

Investigation of *cpa.* and *zpx.* Genes in *Cronobacter sakazakii* Isolation from Clinical Specimens in Thi-Qar Province

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Abstract

Cronobacter sakazakii is a member of Enterobacteriaceae family and it is a food-born pathogenic bacteria which can cause several diseases for human and animals. The present study focused on the isolation of this species from clinical specimens from different sexes and ages in addition to hospital environments specimens and then investigation of *cpa.* and *zpx.* genes. The specimens have been taken from group of hospitals in Al-Nasiriyah city (center of Thi-Qar province- south of Iraq). The identification was done by both phenotypical methods and confirmed by API. 20 E. system. Then investigation of *cpa.* and *zpx.* genes by conventional PCR.

Out of 400 specimens (100 from each Burn humans, stool of patients with diarrhea, urine with UTI. in addition to hospital environments specimens) there were 16 (4 %) of specimens gave positive for *C. sakazakii* included : 4 from 100 burns specimens (4%), 6 from 100 stool specimens (6%), 0 from 100 urine (0%) and 6 from 100 hospital environments specimens (6%). The 16 isolates have been tested for presence of *cpa.* and *zpx.* genes by PCR., a number of 13 (81.25%) and 16 (100%) gave positive for these genes respectively. This species was exist in the clinical specimens and can cause diarrhea and burn infection in the area of study with ratio equal to what obtain by other researchers. Additionally this species considered hazardous because of having the *cpa.* and *zpx.* genes.

Key words: *Cronobacter sakazakii*, *cpa.*, *zpx.*

Introduction

Cronobacter sakazakii is one member of Enterobacteriaceae family which characterized by *gram-negative, rod-shaped*, facultative anaerobic, motile with a peritrichous flagella.⁽¹⁾ Firstly, it was identified and named as “yellow pigmented *Enterobacter cloacae*” by Urmenyi and white-Franklin (1961).⁽²⁾ The Japanese bacteriologist “Riichi Sakazakii” reclassified it (in 1980) as new species -*Enterobacter sakazakii*- based on genotype and phenotype classification.⁽³⁾ In 2007 it has been re-ranged in new genus called *Cronobacter* based on revised taxonomy.⁽⁴⁾ From 2008-2012, the genus *Cronobacter* subjected to more revisions which now consists of 7 species: *C. sakazakii*, *C. dublinensis*, *C. malonaticus*, *C. muytjensii*, *C. condimenti*, *C. turicensis*, and *C. universalis*⁽⁵⁾.

This organism is lactose fermenter bacteria with pink-mucoid colonies on MacConkey agar. It can be

identified with a typical non-diffusible yellow pigment colonies on Tryptic Soy Agar (TSA.) at 25°C and can also be grow on Eosin Methylene Blue (EMB.) and deoxycholate agar.⁽⁶⁾

C. sakazakii can be isolate from different sources such as: environments (e.g., domestic environments and manufacturing plants), clinical sources (e.g., cerebrospinal fluid, blood, and sputum), food (e.g., cheese, meat, and vegetables), and animals (e.g., rats and flies).

C. sakazakii is emerging foodborne pathogen which has been classified as sixth most common cause of nosocomial infections and antibiotic resistant strains.⁽⁷⁾ International Commission of Microbiological Specifications for Foods,⁽⁶⁾ considered *Cronobacter* as pathogenic organisms threatening human live and causing serious diseases,⁽⁶⁾ as well as World Health Organization

(WHO) recognized all *Cronobacter* species a pathogenic microorganisms.⁽¹⁾ The mortality rate with *Cronobacter* infections ranged between 40– 80%. This species is life-threatening for all human age groups (premature neonates, infants and immunocompromised adults) which can cause septicemia, meningitis and necrotizing enterocolitis. Urinary tract infection and diarrhea have also been recorded in addition to neurological sequelae. Patrick *et al.*, (2014).⁽⁸⁾ mentioned that the majority of *Cronobacter* infections are in the adult population, especially those suffering from serious underlying disease or malignancy.

C. sakazakii has a group of virulence factors, but these factors remain poorly studied.⁽⁹⁾ Recent studies by improved DNA-based techniques have identified many virulence factors in *C. sakazakii* such as seven O-serogroups and eleven proteolytic enzymes. Among the virulence-related proteins, outer membrane proteins (*ompA* and *ompX*) are involved in the colonization of the gastrointestinal tract and may have roles in helping the organism penetrate the blood–brain barrier.⁽⁹⁾ *Cronobacter* plasminogen activator (encoded by *cpa*. gene) is an outer membrane protein provides resistance against the bactericidal activity of serum, activates plasminogen, and inactivates alpha2- antiplasmin. Other virulence factor is Zinc-metalloprotease (encoded by *zpx*. gene) which causes cell deformation and cells rounding.

Genes in the area of current study (Thi-Qar province – south of Iraq), though there was a study by.⁽¹⁰⁾ which studied isolation of *C. sakazakii* from different sources and identified by phenotypic methods. Therefore, this work aimed to investigate the incidence of *C. sakazakii* in clinical and hospital environments specimens and detection of *cpa*. and *zpx*. genes.

Methodology

Four hundred specimens (included 100 specimens from each: Burns, Stool with diarrhea, Urine with UTI. and Hospital environment) were collected from Thi-Qar province (south of Iraq) hospitals: Bint-El-Huda Hospital, Al Hussein Educational Hospital and Mohammed Al Moussawi Hospital. The specimens were tacked by media swabs and transported to laboratories of college of sciences- Thi-Qar university in a cool box within 1-2 hours.

Pre-enrichment procedure

The methods of culture and isolation of *C. sakazakii* were done, the specimens were pre-enriched by mixing 25 ml/g sample with Buffered peptone water (BPW). Mixed well and incubated at 37°C for 24.0±2.0 h.

Isolation of *C. sakazakii*

After pre-enriched, all specimens were cultured on MacCokey agar (*C. sakazakii* lactose fermenter) for 37°C for 24 h. Then the pink – mucoid (lactose fermenter colonies) were sub-cultured on Tryptic Soy Agar (TSA) and incubated at 25 °C for 48-72 h. (colonies appeared as yellow pigmented) and chromogenic selective media- *Enterobacter sakazakii* Isolation Agar (ESIA.) was used, *C. sakazakii* appear as green- bright blue colonies.

Identification of *C. sakazakii*

The isolates were tested by microscopic examination under light microscope (which appear gram negative bacilli). Then tested by conventional biochemical methods as Oxidase test, Catalase test, Triple Sugar Iron (TSI. – A/A with gas production and no H₂S), Urease test, IMViC. test (Indole, Methyl-red, Voges-Proskauer and Citrate), Motility test and DNase test. Then this identification was confirmed by API. 20 E. system.

*Note: all used media were Oxiod / England except ESIA. Media.

Detection of *cpa*. and *zpx*. genes

After plasmid extraction, Cpa. and BAM. primers were used to detection of *cpa*. and *zpx*. genes respectively by PCR. technique for all identified isolates. The used primers for this purpose.

The primers were processed according to manufacturing instructions. The total volumes of reagents and premix tubes contents which used in the PCR amplification were 20 µl, included: 1 µl from each forward and reverse primers, 3 µl from DNA. template and 10 µl free water.

The results of virulence genes amplification have been visualized by 1% agarose gel electrophoresis (0.25 gm of Agarose powder in 25 ml of (1x) TBE. buffer) at 70 V. for 45 min, a ladder with 100-2000 bp. Has been used with genes electrophoresis.

Results

Total isolates of *C. sakazakii* were 16 out of 400 (4%) specimens, included : 4/100 burns specimens

(4%), 6/100 stool specimens (6%), 0/100 urine (0%) and 6/100 hospital environments specimens (6%), as shown in table (1):

Table (1): The number and percentages of *C. sakazakii* isolates

Clinical specimens	Number of specimens	Number of <i>C. sakazakii</i> isolates (%)	Percentage / 16
Burn	100	4 (4 %)	25 %
Hospitals environment	100	6 (6 %)	37.5%
Stool	100	6 (6 %)	37.5%
Urine	100	0 (0 %)	0 %
Total	400	16 (4 %)	100%

Phenotypic identification

-Morphological examination:

Isolates of *C. sakazakii* were lactose fermenter, pink color with thick center and mucoid colonies after 24 hrs. of incubation at 37 °C. with MacConkey agar. The isolates showed slight yellow–golden yellow pigmented colonies on TSA. agar and showed two types of colonies with ESIA. agar: typical colonies (small green to blue-green colonies) and non-typical colonies (slightly transparent and violet colonies), as shown in figure (1), The *C. sakazakii* isolates appeared as gram-negative rod shape under light microscope.

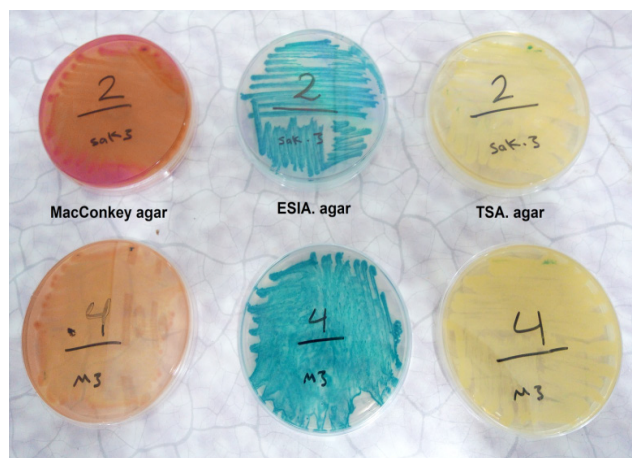


Figure (1): Morphological examination of *C. sakazakii*

Biochemical identification:

Biochemically, *C. sakazakii* isolates have been

identified by a list of testes (TSI., Catalase, Oxidase, Urease, IMViC. & Motility) and confirmed by API. 20E. system. The results of these tests showed in table (2) below:

Table (2): *C. sakazakii* biochemical tests and its results

Tests		Results
TSI.		A/A, G.*
Catalase		+
Oxidase		-
Urease		-
IMViC.	Indole	V*
	Methyl-red	-
	Voges- Proskaur	+
	Citrate	+
Motility		+
DNase		-

*A/A : acidic/acidic, G: gas (O₂). *V: variable.

C. sakazakii is considered a pathogenic bacterium and is necessary to focus on this bacterial species because of their own high virulence factors which constitute a threat to humans and animals. Its diagnosis show high similarity with other Enterobacteriaceae member's (as *Citrobacter* spp. and *Enterobacter* spp.) which lead to difficulty in diagnosis and do not rely on one type of methods; therefore the in present study the identification was done by using of morphological, biochemical and API.20 E..

The current study is the first in the study area, as well as the global studies with such design is very few where most local and global studies have been focused in isolation of the *C. sakazakii* from milk samples and food. There were some local studies isolated these organism from some clinical specimens. The results of current study in comparison with these locally studies indicated to the variation in isolation rates from one region to region and form time to time, where lower than results of study by⁽¹¹⁾ who obtained 16% of *C. sakazakii* isolates from clinical specimens from ALImamain Al-Kadhumain Medical City, Baghdad/Iraq, lower than results of study by⁽¹²⁾ who detected 9% of *C. sakazakii* isolates from clinical specimens from various hospitals of Najaf/Iraq.

The current results slightly higher than⁽¹⁰⁾ in Thi-qar province/Iraq who obtained one isolate (2%) of *C. sakazakii* from 50 specimens from patients stool with diarrhea, other study by⁽¹³⁾ in Thi-qar province reported 2% of *C. sakazakii* from stool of patients and a study by⁽¹⁴⁾ in Al-basrah city obtained 0 % from patients stool. In mexico: 0.33% in Hospitalized Nursing Infants Associated with the Consumption of Powdered Infant Formula (Two Cases of Hemorrhagic Diarrhea Caused by *Cronobacter* .⁽¹⁵⁾

Investigation of *cpa.* and *zpx.* genes :

Figure (2) show the extracted plasmid After DNA, the results of virulence genes investigation by PCR. technique revealed that 13 isolates (81.25) were positive for *cpa.* gene and all 16 isolates (100%) were positive for *zpx.* gene, as shown in figures (3 and 4).

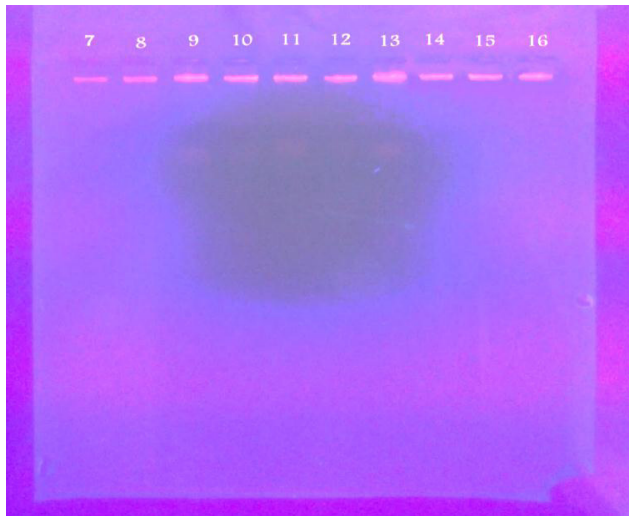


Figure (2): Extracted plasmids DNA.



Figure (3): *cpa.* gene amplification from plasmid

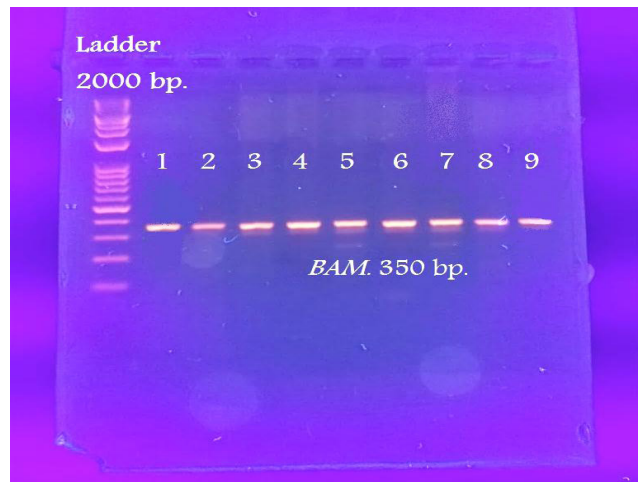


Figure (4): *Zpx.* gene amplification from plasmid

The present study focused on the some important virulence genes which carried on the bacterial plasmids, these are: *cpa.* gene (*Cronobacter* plasminogen activator) and *zpx.* Gene (zinc containing metalloprotease).

A *cpa.* gene encodes by *cpaF* and *cpaR* primer was obtained in 13 (81.25%) of present isolates. This results agree with most previous studies by⁽¹⁶⁾ who recorded 60 % of a novel isolate of *C. sakazakii* have *cpa.* gene using an in vitro blood brain barrier model,⁽¹⁷⁾ 100% of *C. sakazakii* from clinical sample have *cpa.* gene,⁽¹⁸⁾ revealed 100% of *C. sakazakii* from some food and dust samples have *cpa.* gene and Almajed 2015, obtained (85%) of *cpa.* gene in *C. sakazakii* from clinical samples. In contrast disagree with⁽¹⁹⁾ who recorded *cpa.* gene (28%; 12/43) in *C. sakazakii* from different sources.

The pESA3 encodes for the outer membrane protease *Cpa.* (which is responsible for serum resistance), this protease resistance against complement-dependent killing of serum by cleaving complement components C3. and C4b. This process leads to converting plasminogen

to plasmin, which then activate of other proteolytic enzymes, including matrix metalloproteinases, resulting in degradation of the tight junctions of microvascular endothelial cells. The bacteria will be able to migrate to peripheral tissue and invade the CNS.⁽²⁰⁾ Cpa. help bacteria to avoid serum-mediated killing together with the persistence within macrophages and other phagocytic cells gives the organism an advantage so it can survive in the blood stream, multiply, cause bacteraemia, and potentially reach vital organs such as the brain and the meninges. Degrading the components of the tight junctions resulting in the migration of the bacterial cells leading to more damage to the infected organ or tissue.

Zpx. gene encodes by BAM122 and BAM123 primer was obtained in 16 (100%) of isolates. This result agree with⁽¹⁸⁾ who revealed (100%) of *C. sakazakii* from some food and dust samples have *Zpx*. gene.

Zpx. is one of the important virulence factors that cause and associated with many diseases, In 2007, a study performed by.⁽²¹⁾ discovered a cell-bound zinc-containing metallo-protease (*Zpx*.) and showed that it is interact with the protease substrate azocasein, which led to rounding of Chinese hamster ovary (CHO.) cells in tissue culture.⁽²¹⁾ In addition to its a key role in allowing *Cronobacter* to disrupt the cellular junctions of the GI tract or CNS. leading to either NEC. or meningitis, respectively. In 2008, Hunter *et al.* performed a study using an infant rat model that implicated over-expression of interleukin-6 (IL-6) and the *Zpx* protein to playing a key role in the pathogenicity of *Cronobacter* spp. associated NEC infection.⁽²²⁾

Conclusion

C. sakazakii do exist and can be isolated from clinical sources (from sites of infections such as stool of patients with diarrhea and burns) as well as from the environment of hospitals not just from their natural sources. Difficulty of its diagnosis by the conventional methods due to some variations in the results of some recognized biochemical tests as indole according to its strains. There is no limited age for the patients infected with these organism contrary to most references which confirmed that “these bacteria affect newborns and infants only”. *C. sakazakii* in the present study is human-health thereat due to presence of virulence genes.

Source of Funding- Self

Ethical Clearance – Not required

Conflict of interest: **None**

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