicine, Zagazig University

ABSTRACT

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

Cronobacter 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* (Farmer *et al*., 1980). Unlike other members of the *Enterobacteriaceae*, *Cronobacter* possess the enzyme α-glucosidase, and this is exploited as a diagnostic feature in chromogenic media (Forsythe, 2010). 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 (Iversen *et al*., 2004a). *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 (Fiore *et al*., 2008). 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 (Bowen and Braden, 2006).

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 (Noriega *et al*., 1990).

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 (Kandhai *et al*., 2004). *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 (Forsythe, 2010).

MATERIAL 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 ^{TM} 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 (El-Sharoud *et al*., 2009). 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 100 ml 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 (FDA, 2002). 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 24 hr at 37˚C. All colonies were streaked onto Tryptic Soy Agar (TSA) and incubated

for 24-48 hr 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) were 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 Lehner *et al*. (2006).

Preparation of crude cell lysate

Two ml aliquots of *C. sakazakii* cultures with approximately 10^9 cfu/ 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 (Nair and Venkitanarayanan, 2006).

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 (DreamTaq ™ 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	5'-TGAAAGCAATCGACAAGAAG-3'			Lehner *et al*. (2006).
EsgluA r	5'-ACTCATTACCCCTCCTGATG-3'	*gluA*	1680 bp	
ESSF	5`-GGATTTAACCGTGAACTTTTCC-3`			Nair and Venkitanaraya
ESSR	5`-CGCCAGCGATGTTAGAAGA-3`	*OmpA*	469 bp	nan *et al*. (2006).

For *gluA* gene, running condition were as described by Lehner *et al*. (2006). 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).

Detection of PCR products

PCR products were analyzed using 1.5% (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 (*OmpA*) as a virulence factor of *C. sakazakii* using PCR.

The PCR was performed according to the method described by Nair and Venkitanarayanan (2006). 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 were 94 °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 CLSI (2013). The antimicrobial agents discs used are; Streptomycin (S, 10 µg), Norfloxacin (NOR, 10 µg), Ciprofloxacin (CIP, 5 µg), Levofloxacin (LEV, 5 µg), Gentamycin (CN, 10 µg), Rifampicin (RD, 5 µg), Ofloxacin (OFX, 5 µg), Augmentin (Amoxicillin /Clavulanic acid 2:1) (AMC, 30µg), Cephalexin (CL, 30 µg), Naldixic Acid (NA, 30µg), Sulphmethoxazole/ Trimethoprim (SXT, 25µg), Ampicillin (AMP, 10µg), Aztronam (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 Osaili *et al.* (2009). 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 10^8 cfu /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 Osaili *et al*. (2007). 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 10^8 cfu/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)

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 2 showed that powdered infant formula milk exhibited a higher frequency of isolation of the organism (5.2%) compared with powdered infant food (1.6%).

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%)

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 (Figure 1).

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%:).

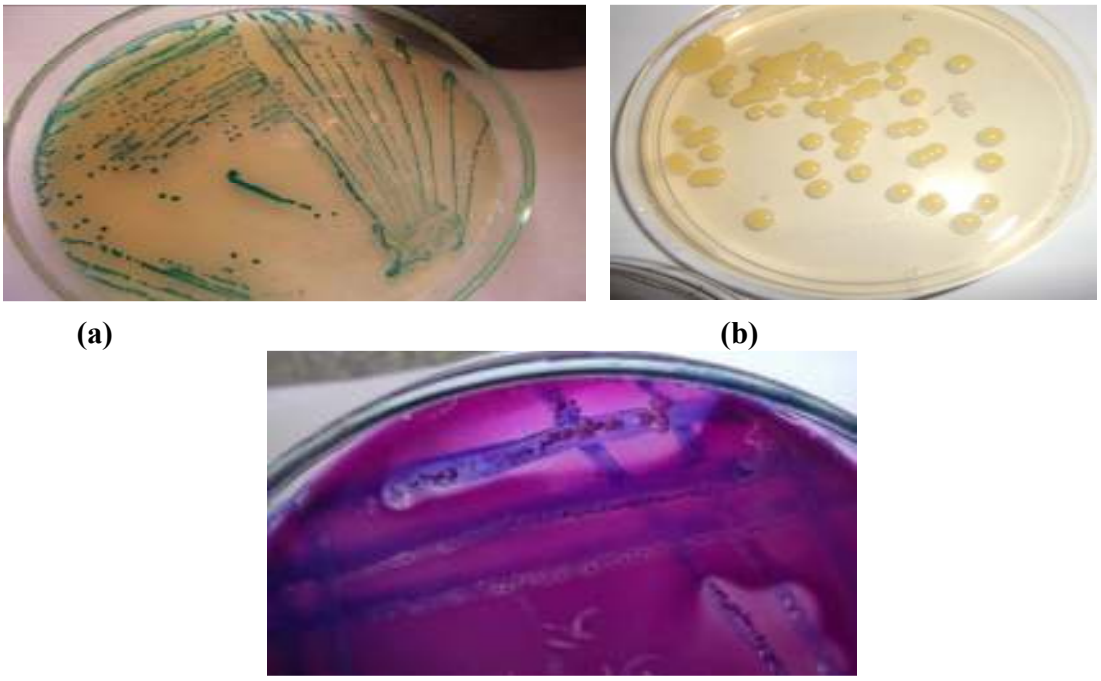

Figure 1. Biochemical identification of *C. sakazaki.*

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.

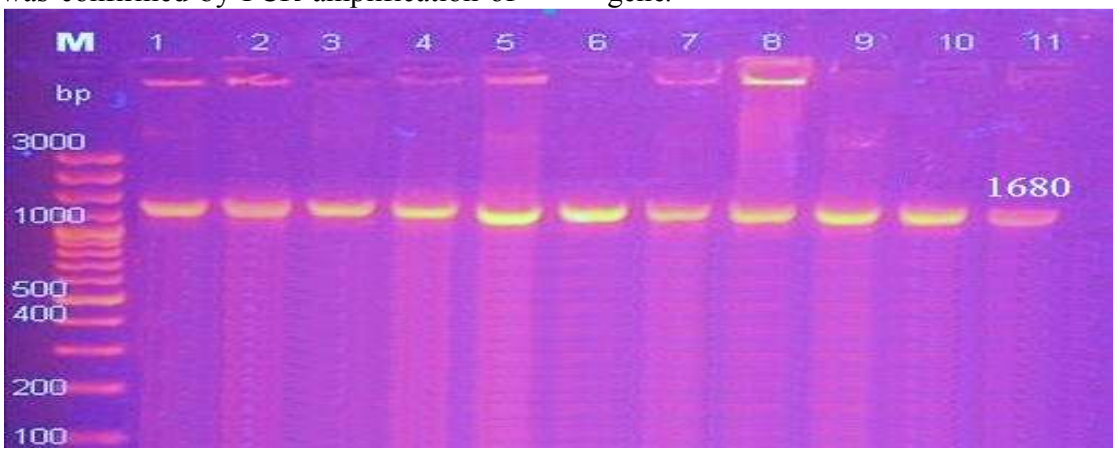

Figure 2. Agarose gel electrophoresis (1.5% w/v) of the PCR products of *C. sakazakii* DNA isolated from powdered infant formula milk and food and blood of septicemic infant revealing that all isolates gave a characteristic band at 1680 bp which was specific for α-glucosidase gene. M: molecular weight 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).

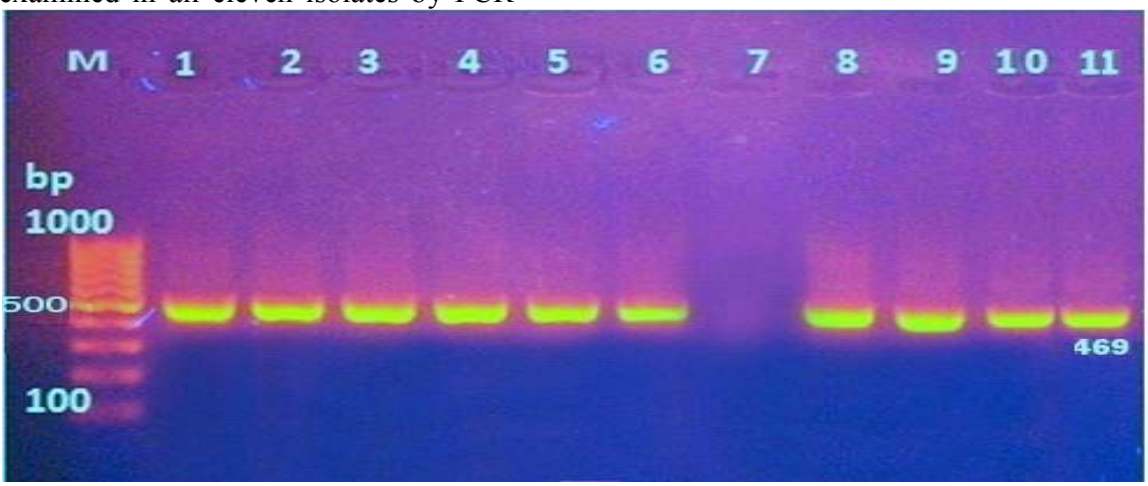

Figure 3. Agarose gel electrophoresis (1.5 % w/v) of the PCR products of *C. sakazakii* DNA isolated from powdered infant formula milk, powdered infant food and blood of septicemic infant revealing that all isolates gave a characteristic band at 469 bp which was specific for *OmpA* gene. M: Molecular 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, naldixic acid, gentamicin, imipenem, ceftazidime, sulphmethoxazole/Trimethoprime (90.9% each), aztronam (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.

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.

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, Levfloxacin; NOR, norfloxacin; OFX, ofloxacin; CIP, ciprofloxacin; NA, naldixic acid; CN, gentamycin, IMP, imipenem; CAZ, ceftazidime; SXT, sulphmethoxazole/ trimethoprime; ATM, aztronam; S , streptomycin; CTX, cefotaxime; AMC, amoxicillin/clavulanic acid (augmentin); CL, cephalexin; RD, rifampicin; AMP, ampicillin.

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 incase 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.

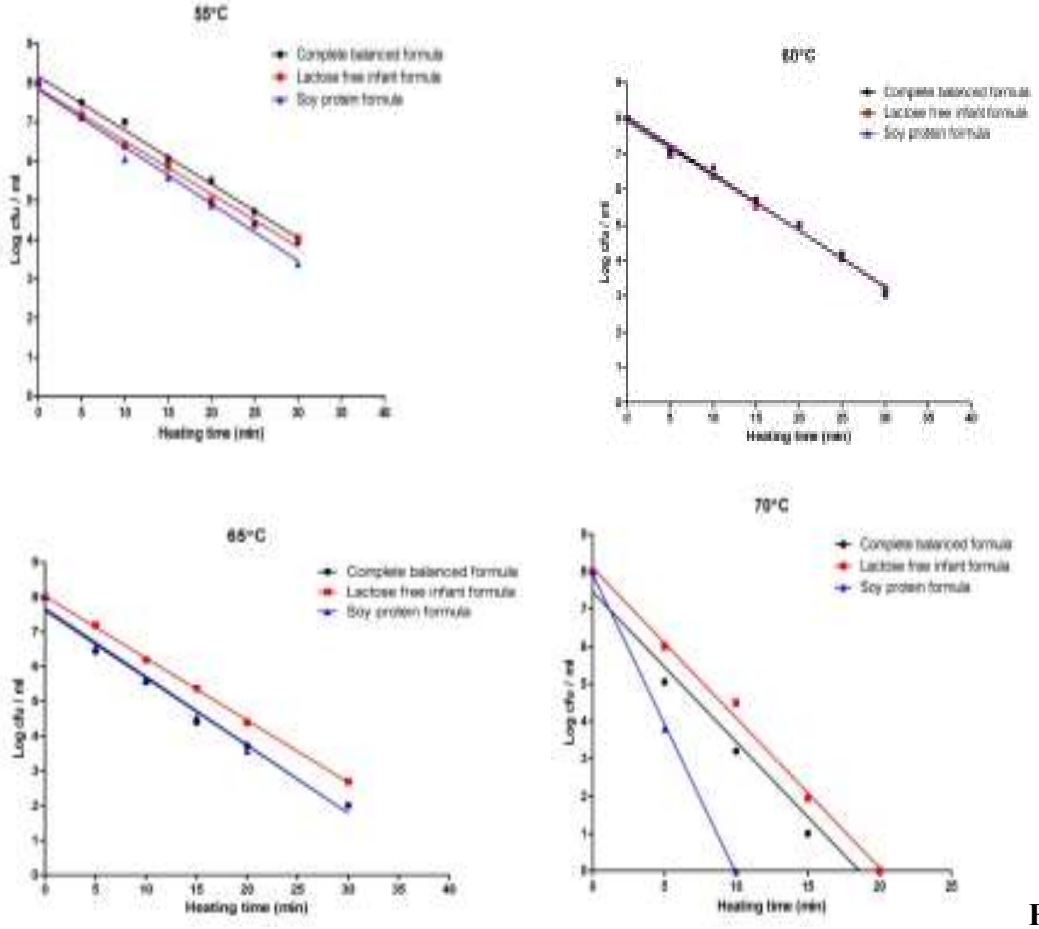

Figure 4. Thermal inactivation of *C. sakazakii* at 55˚C, 60˚C, 65˚C and 70˚C in reconstituted lactose- free infant milk formula (▬), soy protein infant formula (▲) and complete balanced formula (●). Results shown are the means of three replicate experiments.

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

Table 5. 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	25˚C	10	$5.03x10^6$	6.70	7.69
(Low fat).	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
	25˚C	10	$5.3 x10^6$	6.72	7.81
Whole milk powder	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 (Lai, 2001; Van Acker *et al*., 2001; Bar-Oz *et al*., 2001). 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 (Muytjens *et al*., 1988; Simmons *et al*., 1989; Nazarowec-White and Farber, 1997b; Shaker *et al*., 2007) who reported a direct correlation between infant formula and *C. sakazakii.* The obtained percentage was less than that obtained by Muytjens *et al*., 1988; Iversen and Stephane, 2004 and Aigbekaen and Oshoma, 2010, who recorded 14.1%, 24%; and 27.1%, respectively. While our results were consistent with that reported by Nazarowec-White and Farber, (1997b) 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* (Postupa and Aldova, 1984; Van Acker *et al*., 2001 and Block *et al*., 2002). 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 (Iversen *et al*. 2004b).

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 (Nazarowec-White and Farber, (1997a); Iversen *et al*., (2004b)). Also, Iversen and Forsythe, (2003) 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

(Forsythe, 2005). Therefore, hygienic measures and practices must be used during the manufacture of formula to minimize entry of contaminants into the process (Aigbekaen and Oshoma, 2010).

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 Gurtler *et al.*, (2005) and (Al- Holy *et al*., 2008) who reported that Food and Drug Administration (FDA, CFSAN, 2002 and ISO/TS 22964, 2006) methods are 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 (Sani and Yi, 2011).

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 (Iversen, 2007; Lehner *et al*., 2006). The α-glucosidase based PCR, exclusively targets the gene responsible for the α-glucosidase activity in *C. sakazakii* (Lehner *et al*., 2006).

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 (Nair and Venkitanarayanan, 2006; Prasadarao *et al*. 1996; Kim, 2000). The outer membrane protein A, encoded by the *OmpA* gene, is probably the best characterized virulence marker (Nair and Venkitanarayanan, 2006). 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 (Nair *et al*., 2009).

In our study, high sensitivity of *C. sakazakii* was found with levofloxacin, ofloxacin, norfloxacin, ciprofloxacin, gentamycin and sulphamethoxazole. These results are higher than that recorded by Aigbekaen and Oshama (2010), 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 Aigbekaen and Oshama (2010) (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 (Stock and Wiedemann, 2002). These results were compatible with that obtained by Aigbekaen and Oshoma (2010).

Cronobacter sakazakii like other *Enterobacter* species have acquired resistance by inactivating beta-lactam antibiotics due to production of beta-lactamases (Drudy *et al*., 2006).

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 incase of soy protein formula, the decrease was about 6.95 $\log_{10}$, these results are consistent with that obtained by (Osaili *et al*., 2008 b). In previous studies, D-values of *Cronobacter sakazakii* in reconstituted infant milk formula were with wide range. Edelson –Mammel and Buchanan (2004) and Iversen *et al.,* (2004 a, b) 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, Nazarowec-White and Farber (1997c) 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 Osaili *et al*., (2009); Nazarowec-White and Farber, (1997c); Kim and Park, 2007).

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دراسات على انتشار والمقاومة الميكروبية وبقاء كرونوباكتر ساكازاكي

فاطمة عبدالجليل[١]، همت كمال عبداللطيف[١]، أحمد عمار[٢]، فتحي سري[١]

[١] قسم الميكروبيولوجي والمناعة- كلية الصيدلة-جامعة الزقازيق
[٢] قسم الميكروبيولوجي والفطريات والمناعة- كلية الطب البيطري-جامعة الزقازيق

يعتبر ميكروب كرونوباكتر ساكازاكى مسبب طارئ للأمراض فى الأطفال حديثى الولادة حيث أن لديه القدرة على إحداث الالتهاب السحائى والتسمم الدموى والإسهال الشديد بسبب تناول الأطفال الحليب الملوث به. ويعد حليب الأطفال وسط بيئى ملائم له.

.ولذلك تهدف هذه الدراسة إلى توضيح مدى إنتشار كرونوباكتر ساكازاكى في مسحوق حليب الأطفال و الرضع والأطعمة المجففة الخاصة بهم المتوفرة في سوق المواد الغذائية المصرية . وهدفت الدراسة أيضا إلى تحديد العوامل التي تؤثر على بقاء ونمو كرونوباكتر ساكازاكى في مسحوق حليب الرضع من أجل السيطرة على انتشار الميكروب.

تم إجراء هده الدراسة على ٢٣٤ عينة من مسحوق حليب الرضع ومساحيق أغذية الأطفال. وتم اختبار ٧ من العينات السريرية و ٣ عينات بيئية لمعرفة وجود كرونوباكتر ساكازاكى وأظهرت النتائج أنه قد تم عزل الميكروب من ٩ عينات من أصل ١٧٣ عينة من مسحوق حليب الرضع و واحدة من أصل ٦١من أطعمة الأطفال المجفف وكان إجمالي معدل الانتشار بنسبة (٤,٢٧%). وقد لوحظ ارتفاع وتيرة عزل الميكروب من مسحوق حليب الرضع وكانت نسبته (٥,٢%) مقارنة مع أغذية الأطفال المجففة وكانت نسبة العزل (١,٦%).

وأظهرت النتائج أن عينة واحدة فقط من بين ٧ عينات سريرية كانت إيجابية لوجود كرونوباكتر ساكازاكى بها، في حين أنه لم يتم العثور على الميكروب فى العينات البيئية.

ولقد تم إجراء تفاعل إنزيم البلمرة المتسلسل للتأكد من عترات كرونوباكتر ساكازاكى وأكدت النتائج على أن المعزولات المختبرة كانت كرونوباكتر ساكازاكى وذلك لوجود منطقة مميزة عند الوزن الجزيئى ١٦٨٠لجين *gluA*. ولقد تم إجراء تفاعل إنزيم البلمرة المتسلسل لتحديد جين الشراسة (*OmpA*) فى جميع العترات المعزولة، ووجد أن جميع العترات تحمل جين الضراوة (*OmpA*) وذلك لوجود منطقة مميزة عند الوزن الجزيئي ٤٦٩قاعدة مزدوجة.

تم عمل إختبار الحساسية لستة عشر أنواع من المضادات الحيوية.وقد تبين أن الميكروب لديه القدرة على مقاومة الأمبسلين والريفامبسين بنسبة١٠٠ % وتبين أيضا أن الميكروب كان حساسا لليفوفلوكساسين ،نورفلوكساسين وأوفلوكساسن بنسبة١٠٠ % وكان حساس أيضا لجنتاميسن ، سيبروفلوكساسين،سلفاميثوكسازول/ترايميثوبرايم، نالديكسيك أسيد، سيفتازيديم و إيميبنيم بنسبة ٩٠,٩%، بينما كانت حساسيته لأزترونام بنسبة ٨١,٨ % وستربتوميسين ٧٢,٧ % وسيفوتاكسيم بنسبة ٥٤,٥% وأموكسيسيلين/كلافيولانك أسيد بنسبة ٢٧,٣% وسيفلكسين بنسبة ٩,٠٩%. ولقد كانت العترات المعزولة من العينات السريرية أكثر مقاومة لمعظم المضادات الحيويه من العترات المعزولة من بودرة لبن حليب الأطفال.

أوضحت النتائج أن استخدام الماء الساخن عند درجة حرارة ٧٠ درجة مئوية فى تحضير حليب الأطفال من الطرق المؤثرة فى القضاء على الميكروب الموجود ببودرة لبن حليب الأطفال بأنواعه المختلفة (بروتين الصويا، خالي من اللا كتوز والحليب المتوازن المتكامل).