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Phenotypic and Molecular Identification of *Cronobacter sakazakii* Isolated from Clinical Specimens in Thi-Qar Province

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Abstract:

***Cronobacter sakazakii* 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 (Burn humans, stool of patients with diarrhea, urine with UTI. in addition to hospital environments specimens) from different sexes and ages. 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 and molecular methods with using of culture (on enriched, selective and differential media), microscopic examination, biochemical tests, API. 20 E. system and PCR. for amplification of ITS. region (the space between 16s and 23s ribosomal DNA.).Out of 400 specimens (100 from each source) there were 16 (4 %) of specimens give positive for *C. sakazakii* which included: 4/100 burns specimens (4%), 6/100 stool specimens (6%), 0/100 urine (0%) and 6/100 hospital environments specimens (6%). The 16 isolates have been identified by PCR. for the presence of ITS. region with using of SG. Primer (282 bp.), all the 16 (100%) isolates were positive for this gene. 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. There are variation in results of biochemical tests according to its strains which leads to difficulty with diagnosis, furthermore the diagnosis with ITS. showed the same results of phenotypical methods used in the present study.**

Key words: *Cronobacter sakazakii*, ITS. region & Phenotypic and Molecular Identification

Introduction

Cronobacter sakazakii is one of the Enterobacteriaceae family member which firstly identified by Urmenyi and Franklin (1961), and known as yellow pigmented *Enterobacter cloacae*, then in 1980 based on genotype and phenotype classification the Japanese bacteriologist " Riichi Sakazakii" reclassified it as new species and named as *Enterobacter sakazakii* (Farmer *et al*., 1980). From 1980 to 2007 this organism still within the genus *Enterobacter*, in 2007 based on recently revised taxonomy ranged in new genus called *Cronobacter* (Iversen *etal*., 2007; Joseph *etal*.,2011). In 2008 and 2012, this classification subjected to more revisions lead to joining of new species, Currently *Cronobacter* contains seven species including: *C. sakazakii*, *C. dublinensis*, *C. malonaticus*, *C. muytjensii*, *C. condimenti*, *C. turicensis*, and *C. universalis* (Joseph *et al.* 2012; Brady *et al*. 2013).

Cronobacter was recognized worldwide as an emerging foodborne pathogen which considered the sixth most common cause of nosocomial infection and antibiotic resistant strains are observed with increasing frequency (Peters *et al.*, 2000). In 2002, the International Commission of Microbiological Specifications for Foods (ICMSF) classified *Cronobacter* as pathogenic organisms, danger for their lives and causing serious long-term diseases (ICMSF, 2002). World Health Organization (WHO) recognized all *Cronobacter* species as pathogenic microorganisms for human (FAO/WHO, 2008). Among premature neonates, infants and immunocompromised adults these infections can be life-threatening which can cause septicemia, meningitis and necrotizing enterocolitis (Block *et al.*, 2002 ; Bowen and Braden, 2006). Urinary tract infection and diarrhea have also been observed in addition to neurological sequelae which can be permanent and the mortality rate ranged between 40–80% (Lai, 2001). *Cronobacter* infections may occur in the adult population, especially those suffering from serious underlying disease or malignancy (Patrick *et al.*, 2014). This species can isolated from all age groups, with a higher frequency in children less than 14 years of age (Gosney *et al.*, 2006 ; Liu *et al.*, 2013). *Cronobacter* spp. can isolate from clinical

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samples such as throat swabs, urinary tract, tracheal aspirates, bronchoalveolar lavage, cannulae and sputum. Patrick *et al*. (2014) reported that there are large rates of infections with this organism among adult population, especially in urinary tract infections from an earlier period (2003– 2009) (UTIs) (Patrick *et al.*, 2014).

C. sakazakii is bacillus facultative anaerobic bacteria, gram-negative, motile with a peritrichous flagella (ICMSF, 2002; FAO/WHO, 2008). This organism could grow on agar plate with two forms of colonies: glossy or matte. It can grow on MacConkey agar with "pink" colonies because it is able to ferment lactose (Lai, 2001). This bacterium can also grow on Eosin Methylene Blue (EMB) and deoxycholate agar with "Blue-Green" colonies, because it can produce α-glucosidase enzyme, additionally it can be identified with a typical non-diffusible yellow pigment colonies on Tryptic Soy Agar (TSA.) at 25°C (ICMSF, 2002; Lai, 2001). This bacterium, biochemically is positive for both citrate test, Voges-Proskauer test, catalase test and DNase test, but is negative for both oxidase test, Methyl red test, urease test and nitrate reduction with variable results for indole test according to its strains (ICMSF, 2002).

The intergenic spacer (ITS.) region is a gene located between 16S-23S ribosomal RNA. ITS. detected by specific primer called SG. Primer. ITS. gene is more efficient than 16S in diagnosis of *C. sakazakii* , where most previous studies have diagnosed isolates by this region which not diagnosed by the 16s. (Jaradat *et al*., 2009 ; Belal *et al*., 2013; Fakruddin *et al*., 2014; AL-Lami *et al.*, 2015).

There was no previous study about the molecular identification of *C. sakazakii* isolated from clinical specimens in area of study (Thi-Qar province – south of Iraq), though there was a study by Al-Safi (2015) but the *C. sakazakii* isolated from general sources and identified by phenotypic methods only. Therefore, this work aimed to identification of *C. sakazakii* by using conventional microbiological and molecular methods.

Materials and methods

A total of 400 specimens (100 specimens from each of Burns specimens, Hospital specimens, Stool of patients with diarrhea and Urine specimens from UTI. patients) have been collected from Thi-Qar hospitals (Bint El-Huda Hospital, Al Hussein Educational Hospital and Mohammed Al Moussawi Hospital) at the period from April-2018 to January-2019. All specimens were taken before antibiotic therapy started and transferred to bacteria laboratory in college of science - Thi-Qar university by carry-blair media in a cool box within 1-2 hours.

Isolation of *C. sakazakii*

According to Cappuccino and Welsh (2018), the specimens were pre-enriched in the buffered peptone water for 18 h. at 37 °C and then cultured on MacCokey agar (*C. sakazakii* lactose fermenter) at 37 °C for 24 h. The pink – mucoid - lactose fermenter colonies were sub-cultured on Trypticase Soy Agar (TSA) at 25 °C for 48-72 h. (colonies appeared as yellow pigmented) and chromogenic selective media- *Enterobacter sakazakii* Isolation Agar (ESIA.- Conda / Spain) at 37 °C for 24 h., *C. sakazakii* appear as green- bright blue colonies.

Identification of *C. sakazakii*

According to Mahon and Lehman (2015), the selective isolates were tested by microscopic examination under light microscope (which appear gram negative bacilli). Then tested by conventional biochemical methods as Oxidase test, Ctalase 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.

Molecular identification

The DNA extraction was performed by a special extraction kit from Geneaid™ DNA Isolation (Kit / Taiwan) and followed the kit instructions. Then it was visualized by 0.8 agarose gel electrophoresis at 70 V. for 45 min.

Molecular identification was done by Polymerase Chain Reaction (PCR.) for investigation of the ITS. gene (the region between 16s and 23s using for the detection of *C. sakazakii*) which detected by specific primer SG. (SGF. & SGR.) which designated by Integrated DNA Technologies company (IDT. / USA.) as described by Liu *et al*., (2006), as shown in table (1) bellow.

The PCR. mixture was done by using of AccuPower® PCR PreMix (Bioneer/ Korea), as shown in table (2). Thermal cycling parameters and program shown in table (3).

The agarose gel was 1% which has been prepared by dissolving 0.25 gm of Agarose powder in 25 ml of (1x) TBE. Buffer. At the loading stage each well of the gel were loaded with 5 µl of PCR. products except first well which was loaded with 4 µl of DNA ladder 100bp-2000bp (Bioneer/Korea). Electrophoresis products were subjected to 70 volt for 45 min. then the Agarose removed from the electrophoresis tank and visualized with UV. transilluminator and photographed.

Table (1) SG. primer description

primer	Target region	Sequence	Size
SG.-F.	ITS.-G	GGGTTGTCTGCGAAAGCGAA	282
SG.-R.	ITS.-G and ITS-IA	GTCTTCGTGCTGCGAGTTTG	

* SG.: specific gen. * F.: forward * R.: reverse.

Table (2) PCR. mixture preparation

Reagent	Volume
Primer forward	1 µl
Primer reverse	1 µl
DNA template	2 µl
Free water	16 µl
Total Volume	20 µl

Table 3: Thermal cycling parameters and program

Steps	Temperature	Time	N. of cycles
Initial denaturation	94 °C.	2 min.	1
Denaturation	94 °C.	30 sec.	35
Annealing	68 °C.	60 sec.	
Extension	72 °C.	90 sec.	
Finial Extension	72 °C.	5 min.	1

The Results

Out of 400 specimens, *C. sakazakii* has been identified in 16 isolates (4%) distributed on : 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%), as shown in table (4):

Table (4): The number and percentages of *C. sakazakii* isolates

Clinical specimens	Number of specimens	Number of *C. sakazakii* 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%

Bacterial isolates appeared as lactose fermenting, pink color with thick center and mucoid colonies after 24hrs. of incubation at 37 °C. with MacConkey agar, with TSA. agar showed slight yellow – golden yellow pigmented colonies and with ESIA. agar showed typical colonies (small green to blue-green colonies) and non-typical colonies (slightly transparent and violet colonies), as shown in figure (1), then isolates have been examined under the microscope, *C. sakazakii* appeared as gram-negative rod shape.

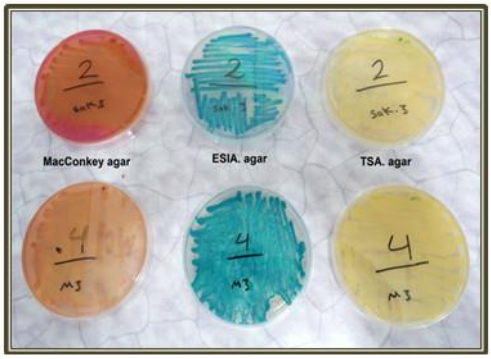

Figure (1): Morphological examination of *C. sakazakii*

B- Biochemical identification:

C. sakazakii isolates were identified biochemically by a list of testes (TSI., Catalase, Oxidase, Urease, IMViC. & Motility) and confirmed by API. 20E. system. These isolates gave a group of results which showed in table (5) below:

Table (5): *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 (O_2). *V: variable.

Molecular identification:

After DNA extraction, all 16 isolates were identified by PCR. technique for the presence of ITS. region, all of 16 isolates (100%) were positive for this gene, as shown in figure (2).

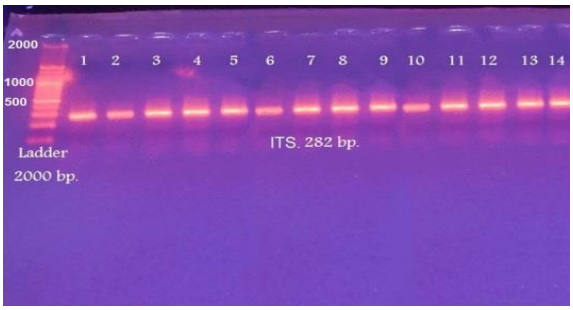

Figure (2): Amplification of ITS. by SG. Primer

1% agarose gel, 70 volt for 45 min.

Discussion

C. sakazakii is one of Enterobacteriacae family which considered a pathogenic or opportunistic pathogenic bacteria 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 Enterobacteriacae member's (as *Citrobacter* spp. and *Enterobacter* spp.) which lead to difficulty in diagnosis and do present study the identification was done by using of morphological, biochemical, API.20 E. and molecular methods.

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 Hassan and Naser (2018) who obtained 16% of *C. sakazakii* isolates from clinical specimens from ALImamain Al-Kadhumain Medical City, Baghdad/Iraq, lower than results of study by Hussain and Al-ammar (2013) who detected 9% of *C. sakazakii* isolates from clinical specimens from various hospitals of Najaf/Iraq.

The current results slightly higher than Al-Safi (2015) in Thi-qar province/Iraq who obtained one isolate (2%) of *C. sakazakii* from 50 specimens from patients stool with diarrhea, other study by Jaber *et al.,* (2015) in Thi-qar province reported 2% of *C. sakazakii* from stool of patients and a study by Radhi, (2016) in Al-basrah city obtained 0 % from patients stool. Flores *et al.,* (2011) in mexico : 0.33% in Hospitalized Nursing Infants Associated with the Consumption of Powdered Infant Formula (Two Cases of Hemorrhagic Diarrhea Caused by *Cronobacter*).

A PCR technique was performed by using primers (SG-F & SG-R) to determine whether a 282 bp., where all diagnosed isolates (16- 100%) were showed positive when assay for ITS. amplification. Many previous studies used ITS. regions for detection of *C. sakazakii* as Rashidat *et al.,* (2013) who detected it in 3 (1.95%) from 154 Powdered Infant Formulae retailed samples in Nigeria, AL-Lami *et al.,* (2015) identified 11 (9.16%) *Cronobacter* spp. from 120 different food samples in Iraq by ITS. Rajani *et al.,* (2016) diagnosed *C. sakazakii* from 93 food and environment samples in India. Other studies also used ITS. for detection of *C. sakazakii* as Jaradat *et al*., (2009) and Belal *et al*., (2013).

ITS. region is more efficient than 16S in diagnosis of *C. sakazakii* as most previous studies have confirmed, where it diagnosed isolates which not diagnosed by the 16s , AL-Lami *et al.,* (2015) showed that 16s identified only 6 isolates while ITS. identified 11 isolates, Fakruddin *et al*., (2014) showed that 16s gene was found in three *C. sakazakii* food isolates, and negative in other three isolates, also Jaradat *et al*., (2009) and Belal *et al*., (2013) proved that. This meaning that it gave diagnostic ratios more than 16S, this may be due to it's a large degree of sequence and length variation at the level of genus and species which make it more stable with detection by PCR. and more stable against to mutations.

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 ".

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