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Isolation and molecular identification of *Cronobacter sakazakii* isolated from different foodstuffs and infant stools

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ABSTRACT

Cronobacter sakazakii is a foodborne pathogen that can cause serious infections (enterocolitis and meningitis) with high fatalities in neonatal infants. This work was designed to isolate *Cronobacter sakazakii* and explore its molecular identification and prevalence rate. The present study was performed on 225 random samples of powdered milk infant formula, milk powder, minced meat, fresh cow milk, and diarrheic infant stool (45 for each) collected from different shops and hospitals in Cairo, Kaliobia, and Monofia Governorates, Egypt. *Cronobacter sakazakii* isolates were characterized by conventional phenotypical methods and MICROBACT™ kits, then genotypic identification of the common 16S rRNA gene by PCR. Out of the fifteen *Cronobacter sakazakii* isolates obtained, the majority were isolated from powdered milk infant formula samples 5 isolates (11.1%), 4 from milk powder (8.9%), 3 from diarrheic infant stool (6.7%), 2 from minced meat (4.4%), and 1 from fresh cow milk samples (2.2%). The *Cronobacter sakazakii* 16S rRNA gene was detected in all tested isolates. Therefore, the results of this work highlight the importance of *Cronobacter sakazakii* as a high public health concern in newborns, indicating the potential risk of infant infection by this bacteria from mostly all sources of infant food.

1. INTRODUCTION

Cronobacter sakazakii (*C. sakazakii*) is a Gram-negative rod-shaped, facultatively anaerobic, motile, non-spore-forming bacterium with peritrichous flagella that belongs to the Enterobacter genus and Enterobacteriaceae family (Al-Aawadi and Weda, 2020). *Cronobacter sakazakii* is a lactose fermenter, catalase positive and oxidase negative bacteria, it has pink mucoid colonies when isolated using MacConkey. It can be recognized by a characteristic non-diffusible yellow pigment colony on Tryptone Soy Agar (TSA) at 25°C. It can also grow on deoxycholate agar and Eosin Methylene Blue (EMB) (Da Silva *et al*., 2018). *Cronobacter sakazakii* has many adaptations, including its ability to build biofilms, tolerate a broad range of growth temperatures, and tolerate ionic strength and dryness, in addition to, its resistance to antibiotics. These adaptations are crucial for the bacterium's survival in harsh settings (Singh *et al*., 2015; Holý *et al*., 2019).

Cronobacter sakazakii is an opportunistic foodborne pathogen that has been isolated from powdered milk infant formula (MF), milk powder (MP), fresh milk, cheese products, minced beef, sausages, dry cereals, and different vegetables (Ueda, 2017; Abebe, 2020; Csorba *et al*., 2022). Because of its polysaccharide capsule, resistance to desiccation, and ability to produce a yellow carotenoid pigment that protects it from oxygen radicals, *Cronobacter sakazakii* may be found in a range of environments (Iversen and Forsythe, 2003). *Cronobacter sakazakii* infection usually attacks all age groups, especially prematurely born neonates and infants, via consuming contaminated powdered infant formula (MF) and milk powder, causing bacteremia, sepsis, necrotizing enterocolitis, meningitis and acute diarrhea, with a high mortality rate of up to 80% (Chen *et al*., 2018; Kadlicekova *et al*., 2018; Henry and Fouladkhah, 2019). Polymerase chain reaction (PCR) and other genotyping techniques have been seen as useful methods for performing epidemiological monitoring to determine the similarity of bacterial pathogens isolated from dietary, environmental, and clinical samples. Although culture methods are important for Cronobacter spp detection, they are time-consuming and involve complex steps (Chen *et al*., 2018; Armstrong *et al*.2019). The molecular characteristics of *C. sakazakii*, which is isolated from powdered milk infant formula and other sources, can be revealed through genotyping, which can also aid in the pathogen's prevention and source tracing (Ling *et al*., 2021, Fei *et al*., 2018, Fei et al., 2022).

It is now believed that *Cronobacter sakazakii* is a newly discovered opportunistic bacteria that is frequently isolated from powdered milk infant formula and other foods that usually cause severe morbidity and mortality through some infectious diseases in neonates, infants, and other age groups, and so far, especially in Egypt, limited studies are

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available on its prevalence rate in foodstuff. Therefore, the present study was performed to determine the prevalence rate, phenotypic characterization, and molecular identification of this pathogen in different foodstuffs and infant stools.

2. MATERIAL AND METHODS

2.1. Collection of samples

A total of 225 random samples of powdered milk infant formula, milk powder, minced meat, fresh cow milk, and diarrheic infant stool (45 for each) were collected from various local stores, supermarkets, and medical facilities in Cairo, Kaliobia, and Monofia Governorates, Egypt, during the period from March 2022 to May 2023. Every sample was collected separately in sterile plastic bags, stored in an icebox, and sent as quickly as possible to the lab for bacteriological analysis under aseptic circumstances.

2.2. Preparation of samples

2.2.1. Powdered milk infant formula; milk powder; fresh cow milk and minced meat samples

Aseptically, (10 ml or g of each fresh raw milk, powdered milk infant formula, milk powder, and minced meat) were taken and put separately in a sterile stomacher bag containing 90 ml of sterile buffered peptone water (Oxoid CM9). After two minutes of homogenization at the stomacher (M A 106402, France) at 450 to 640 strokes per minute, the mixture was left to stand at room temperature for five minutes. After that, they were put into sterile flasks, shaken well, and incubated for 24 hours at 37 °C to facilitate primary enrichment (FDA, 2002; Da Silva *et al*., 2018).

2.2.2. Stool samples

For primary enrichment, two grams of each stool sample were homogenized in eighteen milliliters of sterile pure water. One milliliter was taken and put into a universal bottle containing nine milliliters of sterile buffered peptone water and the mixture was incubated for twenty-four hours at 37 ºC. It was assumed that the presence of turbidity in enrichment cultures indicated a favorable outcome (presumptive positive result). Ten ml of each mixture for all samples were suspended in 90 milliliters of Enterobacteriaceae enrichment broth "EE broth" (High Media) and incubated at 37°C for the entire night (FDA, 2002).

2.3. Isolation and phenotypic identification of Cronobacter sakazakii

A loopful of each enrichment culture was streaked onto both Violet Red Bile Glucose Agar (VRBGA) (HighMedia, India) and MacConkey's agar (Oxoid No. CM115) and incubated at 37 °C for 24 hours. Then the pink-red colonies were sub-cultured on Tryptone soya agar (TSA) (HighMedia, India) and incubated at 25 °C for (48-72 h.), suspected yellow colonies were purified on TSA, and then kept in semi-solid agar for biochemical identification. The *suspected C. sakazakii* colonies were confirmed morphologically by Gram's stain and motility test. Then, they were identified biochemically by conventional biochemical methods such as Oxidase, Catalase, H2S, Urease, Nitrate reduction, Indole, Methyl-red, Voges-Proskauer, and Citrate tests. In addition, they were resuspended in physiological saline and subjected to subsequent biochemical characterization using MICROBACT^{TM} identification kits (Oxoid) according to the manufacturer's instructions. Finally, the purified isolates were preserved in Tryptone soya broth (TSB) (Oxoid-CM0129) supplemented with 20% glycerol for PCR identification. FDA (2002); Da Silva *et al*. (2018) and Pakbin *et al*. (2022).

2.4. Genotypic identification of some C. sakazakii isolates by PCR

Five selected *C. sakazakii* isolates (one isolate from the examined samples of powdered milk infant formula, milk powder, minced meat, cow milk, and diarrheic infant stool) were tested for detection of Cronobacter 16S rRNA gene by using QIAamp® DNA Mini Kit instructions (Qiagen, Germany, GmbH), Emerald Amp GT PCR mastermix (Takara, Japan). PCR technique was done by the primer as shown in table (1) and running conditions: one cycle of denaturation at 94℃ for 5 min, followed by 35 cycles of 94℃ for 30 s, 52℃ for 40 s, and 72℃ for 1min. PCR was completed with a final extension at 72C for 10 min. Products of PCR were separated by 1. 5% agarose gel electrophoreses (Sambrook *et al.,* 1989). The gel was photographed by a gel documentation system.

Table 1 Primer used for detection of 16S rRNA gene in *Cronobacter. sakazakii* isolates

Target gene		Primer sequence (5'-3')	Amplified product	References
Cronobacter 16S rRNA	F	ACAGGGAGCAGCTTGCTGC		Jaradat *et al.,* 2009
	R	TCCCGCATCTCTGCAGGA	952 bp	

These procedures was approved from the Animal Ethical Committees of Benha University, with the ethical approval number (BUFVTM 32-09-23)

3. RESULTS

3.1. Prevalence of *Cronobacter sakazakii* isolates in examined samples.

Out of 225 samples, 15 *Cronobacter sakazakii* isolates were recovered with a percentage representing (6.7 %). For each sample type, the rate of isolation was (11.1%) from powdered milk infant formula, (8.9%), from milk powder, (6.7%), from diarrheic infant stool, (4.4%) from minced meat, and (2.2%) from fresh cow milk samples, as in Table (2).

Table 2 Prevalence of *Cronobacter sakazakii strains* isolated in examined samples.

Samples	Number of samples	Positive samples	
		No.	%
Powdered milk infant formula	45	5	11.1
Milk powder	45	4	8.9
Minced meat	45	2	4.4
Cow milk	45	1	2.2
Diarrheic infant stool	45	3	6.7
Total	225	15	6.7

3.2. Identification of *Cronobacter sakazakii* isolates

3.2.1. Phenotypic identification

Cronobacter sakazakii colonies on VRBGA medium appeared as glucose fermenter, purple-red colonies, surrounded by halo zone of precipitated bile acids, and they appeared as lactose fermenter, pink, mucoid colonies with thick center on MacConkey agar. Moreover, on TSA agar, they showed yellow pigmented colonies. The morphological characters of *C. sakazakii* isolates were Gram-negative, motile and non-spore forming rods. All 15 isolates had the same *C. sakazakii* biochemical characters as positive results for catalase, Voges-Proskauer, nitrate reduction, and citrate utilization tests. And negative results for oxidase, methyl red, Indole, H2S, and Urease tests. Moreover, the results of the MICROBACTTM identification kits showed an identical biochemical reaction to *C. sakazakii*, according to the Oxoid manufacturer's instructions.

3.2.2. Genotypic identification

All five selected *C. sakazakii* isolates were positive for the *C. sakazakii* 16SrRNA gene, yielding positive bands at 952 bp. (Fig. 1).

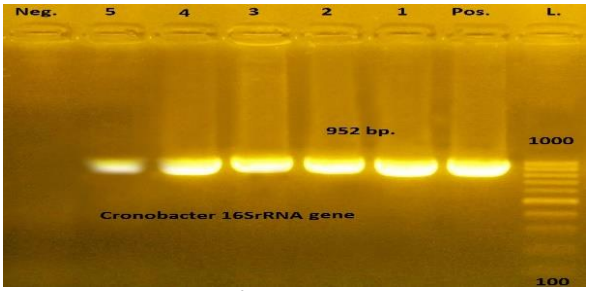

Fig. 1 Agarose Gel electrophoresis of *C. sakazakii* (16S rRNA) gene.
Lane L: 100-1000 bp. DNA Ladder. Neg.: Negative control (*E. coli* ATCC 25922). Pos.: Positive control (*C. sakazakii* ATCC®29544™ at 952 bp.). Lanes (1 - 5): number of *C. sakazakii* isolates from each sample (1 powdered milk infant formula, 2 Milk powder, 3 Minced meat, 4 cow milk, and 5 diarrheic infant stool), all five isolates showed positive bands for 16S rRNA gene at 952bp.

4. DISCUSSION

Since *Cronobacter sakazakii* is a newly discovered opportunistic bacterium, it is important to regularly check for its spread throughout various food sources. More research is becoming necessary due to its possible risk of causing severe morbidity and mortality in newborns, infants, and other age groups.

In the current work, the overall *C. sakazakii* isolation rate of (6.7 %) is nearly similar to that has been recently reported (Al-Aawadi and Weda, 2020; Csorba *et al*., 2022; Pakbin *et al*., 2022).

The prevalence of *C. sakazakii* in powdered milk infant formula was (11.1%) in harmony with those recorded by (Mardaneh et al., 2017; Csorba et al., 2022; Pakbin et al., 2022). But there is disagreement with those obtained (Zhang et al. 2017, Tayeb et al. 2020, and Fei et al. 2022) who reported lower *C. sakazakii* prevalence ratios of 1.60%, 3.1%, and 0.75%, respectively, in commercial powdered milk infant formula, with those of Elkhawaga *et al.* (2020) who recorded a higher incidence of 17.5 %, and with Jošić *et al.* (2017) and Jebur and Abood (2018), who did not find *C. sakazakii* in powdered milk infant formula samples.

Meanwhile, its prevalence in milk powder was (8.9%), similar former report (Parra-Flores *et al*., 2015). However, this result disagrees with those of Holý *et al*. (2021), who recorded a lower incidence of 0.41%, and with Abd El-Tawab *et al*. (2019), who reported zero incidence of *C. sakazakii* from milk powder samples.

As *Cronobacter sakazakii* can form biofilms, heat-tolerant, and resistant to desiccation, it represents a risk to food safety when it comes to powdered milk and its products, particularly powdered infant formula products. As a result, it might endure a long period in the processing environment. (Ling et al., 2020; Wu et al., 2021).

Others reported that because liquid milk is exposed to high temperatures to evaporate the water to turn into powdered milk (spray drying), *Cronobacter sakazakii* appearance in samples of milk powder is unusual. This is regarded as a successful step in getting rid of Cronobacter infection (Iversen and Forsythe, 2004; Shaker et al., 2007).

Moreover, the prevalence of *C. sakazakii* in minced meat was 4.4%, in concurrence with the results of Wang *et al.* (2012), whereas it is lower than that reported by Mohammed *et al.* (2015), who isolated it with an incidence of 28%, and higher than that of Aksu *et al*. (2018), who did not detect *C. sakazakii* in minced meat samples. The presence of *C. sakazakii* in minced meat, particularly given how popular this product is in fast food restaurants where contamination from insufficient cooking or post-heat treatment may happen.

The prevalence of *C. sakazakii* in fresh cow milk samples was (2.2%), in contradiction with that reported by Berhilevych and Kasianchuk (2017), who isolated *C. sakazakii* from raw milk samples with a higher incidence of (19.4%), and with that reported by Lehner *et al*. (2010) and Aksu *et al*. (2018), who did not detect *C. sakazakii* in raw milk samples.

In addition, the prevalence of *C. sakazakii* in diarrheic infant stool was (6.7%). This agreed with a previously reported isolation rate by Al-Aawadi and Weda (2020). Meanwhile, the prevalence rate of *C. sakazakii* in the current study is far lower than a previously reported isolation rate by Hassan and Naser (2018) (16%). The isolation rate of *C. sakazakii* in the current study is higher than in other studies, which showed that the prevalence rate of this bacteria was only 2% (Jaber *et al*. 2015) and nil (Radhi, 2016) from diarrheic infant stool samples.

Regarding the colonial appearance (characteristic, yellow-pigmented colonies on Tryptic soya agar at 25 °C for (48-72 h) and the biochemical profile and commercial MICROBACTTM profile of *C. sakazakii* isolated, it was similar to those previously reported by Da Silva *et al*. (2018), Al-Aawadi and Weda (2020), Tayeb *et al*. (2020), and Pakbin *et al*. (2022).

A potent method for the quick and accurate identification of *C. sakazakii* and other Cronobacter species is the PCR detection of the 16S rRNA gene. (Bergilevich *et al*., 2015; Chen *et al*., 2018). In this study, five isolates were tested with PCR, and all were accurately identified as *C. sakazakii.* This gene was amplified in all tested strains, giving products at 952 bp. This result came in agreement with those of Berhilevych and Kasianchuk (2017) and Tayeb *et al*. (2020).

5. CONCLUSIONS

The current study revealed the contamination status and characterization of *C. sakazakii* in many different sources, such as powdered milk infant formula (11.1%), milk powder (8.9%), minced meat (4.4%), fresh cow milk (2.2%), and diarrheic infant stool samples (6.7%). The study concluded that *C. sakazakii* strains are food-borne pathogens. Powdered milk infant formula is the main source and reservoir of *C. sakazakii* (11.1%), which may be the causative agent of diarrhea in infants. So, it is a public health concern, and further studies are necessary.

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