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Isolation and identification of Cronobacter species from some animal products

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ABSTRACT

This work was carried out to study the bacteriological importance of *Cronobacter sakazakii*as a potential foodborne emerging pathogen involved in severe illness and deaths in humans, especially neonates due to consumption of contaminated infant powdered infant formula (PIF) (PIF milk and food). A total of 100 samples [PIF milk (n=55), PIF food (n=15), milk powder (n=15) and milk powder products (n=15) were collected and subjected to bacteriological examination for the presence of *Cronobacter*. Six samples out of 100 examined (6%) were found positive for *Cronobacter* spp. The isolation rates were 4% in PIF milk and 1% in PIF food. The identity of the isolated organism was confirmed as *Cronobacter* spp. by subjecting the bacteriologically positive samples to PCR technique using 16S rRNA species specific primers. *Cronobacter* specific 16S rRNA was detected respectively in 3/5 and 1/5 of bacteriologically positive PIF milk and PIF food examined. All positive 16S rRNA (n=4) were examined for the presence of *C. sakazakii*. *C. sakazakii*were confirmed in an isolate from 3 isolates of PIF milk and one of PIF food. The outer membrane protein A (*omp*A) gene was detected in 2 identified *C. sakazakii* isolates, while gene encoding for zinc-metaloprotease (*zpx*) was only identified in 3 *C. sakazakii* isolates. These results lead to the conclusion that examined milk products could be a potential source of *C. Sakazakii*, so the retailed powdered milk could have public health significance. Further studies are needed to study control strategies of *C. sakazakii*.

1. INTRODUCTION

Cronobacter spp. are emerging foodborne opportunistic pathogens that can infect neonates and infants resulting in necrotizing enterocolitis, bacteremia, and meningitis, with a 40–80% mortality rate (Holy and Forsythe, 2014; Li *et al*., 2016). *Cronobacter sakazakii* has been previously known as *Enterobacter sakazakii*. It is Gram-negative bacteria, motile peritrichous, rod-shaped, nonspore forming sakazakii bacteria belonging to the family Enterobacteriacea. In addition, these bacteria are typically facultative anaerobic, oxidase-negative, catalase positive; it also produces a yellow pigment (Iversen *et al.*, 2007). A high mortality rate particularly in infants has been documented due to consumption of contaminated PIF (Lampel and Chen, 2009). The genus Cronobacter has been divided into seven species: *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter turicensis*, *Cronobacter muytjensii*, *Cronobacter dublinensis*, *Cronobacter universalis*, and *Cronobacter condimenti* (Joseph *et al*., 2012[a,b]; Yao *et al*., 2012). Infant milk powder was implicated as the main source of Cronobacter resulting in 50–80% of infections (van Acker *et al*., 2001; Ye *et al*., 2010).

However, various food and environmental samples have also been documented as sources of infection (Iversen and Forsythe, 2004;Kandhai*et al*. 2004 & 2010;Friedemann, 2007). Severe meningitis, meningo-encephalitis, necrotizing enterocolitis, and sepsis caused by *C. sakazakii* have been reported in neonates consuming infant powdered milk (Caubilla-Barron *et al*., 2007). Moreover, surviving patients were reported to suffer severe neurological signs including hydrocephalus and developmental delay (Lai, 2001). Infection in elderly immune-compromised individuals consuming contaminated milk powder have also been reported (Lai, 2001; van Acker *et al.*, 2001). Virulence associated genes in *Cronobacter spp*. have a role in the potential of these bacteria to produce pathogenic effect in infected persons. The outer membrane protein A associated gene (*omp*A) and the gene encoding for zinc-metaloprotease (*zpx*) are both considered important virulence factors (Mohan Nair and Venkitanarayanan, 2006). The main objective of this study is to investigate the isolation and identification of *Cronobacter sakazakii* in different samples including powdered infant formula (milk and food), milk powder, and some milk powder products. Identification of virulence associated genes of the isolates were carried out. In addition of antibiotic sensitivity of cronobacter isolates.

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2. MATERIAL AND METHODS

2.1. Sampling

One hundred samples of PIF milk (N=55), PIF food (N=15), milk powder (N=15) and some milk powder products (N=15) at different localities in El Sharkia Governorate, Egypt during the period of April 2016 to February 2017.The collected samples were aseptically transported as soon as possible to the laboratory of Veterinary Hospital, Faculty of Veterinary Medicine, Zagazig University, for bacteriological examination.

2.2. Isolation and identification of Cronobacter species

Pre-enrichment of the samples was performed in buffered peptone water at 37 C for 24h (ISO 2006). A portion (0.1mL) of the culture was then transferred to 10mL of Cronobacter screening broth (CSB 38948, Sigma) and incubated at 42 C for 24h, and then examined for changing of color to yellow (Iversen et al., 2008). A loopful from the enriched CSB culture was streaked directly onto Cronobacter species isolation chromogenic agar (CSIA; Sigma, 14703; Hichrome CISA) and incubated at 44 °C for 24h.

2.3. Identification of Cronobacter species

2.3.1. Microscopic examination

Films were prepared from the pure cultures of the isolated organism. Each film was stained with Gram's staining technique and examined microscopically to verify the presence of characteristic features of the organism and to confirm the specificity of the colonies (Cruickshank *etal*., 1975).

2.3.2. Biochemical identification of bacterial isolates

Biochemical identification of Cronobacter spp. was carried out using catalase, oxidase, indole, and urea hydrolysis and citrate utilization tests as previously described by Cruickshank *etal*. (1975).

2.3.2.1 Catalase test: A loopful from three days old culture was mixed with a drop of hydrogen peroxide 3% (H_2O_2) on a clean sterile slide. Appearance of gas bubbles indicates catalase production.

2.3.2.2 Oxidase test: A loopful from the suspected colonies was rubbed on the surface of oxidase discs (Hi media, DD018). The appearance of intense deep purple color within few seconds indicates oxidase production.

2.3.2.3 Indole test: Kovac's reagent (0.5 ml) was added to 24 h peptone water culture and incubated at 37°C. The development of rosy color ring indicates the presence of indole.

2.3.2.4 Urea hydrolysis test: The isolated microorganism was streaked onto the surface of Christensen's medium (Hi media, M112) and incubated at 37°C for 24-48 h. The negative tubes were re-incubated and re-examined daily up to 7 days. A purple pink color indicates urea hydrolysis.

*2.3.2.5 Citrate utilization test*Simmon's citrate agar (Hi media, M099) slopes were streaked with a loopful of the suspected isolates and incubated at 37°C for up to seven days. The development of blue color indicates citrate utilization.

2.4. Molecular identification of Cronobacter species

The DNA from biochemically suspected Cronobacter isolates was extracted using QIAamp DNA Mini Kit (Cat. no. 51304, Qiagen) according to the manufacturer's instructions. Amplification of 16S rRNA specific for *Cronobacter spp*. was carried out using conventional PCR (Hassan *et al*., 2007). The virulence associated genes ompA (Mohan NairandVenkitanarayanan,2006) and *zpx*(Kothary*et al*.,2007) were also identified by PCR. A positive control of *C. sakazakii* was kindly provided by the Biotechnology Unit, Reference laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Dokki, Giza, Egypt. For further confirmation of *C. sakazakii* isolates identified by 16S rRNA PCR from buffalo fecal samples, sequencing of the amplified products was carried out. QIAquick Gel Extraction Kits (Qiagen, S. A. Courtaboeuf, France) were used for amplicon extraction from gel according to the manufacturer's guidelines. The purified products were sequenced with Big dye Terminator V3.1 Cycle Sequencing Kits (PerkinElmer, Foster City, CA) as described by the manufacturer. DNASTAR software (Lasergene ver. 7.2, DNASTAR, Madison, WI) was used to analyze two sequences that were then submitted to the GenBank, which provided the two accession numbers of KY210879 and KY210880. Identity of the two isolates with other C. sakazakii isolates on the GenBank was determined.

2.5. Antibiotic susceptibility testing

The PCR confirmed 4 Cronobacter isolates (*C. sakazakii*) were subjected to antibiotic susceptibility testing against eight commonly used antibiotics. The standard Kirby-Bauer disk diffusion method according to the National Committee for Clinical Laboratory Standards was used to determine the antimicrobial sensitivity profiles of Cronobacter isolates (NCCLS, 2013). These antibiotics with the respective disk concentrations were Streptomycin (5 mg), Gentamycin (10 mg), Meropenem (10 mg), Rifampicin (10 mg), Cefotaxime (30 mg), Lincomycin (10 mg), Cefoperazone (30 mg) and Ciprocin (5 mg). The antibiotic disks were placed on the inoculated plates, and then the plates were incubated at 35°C for 18-20h. The plates were examined for the presence of zones of inhibition of bacterial growth around the antibiotic discs which indicate the susceptibility of the isolated strains to these antibiotics. The zones of inhibition were then measured with a caliber and recorded (CLSI, 2014).

3. RESULTS

Table1PCR results of 5 *Cronobacter* isolates recovered from PIF milk and PIF food**.**

Sample	16S rRNA	*omp*A	*zpx*
PIF1 (milk)	-	Not done	Not done
PIF2 (food)	+	-	+
PIF3 (milk)	+	+	-
PIF4 (milk)	+	+	-
PIF5 (milk)	+	-	-

Table 2 Isolation of *Cronobacter* spp. isolated from different sources (PIF milk PIF food, powdered milk and powdered milk products) by bacteriological and PCR.

Sample types	Number examined	Cronobacter spp. isolates based on			*C. sakazakii* (*omp*A -)	+ve (%)	*C. sakazakii* (*zpx*-)	+ve (%)
		Growth +ve (%)a	16S rRNA PCR					
			No. (%)a	No. (%)b	No. (%)a	No. (%)c	No.(%)a	No.(%)c
PIF milk	55	4 (7%)	3 (5%)	3 (6%)	2 (3.6%)	2 (50%)	1 (18%)	1 (25%)
PIF food	15	1 (6%)	1 (6%)	1 (2%)	1 (6%)	1 (25%)	0	0
MP	15	1 (6%)	0	0	0	0	0	0
MPP	15	0	0	0	0	0	0	0

MP: Milk powder. MPP: Milk powder product. (a) The percentage in relation to the total examined samples (b) The percentage in relation to the bacteriologically +ve*Cronobacter spp*. (c)The percentage in relation to the 16S rRNA +ve*Cronobacter* spp.

Table 3 Antibiotics susceptibility of *Cronobacter sakazakii* isolates recovered from different sources.

Antibiotic class	Antibiotic	Cronobacter isolates (n=4)			ARI index= Y/nX
		S(ratio)	I(ratio)	R(ratio)	
Quinolones/Fluoroquinolones	Ciprocin (CIP)	4/4	0	0	0.00
Cephalosporins (Third generation)	Cefoperazone (CFP)	2/4	1/4	1/4	0.03
	Cefotaxime (CTX)	¾	0	1/4	0.03
Beta lactam	Meropenem(MEM)	3/4	0	1/4	0.03
Aminoglycosides	Gentamicin (CN10)	3/4	0	1/4	0.03
	Streptomycin (S)	0	1/4	3/4	0.09
Rifamycin	Rifampicin (RF)	0	1/4	3/4	0.09
Lincosamides	Lincomycin (L2)	1/4	1/4	2/4	0.06

S: Sensitive. I: Intermediate. R: Resistant. ARI: Antibiotic Resistance Index. Y = the number of resistant isolates to certain antibiotics.N = the total number of tested isolates.X = the total number of tested antibiotics.

Table 4 Antibiotic resistance patterns of *Cronobacter* isolates from different sources (no=4)

Patterns	Isolate code	Isolate source	Resistance profile	N	MARindex (a/b)
I	PIF1	Powdered infant milk	R, L, S	3	0.38
II	PIF2	Powdered infant milk	R, S	2	0.25
III	PIF3	Powdered infant milk	R, L, S	3	0.38
IV	PIF4	Powdered infant food	M, F, G, C, R, L, S	7	0.88

Average MAR = 0.47. F (Cefoperazone), C (Cefotaxime), I (Ciprofloxacin), G (Gentamicin), R (Rifampicin), S (Streptomycin), M (meropenem),L (lincomycin).N = Number of antibiotics showing resistant. a: the number of antibiotics to which the isolates are resistant. b: the total number of tested antibiotics (8).

Table 5Data of the isolated strains of *C. Sakazakii* in the current study.

Sample number	Date of isolation	Locality	Source	Biochemical identification					PCR			
				Oxidase	Catalase	Citrate	Urea	Indole	16SrRNA	OmpA	zpx	Antibiotic sensitivity
1	6/5/2016	Zagazig, Sharkia	PIF milk	-ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	Not done
2	10/5/2016	Fquos, Sharkia	PIF milk	-ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	R, L, S
3	10/5/2016	Al hussainia , Sharkia	PIF milk	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	R, S
4	1/5/2016	Zagazig, Sharkia	PIF milk	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	R, L, S
5	17/5/2016	Abokabir, Sharkia	PIF food	-ve	+ve	+ve	-ve	-ve	+ve	-ve	-ve	M, F, G, C,R, L, S
6	17/5/2016	Zagazig, Sharkia	PIF milk	-ve	+ve	+ve	+ve	-ve	Not done	Not done	Not done	Not done

F (Cefoperazone), C (Cefotaxime), I (Ciprofloxacin), G (Gentamicin), R (Rifampicin), S (Streptomycin), M (Meropenem),L (Lincomycin).

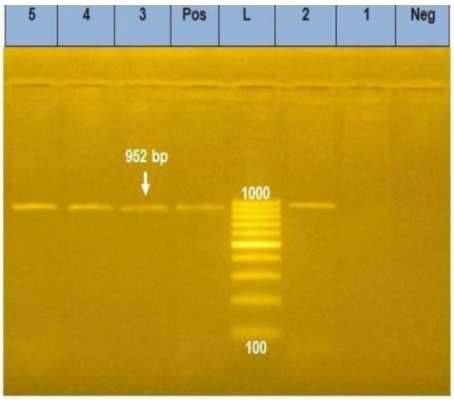

Figure 1 Amplification of 168 rRNA gene for the molecular identification of *cronobactersakazakii* isolates recovered from different sources with an amplicon size 952 bp . L: 100 bp ladder, Pos*: C. sakazakii* positive control, Neg: Negative control. (2,3,4,5) positive samples, (1) negative sample.

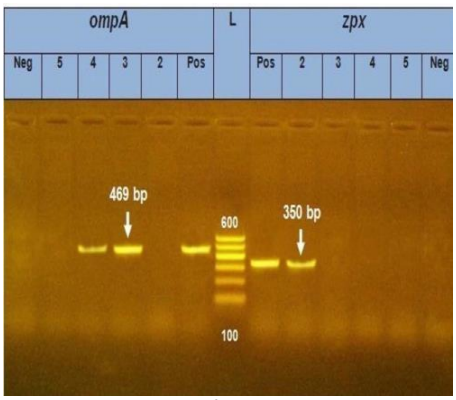

Figure 2 Agarose gel electrophoresis of *omp*A and *zpx* virulence associated genes amplification in *Cronobacter* isolates from different sources with amplicon size of 469 bp for *omp*A and 350 bp for zpx gene. L: 100 bp ladder, Pos: *C. sakazakii*positive control, Neg: Negative control, (3,4 left): Positive samples for *omp*A. (2 right): positive sample for *zpx* gene.

4. DISCUSSION

Isolation and identification of *Cronobacter spp*. In the current study, showed that out of 100 examined samples, 6 (6%) were bacteriologically suspected as *Cronobacter species*. However, only 4 (4%) were contaminated with *C. sakazakii* confirmed by PCR amplification of 16S rRNA sequence specific for *Cronobacter species*.This result is supported by Saleh (2016) who found that 2 (2%) of the examined PIF samples were positive for *Cronobacter spp*. and only 1 (1%) was identified as *C. sakazakii*, this agreement may be due to single source of contamination. Nearly similar results of Cronobacter isolation were previously reported in PIF samples, for instance, 1.5% in Iraq (Al-Timimi, 2007), 1.9% in Nigeria (Rashidat *et al*., 2013), 2.4% in different countries (Iversen and Forsythe, 2004), 2.5% in Turkey (Heperkan *et al*., 2017), 3% in France (Proudy*et al*., 2008) and 3.1% in Bangladesh (Hoque et al., 2010). However, higher isolation rates of *Cronobacter spp*. ranging from 6.6% to 25% were also documented, 6.6% in Japan (Oonaka *et al*., 2010), 8.7% in Netherlands (Kandhai *et al*., 2010), 13% in Ivory Coast (Yao *et al*., 2012), 13.3% in Egypt (El-Sharoud*et al*., 2009), 18% in Iraq (Jaber et al., 2015), 24% in Egypt (El-Gamal et al., 2013), 24% in Nigeria (Moses David *et al*., 2013) and 25% in Jordan (Shaker *et al*., 2007). In the present study, four of the milk powder samples were positive for the presence of *Cronobacter spp*. The presence of *Cronobacter spp*. in milk powder samples is unusual due to normal pasteurization of liquid milk before manufacturing of milk powder products and the pasteurization process is considered an effective step for elimination of Cronobacter contamination (Iversen and Forsythe, 2004; Shaker *et al*., 2007). Molecular amplification of *omp*A gene revealed that 2 out of 4 (50%) Cronobacter isolates harbored *omp*A associated gene (isolates from PIF), while, only one isolates of PIF were positive for *zpx* associated gene.

In accordance, Cronobacter isolates from PIF were positive for *omp*A gene (Cai et al., 2013; Fei et al., 2015). Sequencing of 16S rRNA was carried out on *C. sakazakii* isolates which revealed that 4 *Cronobacter spp* were positive 16S rRNA and one negative sample (non-*C. sakazakii*). The 16S rRNA sequencing is a powerful technique for rapid and specific identification of different *Cronobacter species* (Lehner *et al*., 2006; Hassan *et al*., 2007; Jaradat*et al*., 2009). in the current study, Cronobacter isolates were highly sensitive to Ciprocin (100%) followed by Gentamycin (75%), cefotaxime (75%) and meropenem (75%), while they were less sensitive to cefoperazone (50%) and poorly sensitive to lincomycin (25%). Meanwhile, the isolates were resistant to Rifampicin (75%) and Streptomycin (75%). The high sensitivity of Cronobacter isolates to ciprofloxacin is in accordance with other reported studies (Stock and Wiedemann, 2002; AlTimimi, 2007; Kim *et al*., 2008a; Hoque *et al*., 2010; Oonaka *et al*., 2010; Hochel *et al*., 2012; Jaber *et al*., 2015; Oloninefa *et al*., 2015). The susceptibility of Cronobacter isolates to cephalosporins (ceftriaxone, cefotaxime and cefoperazone in the current study is in agreement with those previously documented (Muytjens and van der Ros-van de Repe, 1986; Al-Timimi, 2007; Terragno*et al*., 2009; Aigbekaen and Oshoma, 2010; Hoque *et al*., 2010; Oonaka *et al*., 2010; Hochel *et al*., 2012; Oloninefa *et al*., 2015). The calculated antibiotic resistant index (ARI) in our work ranged from zero to 0.09 this result reveals the diversity of anti-biogram profile of isolated *C. sakazakii* strains. The MAR index ranged from 0.25 to 0.88, with the highest MAR index (0.88) found in *C. sakazakii* strain recovered from powdered infant formula food. The lowest MAR index (0.25) was found in the isolates from PIF milk. *C. sakazakii* isolate number 4 was multi-resistant strain showing resistance to 7 antibiotics, isolates number 1and 3 were resistant to 3 antibiotics, and isolate number 2 was resistant to only 2 antibiotics. The isolates were retrieved from different localities in Sharkia governorate and various date of isolation from the same PIF source; this may be due to various source of contamination. Nevertheless, the isolate number 3 was resistant to 2 antibiotics in different sources (PIF, milk and food) this may refer to single sources of contamination. Isolate number 6 was morphologically suspected *C. sakazakii* in plate on selective media but biochemically was negative.

4. CONCULOSION

From the obtained results it could concluded that, powdered infant formula is considered as the most important reservoirs for *Cronobacter spp*. especially for *C. sakazakii*. Reconstitution and consumption of powdered infant formula is regarded as a risk for infection in infants. Quinolones and Cephalosporin's is the drug of choice for treatment of Cronobacter infected cases in Sharkiagovernorate.

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