

## Incidence rate and antibiotic resistance profile of *Cronobacter sakazakii* isolated from various food products

\*Tayeb, B.A., Mohamed Sharif, Y.H. and Ameen, A.M.

Food Microbiology Unit, Department of Laboratory, Directorate of Veterinary in Duhok, Jn 1,1000 Galy Way, Duhok, Kurdistan region of Iraq

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### Abstract

*Cronobacter sakazakii* (*C. sakazakii*) is a foodborne pathogen which has been linked to various food outbreaks. The current study was aimed to isolate and identify this pathogen from different kind of food products and herbs as well as to reveal its antibacterial resistant profiling. A total of 235 samples were collected, including 130 Powdered Infant Formula (PIF), 55 spices and herbs and 50 samples from Bouillon Flavoured Powder (BFP). These samples were obtained from local markets in Duhok city of Iraq from February to August 2019. The collected items were checked for the presence of *C. sakazakii* by using conventional biochemical tests and confirmed through molecular techniques. The findings showed that 3.1% of PIF, 24% of BFP and 78.2% of spices and herbs were positive for *C. sakazakii*. This bacterium expressed a complete susceptibility to each of ampicillin, tetracycline, gentamycin and chloramphenicol and showed high to mild resistance to the rest of the examined antibiotics. Microbiological evaluation of imported food is crucial for safeguarding public health. The high contamination rate and high drug-resistant *C. sakazakii* in the examined food products declared prospective hazards to endangered people.

## 1. Introduction

*C. sakazakii*, formerly named as yellow-pigmented *E. cloacae*, is one of the seven species of *Cronobacter* spp, and it was first discovered as a new species in 1980 (Farmer *et al.*, 1980). It is a gram negative, rod shape, non-spore forming bacteria and belongs to the family *Enterobacteriaceae*. This bacterium has strong association with foodborne diseases (Iversen and Forsythe 2004). *C. sakazakii* causes severe infection like meningitis and entero-colitis in neonates and infants especially in premature babies (Bar-Oz *et al.*, 2001). The International Commission for Microbiological Specifications for Foods (ICMSF, 2002) has categorized *C. sakazakii* as 'severe hazard for restricted populations, life-threatening or substantial chronic sequelae or long duration'. This could be due to the fact that this organism is considered as a new emerging and an aggressive pathogen in endangered people such as children and elderly (Hunter and Bean 2013; Hariri *et al.*, 2013). In fact, the reports show that the prevalence and severity of the disease are much higher in children than in any other groups (Hariri *et al.*, 2013).

*C. sakazakii* has been isolated from many different

food items such as fruits and vegetables, starches, herbs, and spices. Moreover, other food products have also been reported to be contaminated with this organism like animal foods, milk, fish, meat and other sub-productions like powdered infant food (PIF) and these food sources have been associated with *C. sakazakii* outbreaks (FAO/WHO 2008; Lee *et al.*, 2012).

Nowadays, there is more food safety awareness about *C. sakazakii* worldwide as the globalization of food chain brings a wider range of foodborne pathogens (Shukla *et al.*, 2016). The pressing problem with *C. sakazakii* is the development of antibiotic resistance, which has been confirmed (Lai, 2001). The antibiotic treatment of this dangerous pathogen is quite limited. Furthermore, the infective dose for *C. sakazakii* infection is also low compared to other foodborne pathogens which making it some kinds of difficulty to control or minimize the infection. Therefore, the actions should be taken for alternative antibiotic therapy or biocontrol and tough monitoring is essential for this bacterium (Lehner *et al.*, 2004).

In recent years, various detection methods have been applied and developed for isolation and identification of

\*Corresponding author.

Email: [drbat25@yahoo.com](mailto:drbat25@yahoo.com)

*C. sakazakii* in food and environmental samples (Shukla et al., 2016). Traditional culture-based procedures, like sample preparation, pre-enrichment, plating out, and conventional biochemical tests are widely used for the isolation and identification of this pathogen in food products and environmental samples. However, the drawback of this technique is labor-intensive. Nevertheless, traditional cultural methods are reliable and sensitive enough so that it is still used in many different laboratories. Some other emerging new techniques are now being used for the detection of *C. sakazakii*. Traditional Polymerase Chain Reaction (PCR) or Real-time PCR (qPCR), are both forms of molecular techniques used for identification of this pathogen. They are more sensitive, specific and can detect the organism in reasonably low abundance in a very short time (Si et al., 2016).

The current study was aimed to isolate, identify and reveal the antimicrobial susceptibility of *C. sakazakii* in different type of imported food including PIF, Bouillon Flavoured Powder (BFP) and herbs and spices by using cultural- and PCR-based techniques in Duhok city, Iraq.

## 2. Materials and methods

### 2.1 Sample collection

A total of one hundred thirty samples of PIF were collected from different retail markets in Duhok city. Those PIF items were imported from abroad and the cans belong to famous international trademarks. The physical condition, whether leaked or rusted and the expiry date of the cans was checked. Any cans within three months of expiry dates were excluded from the study. In addition, 55 samples from a different type of herbs and spices were obtained from different shops in the city center of Duhok city. These spices include Cardamom (n=10), Wild thyme (n=5), Cummins (n=8), Oregano herb (n=8), Turmeric powder (n=12), Crushed peppers (n=12). Furthermore, fifty samples of Bouillon Flavoured Powder (BFP) in two shape forms either cubes (n=25) or powdered (n=25) originating from six different countries including Turkey, India, China, USA, Syria and UAE were also collected from various local shops for isolation of *C. sakazakii*.

### 2.2 Enrichment

The method that used for the isolation and identification of *C. sakazakii* in this research was the official techniques of the International Standard Organization (ISO) 22964:2017(E) with a minor modification. Briefly, 10 g of each of the collected sample weighed separately and aseptically transferred into a bottle containing 90 mL of pre-warmed buffered peptone water (BPW), (HiMedia Laboratories, India).

The aliquots were mixed well by vortex for 30 s and incubated at 37°C for 24±2 hrs. From the previous step (cultured buffered peptone water), 0.1 mL was transferred to a tube containing 10 mL of modified Lauryl Sulphate Tryptose Broth Base (mLST) (Oxoid, England) containing vancomycin (5mg/ 100 mL). The selective suspension (mLST suspension) was then incubated at 44±0.5°C for 24±2 hrs.

### 2.3 Isolation of *C. sakazakii*

About 10 µL from the enriched step was taken and inoculated on *Enterobacter sakazakii* Isolation Agar (ESIA), (Oxoid, England) and the plates were incubated at 44°C±0.5°C for 24±2 hrs (Miranda et al., 2017). The typical colonies of *C. sakazakii* on ESIA give bluish green in color (Weber et al., 2009). Therefore, the presumptive colonies were selected to inoculate of Trypticase Soy Agar (TSA) (HiMedia, India) and incubated overnight at 37°C. *Cronobacter sakazakii* produces yellow pigmentation on this agar (Weber et al., 2009).

### 2.4 VITEK-2 Systems

The suspected colonies of *C. sakazakii* were picked and suspended into a tube containing 3.0 mL of 45% sodium chloride solution. The bacterial suspension turbidity was adjusted by using VITEK Densicheck (bioMerieux) to become 0.5 McFarland standards. After that, the ID-Gram Negative (ID-GN) was used by using VITEK-2 system following manufacturer manual steps (BioMerieux, France) (Ling et al., 2003).

### 2.5 Molecular confirmation

The presumptive colonies of *C. sakazakii* from the TSA plates were confirmed by using PCR techniques according to HumPCR™ *Cronobacter sakazakii* Detection Kit (Bioingentech, Chile).

#### 2.5.1 DNA extraction

DNA was extracted from cell suspension using QIAamp DNA Mini kit (Qiagen, Germany). Briefly, a loopful of suspected colonies grown on the agar plates was picked and suspended into 2 mL of distilled water in an eppendorf tube. The suspension was then centrifuged for 5 mins at 2000 rpm and the supernatant was then removed without disturbance of the cell pellet. The pellet was resuspended with 200 µL of distilled water then 20 µL of QIAGEN proteinase K was added. The suspension was incubated in a water bath at 56°C and then 200 µL of the absolute ethanol was added to the mixture. After centrifugation, the mixture was placed in a QIAamp Mini spin column clean 2 mL collection tube and again centrifuged at 8000 rpm for 1 min. The collection tube

was removed and replaced by a new one and then 500µL of Buffer AW1 was added to the mixture and microcentrifuged at 8000 rpm for 1 min. Five hundred µL of Buffer AW2 was added to the tube and centrifuged for 3 min at 14,000 rpm. Before adding the 200 µL of elution buffer from the Tris-EDTA (TE), the collection tube was replaced by a new one and centrifuged at 8000 rpm for 1 min and used as a DNA.

### 2.5.2 Amplification of bacterial DNA

The PCR amplification was carried out in a final volume of 15 µL consisting of 6 µL of *C. sakazakii* premixture (MasterMix), 3 µL of extracted DNA from *C. sakazakii* positive colonies and 6 µL DNase free water. The positive, negative and internal controls were also used at each running time and composed from the same volume as previous. Each reaction tube was covered with 5 µL of mineral oil. The tubes were placed in a thermo cycler (Applied Biosystems, USA) and the PCR cycling conditions are shown in Table 1.

Table 1. PCR cycling condition

No. of cycles	PCR cycle	Temperature	Time
1 cycle	Initial denaturation	94°C	2 min
	Denaturation	94°C	30 s
30 cycle	Annealing	57°C	30 s
	Extension	72°C	30 s
1 cycle		72°C	5 min

### 2.5.3 Gel electrophoresis

The PCR products were analyzed in 1.5% of agarose gel prepared with 1× Tris-acetate-EDTA (TAE) buffer and stained by red safe DNA staining solution (GeNetBio, Korea). The electrophoresis was run in 1X TAE buffer by 100V for a period of 40 mins. The amplified products were identified under ultra-violet (UV) trans-illuminator.

### 2.6 Antimicrobial susceptibility test

The antibiotic susceptibility test of *C. sakazakii* that isolated from food samples was determined using Kirby-Bauer disc diffusion method (Bauer *et al.*, 1966) on Mueller Hinton Agar (HiMedia, India). All the isolates were tested for susceptibility against 10 antibiotics including ampicillin (10 µg), cephalothin (30 µg), chloramphenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), streptomycin (10 µg),

tetracycline (30 µg), erythromycin (15 µg) and norfloxacin (10 µg). The diameters of the inhibition zone for each antibiotic was measured and analyzed according to the clinical and laboratory standards institute (CLSI, 2015).

## 3. Results

In the current research, a total of 130 PIF samples were screened for the occurrence of *C. sakazakii*. Only four samples (3.1%) have shown to be contaminated with this pathogen. The other species belong to Cronobacter family such as *C. malonicus*, *C. Dublinensis* and others were also identified in the PIF samples. There were different kinds of spices and herbs included in the present study. In a total of 55 spice and herb samples, only 43 (78.2%) of them showed positive for *C. sakazakii* by both culture types used in this study (Table 2). The colonies of *C. sakazakii* gave bluish green in color on ESIA and yellowish pigmentation on TSA (Figure 1 A and B). A total of 50 BFP samples, from the cubes or powder bouillons were examined for the presence of *C. sakazakii*, 12 of them (24%) showed positive for *C. sakazakii*. The suspected samples were confirmed as positive by traditional culturing methods and biochemical as well as the molecular techniques.

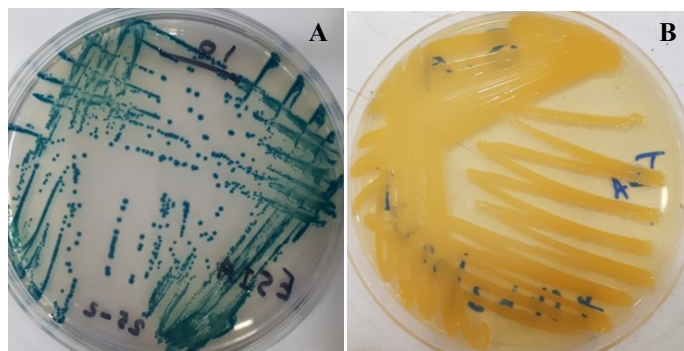


Figure 1. *Cronobacter sakazakii* colonies on ESIA agar showed blue-green colonies (A), TSA shows yellow pigmentation (B)

### 3.1 Confirmation by VITEK-2 System

This system showed the confidence as excellent identification of selected organism of probability 99% as *C. sakazakii* (Figure 2) from most selected colonies. All other isolates from the suspected colonies revealed identification value between 93-98% of the pathogen.

Table 2. The results of examination of food products with dried herbal and spices for the presence of *Cronobacter sakazakii*

Samples	Number of analysed samples	Positive		Negative	
		n	%	n	%
Powdered infant formula (PIF)	130	4	3.1	126	96.9
Herbs and spices	55	43	78.2	12	21.8
Bouillon Flavoured Powder (BFP)	50	12	24	38	48
Total	235	59	25.1	176	74.9

Selected Organism	99% Probability	<i>Cronobacter sakazakii</i>	Excellent identification
	Bionumber: 0625730053322010		Confidence:

Figure 2. VITEK-2 system photomicrograph shows the excellent identification of 99% probability of *Cronobacter sakazakii* in different food items

### 3.2 Confirmation by PCR

Interestingly, all the suspected colonies were identified as positive for *C. sakazakii* by PCR (Figure 3). Moreover, these colonies were also examined by using VITEK2 system and they showed more than 95% as *C. sakazakii*.

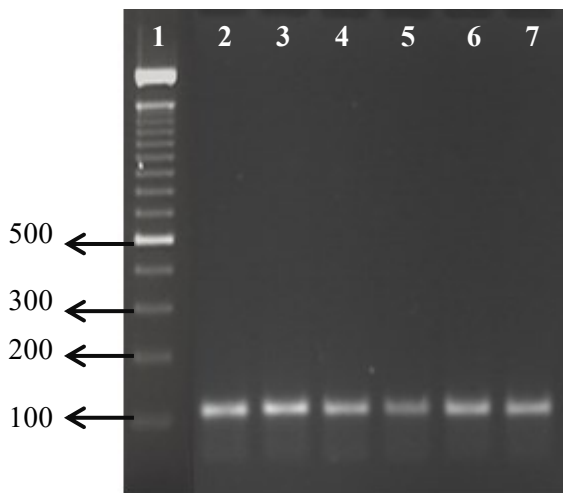


Figure 3. PCR confirmation of *C. sakazakii* isolates, agarose gel electrophoresis of PCR amplified products from suspected *C. sakazakii* colonies ~115bp using 1.5% agarose gel at 100V for 40 min. Lane (1): 100 bp DNA ladder, Lane (2-7): PCR products of PCR product size suspected as *C. sakazakii* gene

### 3.3 Antibiotic resistance test

According to antibiotic resistance profiling, most of the tested isolates were shown to be resistant to the tested antibiotics. However, this bacterium was quite susceptible to ampicillin (83.5%), tetracycline (68.7%), gentamycin (79.8%) and chloramphenicol (82.8%).

Table 3. Antibiotic susceptibility of *Cronobacter* spp. of food isolates

Antibiotics	Concentration µg/disc	Isolates %		
		S	R	I
Ampicillin	10	84	17	-
Chloramphenicol	30	83	17	-
Cephalothin	30	16	66	18
Erythromycin	15	24	46	31
Gentamicin	10	80	17	2.9
Kanamycin	30	15	63	22
Nalidixic acid	30	15	19	67
Streptomycin	10	21	50	28
Tetracycline	30	69	25	-
Norfloxacin	10	20	43	37

S: sensitive, R: resistance, I: intermediate.

*sakazakii* found to be high to mild resistance to the rest of the examined antibacterial (Table 3).

## 4. Discussion

*C. sakazakii* is responsible for severe and deadly infection caused by consumption of contaminated food, even though the lower dose of this bacterium could be life-threatening especially in neonates (Bar-Oz *et al.*, 2001). Additionally, *C. sakazakii* isolates might exist in a various kind of food and environmental samples (Lee *et al.*, 2012). There have been several discovered traditional methods described for the identification of *C. sakazakii* (Shukla *et al.*, 2016). It is well known that the use of conventional chromogenic agar media accompanied with confirmatory based PCR test was confirmed to be rapid and useful for screening and isolation of *C. sakazakii* (Chen *et al.*, 2010; Miranda *et al.*, 2017). Therefore, the combination of both chromogenic agar and molecular methods were used in this study to improve the detection efficiency.

Since Iraq is relatively a consumer country and most of its commodities come from abroad, special attention and microbiological quality should be considered related to food safety. According to our knowledge, there is no data available for the existence of this bacterium from food products in our area. Therefore, the current study was aimed to isolate and identify *C. sakazakii* and assess its antibiotic-resistant profile.

PIF is the most potential source contaminated by *C. sakazakii* (Iversen and Forsythe, 2004). A total of 130 PIF samples, only 3.1% showed positive by traditional culturing methods and by conventional PCR techniques. The occurrence of *C. sakazakii* in his sample type was relatively low compared to other food products examined in this study. This result was different from previous studies that carried out in Iraq. Two different studies were conducted on imported PIF obtaining from local Iraqi markets, one of them showed a high rate of contamination (61.8%) while other was unable to identify this pathogen in this kind of food (Al.Mossawi and Al.joubori, 2015; Jebur and Abood, 2018).

Moreover, the incidence of *Cronobacter* spp. in PIF showed variance according to geographical areas. For instance, a research from China obtained approximately 2282 PIF and follow-up milk samples and from this only 1.1% of the examined samples were positive for *Cronobacter* spp. (Pei *et al.*, 2016). Another previous study performed in the same country which collected about 400 PIF samples and revealed that the positive rate was 11.5% (Pan *et al.*, 2014). A further experiment performed in the UK on a total of 82 PIF samples only two of them showed positive for *C. sakazakii* (Iversen

and Forsythe, 2004). The differences in contamination rates between our study and the previous one could be due to the fact that the quality of the imported food to our region is not good enough to be complying with international standards.

The findings of the current study showed the highest incidence rate (78.2%) of *C. sakazakii* in herbs and spices compared with other food products included in the study. This rate was higher than that of a previous study which only 15% of spice and herb samples containing *Cronobacter* spp. (Ueda, 2017). The results of the present study was on the contrary with those found in a study that analysed 22 samples of spices and only one sample showed positive for this bacterium (Li et al., 2014). On the other hand, a study by Jaradat et al. (2009), has found the highest percentage of *Cronobacter* spp. in spices and herbs compared to other tested food products but there were no positive results in powdered milk (Jaradat et al., 2009). Another experiment showed that half of the examined spice and herb samples were positive for *C. sakazakii* (Belal et al., 2013). The high contamination rate of this bacterium in spices and herbs samples found in this study could be due to that the bacterium has the ability to survive in extremely dry conditions and high temperatures (Breeuwer et al., 2003; Stojanović et al., 2011; Hochel et al., 2012).

BFP cubes or powders are made from dehydrated meat or vegetables and containing of some nutrients like sodium, potassium, carbohydrates (fat) and some vitamins. This kind of food could be used to flavour other dishes like soups, gravies and even add in dry foods such as cooked rice (Morretti et al., 2018). Since it is highly consumed in our country, it should be checked and evaluated for their microbiological quality.

To our knowledge, the BFP has not been evaluated for its contamination rate by microorganisms. This is considered as a part of the objective of the current study for determining the contamination rate in this particular food. Hence, the current study has revealed a relatively high incidence rate of this bacterium in BFP samples. Interestingly, from the total examined of BFP samples, twelve (24%) showed positive for *C. sakazakii*. This lower microbiological quality of the BFP is an indication that BFP was probably contaminated during or after the manufacturing process. Some researchers have proven that *C. sakazakii* is responsible for meningitis in endangered people like neonates and elderly (Bar-Oz et al., 2001); therefore, microbiological quality of BFP should not be underestimated.

Several previous researchers have studied and confirmed that the *Cronobacter* spp., including *C. sakazakii*, can be easily eradicated by antibacterial

administration (Pérez et al., 2007; Fei et al., 2017). However, there are some factors such as long-term application, quantity and underestimated or sub-lethal dose might lead to the development of antibiotic resistance. Another reason behind the development of antibiotic-resistant is the acquisition of antibiotic-resistant genes (ARGs) from the surrounding environment or due to the mutational changes in these genes (Langdon et al., 2016; Kardos, 2017). The current study showed that this bacterium was highly sensitive to only four of the examined antibiotics namely ampicillin, tetracycline, gentamycin and chloramphenicol (Table 3). A previous study worked on 19 strains of *Cronobacter* spp. and revealed that this pathogen is highly resistant to ampicillin which is totally opposite with the data from this study (Fei et al., 2017). However, the findings of the current study are agreed to other previous studies (Molloy et al., 2009; Parra-Flores et al., 2018).

## 5. Conclusion

The findings of the current study revealed that the contamination rate of *C. sakazakii* in food products is high enough to take actions. The microbiological significance evaluation of BFP is not less than PIF samples because of its high incidence rate of *C. sakazakii*. Several methods are needed for isolation, identification and confirmation of this pathogen as none of the traditional culturing, biochemical or molecular techniques are reliable because false negatives or false positives results are inevitable. The evaluation of antibacterial profiling for microorganisms is important to individualise therapy strategies for those who are infected particularly endangered people like neonates and the elderly. If this happened, safety strategy complementation would be easier and control of the disease would be manageable. Consequently, risk assessments of food products, contamination levels and prevent transmission of this bacterium could be evaluated and brought about safer food for communities.

## Conflict of interest

The authors declare no conflict of interest.

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