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Isolation and Detection of *Cronobacter sakazakii* from Infant Dried Milk using PCR and RT-PCR techniques

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Abstract: The present study is an attempt for detection of *Cronobacter sakazakii* by conventional PCR methods using species-specific primers and to detect the gene expression of *thermotolerance* gene (Tt gene) as virulence factor. 105 samples of powdered infant formula (PIF) and infant's food were collected from Baghdad Governorate and Mesan Governorate markets from November 2016 to June 2017. The samples were cultured on the selective media including *Enterobacter sakazakii* Isolation Agar(ESIA) and trypton soy agar(TSA). Sixty eight (64.76%) isolates appeared with yellow pigment colonies when cultured on TSA and gave bluish - green colonies on chromogenic media (ESIA). After the growth of bacteria, isolates were identified by microscopic examination and biochemical tests. The identification of *C. sakazakii* confirmed that twenty one isolates identified as *C. sakazakii* and the others identified as *Enterobacter spp*. by using systems API 20E and VITEK2 systems. In addition, a molecular identification has been done by PCR technology utilizing *16S Rrna* gene. The results showed that 42(61.76%) from 68 isolates were identified as *C. sakazakii and* 26(38.24%) were *Enterobacter spp*. The isolated *Enterobacter spp* include *E. cloacae ssp. Cloacae, E. cloacae ssp. dissolvens, E. hormaechei* and *E . kobei, E. ludwigii* .

Keywords: *Thermotolerance* gene ,*Cronobacter sakazakii*, Infant Dried Milk , *PCR. RT-PCR.*

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Introduction:

Cronobacter sakazakii is an emerging opportunistic food borne pathogen, causing often fatal infection of the blood stream and central nervous system to infants with weakened immune systems, particularly premature infants, although the bacteria have caused illnesses in all age groups(1). Neonatal infections have been reported to be one rise via contact with *C.sakazakii* in the birth canal or through post-birth environmental sources. Although most documented cases involve infant reports, infections in adult have been also reported (2).

The primary origins of *Cronobacter spp.* remain unknown. Due to its ubiquitous nature so, these microorganisms can be found in broad range of food including powdered infant formula (PIF), cheese, meat, vegetables, grain, herbs, spices, tomato, water and households. Also *C. sakazakii* is ubiquitously found in air, soil, floor drain and dry product processing environment. It has been isolated from hospitals, clinical materials and cutting fluids(3).

Most cases of *C. sakazakii* come from powdered infant formula contaminated with the bacterium. Powdered infant formula is most likely contaminated after production, since the pasteurization process is normally adequate to kill *Cronobacter sakazakii* bacteria. However, if the powder is

produced using the dry blending process, and not heated so, these bacteria can survive in the formula(4).

*Cronobacter sakazakii*is found to be resistant to multiple antibiotics like vancomycin, penicillin, oxacillin, and lincosamides (5) and required prolonged treatment with broad spectrum antibiotics(6). The International Commission on Microbiological Specifications for Food has ranked *C. sakazakii* as “severe hazard for restricted populations” because of its resistance to certain antibiotics(7).

In Iraq, *C. sakazakii* was isolated from powdered infant formula milk and gave a positive result to paw Oedema test for detection of heat-labile enterotoxin of *C. sakazakii*(8). Both; the source of *Cronobacter* and vehicle of transmission are not always clear.aim of the study Identification of *C. sakazakii* from different types of food samples by conventional biochemical tests, Api 20E, VITEK 2 system and simplex PCR technique.

Determination the gene expression of thermotolerance gene for *C. sakazakii* using Real Time polymerase chain reaction (RT-PCR).

Material and methods:

Milk samples:

One hundred and five samples of powder infant formula milk (PIFM) were collected from the local markets of Baghdad city and Mesan city which belongs to trade markes (Nido, Dialac, Celia, Novolac, Sunny baby, Biomil, Geguiz, Dovolac , Naktalia, Lery, Pediashour.

Isolation of *cronobacter sakazakii* :

0.5 g.of powder milk samples were added to 4.5 ml of buffered peptone water and then incubated at 37ºC for 18-24 hrs.50 µl aliquot was then incubated in 5 ml modified luryl sulphate treptose broth \Vancomycin at 44ºC for 24hrs. 10µl from of culture (mlst/vancomycin) on ESIA at 44ºC for 24hrs.up to five presumptive *C.sakazakii* colonies that exhibited during culture on ESIA were selected for culture on TSA at 25ºC for 48-72 hrs(9).

Identification of *C. Sakazakii*

Growth on tryptic soy agar:

Tryptic soy agar (TSA) plates were streaked with a pure colony of testing bacteria, and incubated at 25ºC for 72hrs *Cronobacter sakazakii* produces yellow pigmented colonies(10) .

Growth on *Enterobacter sakazakii* Isolation Agar:

Enterobacter sakazakii Isolation Agar (ESIA) plates were streaked with a pure colony of testing bacteria and incubated at 44ºC for 42hrs *Cronobacter sakazakii* colonies give bluish green color(10).

VITEK-2 System:

The bacterial isolates were inoculated onto MacConky agar plates and then incubated overnight at 37°C. A single colony was then taken and suspended into solution. The turbidity of the bacterial suspension was adjusted with VITEK Densichek (bioMerieux) to match the McFarland 0.5 standard in 0.45% sodium chloride. Then the VITEK 2 ID-GN (Gram Negative) card and the bacterial suspension tubes were manually loaded in to the VITEK-2 system. Following steps on the software were done according to

the manufacturers instructions (Bio Merieux, France) (11).

Reading of the API 20E strip:

After 24 hours of incubation, the strip was read by referring to the interpretation table. For the Voges-Proskauer test (VP test), 1 drop of VP1 and VP2 reagents were added and the result were appears after 10 min and recorded. For the Tryptophan deaminase (TDA test), 1 drop of TDA reagent was added the result was appears immediately and recorded. For the indole test, 1 drop of indole reagent was added and the result was recorded after 2 minutes. Results were compared and recorded on the report sheet.

Molecular diagnostics:

DNA Extraction:

Several genomic DNA extracts were used.

The company has its own Genomic DNA mini kit. Thailand (Genaid) for bacterial samples Isolated. The initiator used in polymerization reaction.

PCR Use the prefix shown in the table (1) (12).

Table (1): Name, sequences and the expected product size of primers used to detect *16SrRNA* gene of *C.sakazakii*.

Gene name	Name of primer	Sequence of primer	Expected product size (bp)
16S rRNA	F	5'-TTTATCAACTTGTCAC ACCAG A-3'	~282
	R	5'-ATCCCGCCTTACCACT ACCG-3'	

Table (2): Reaction mixture of PCR working solution for detect the 16S rRNA genes of *C.sakazakii* isolates.

Component	Concentration	Amount (µl)
GoTaq Green Master Mix	2X	12.5
F primer	10 µM/ µl	2
R primer	10 µM/ µl	2
Nuclease free water	-	4.5
DNA sample	-	4
Total volume	-	25

Table (3): Condition of PCR reaction for 16S rRNA genes detection of *C. sakazakii*

No.	Step	Temp. (°C)	Time	No. of Cycles
1	Initial denaturation	96	5 min.	1
2	Denaturation	94	30 sec.	35
3	Annealing	55	30 sec.	
4	Extension	72	30 sec.	
5	Final extension	72	15 min.	1

Electrical Relocation:

Perform the electrical transfer of the extracted DNA and to separate its molecules of different size as stated in (13).

Extraction of RNA:

The RNA was extracted from the *C.sakazakii* isolates using a commercial TRIzol extraction kit (Invitrogen, USA) according to the manufacturer's instructions.

RT-PCR primers:

Primers selection:

To select RT-PCR primers that can detect the gene expression of *thermotolerance* gene for the *C.sakazakii* isolates, the species-specific primers; and the *rpoD*-F and *rpoD*-R primers of the housekeeping *rpoD* gene for the *C.sakazakii* were also used (Table 4), then the general properties of these primers were checked by using Oligocalc Oligonucleotide Porperties Calculator program.

Table (4): Primers pairs for RT-PCR.

Name of primer	Sequence of primer	Expected Product size(bp)
thr–F	5'-GAT CGG ATT GGA GAA CCA GA –3'	282
thr–R	5'- ATT TCT GAC CGC ATT TCC AT -3'	282
rpod–F	5'-TCCGCAGGTAGCACTCA GTTC-3'	
rpod–R	5'-AAGCCGGATTCATAGGTGGTG-3'	

RT-PCR reaction component:

The RT-PCR reaction component to detect the gene expression of the *thr* gene was prepared; thus, the following mixtures was adopted *C. sakazakii* (Table 5).

Table (5): RT-PCR mixture for detect the *thr* gene expression of *C. sakazakii* isolates.

Component	Concentration	Amount (µl)
Master Mix	-	5
thr-F primer	10 µM/ µl	0.5
thr-R primer	10 µM/ µl	0.5
Nuclease free water	-	1.75
RNA sample	-	2
Rt mix	-	0.25
Total	-	10

The RT-PCR program for detect the gene expression of the *thr* gene was set; thus, the following RT-PCR program was adopted for *C.sakazakii* as in table (6).

Table (6): RT-PCR program for detectthe*thr* gene expression of *C.sakazakii* isolates.

Step	Temperature	Time	Cycles
Reverse Transcription	37 °C	15 minutes	
RT inactivation/Hot -start activation	95 °C	10 minutes	
Step qPCR			
a. Denaturation	95 °C	20 seconds	40
b. Annealing	60 °C	20 seconds	
c. Extention	72 °C	30 seconds	
Dissociation	72 °C	30 seconds	
	95 °C	30 seconds	

Results and Discussion:

The culture on tryptic soy agar:

All positive samples that are grown on in *Enterobacteriaceae* Enrichment broth (EE broth) were culture on tryptic soy agar (TSA). The result showed that 68 bacterial isolates were grown on TSA, only 42 isolates (61.76 %) gave typical colonies of *(Cronobacter)* that

appeared as yellow-pigmented colonies on TSA (Figure 1), these includes 41(96.62%) powdered infant milk, and only one (2.38%) from dried infant food.

Figure (1): Colonies of *C.sakazakii* culture on TSA at 37ºC for 72hrs

The Culture of *C.Sakazakii* on *Cronobacter sakazakii* Isolation Agar:

A total of 68 bacterial isolates grown on TSA were further purified by the ABC streaking method and culturing on *Enterobacter sakazakii* isolation agar (ESIA). The results showed that 42 bacterial isolates (61.76%) from 68 total isolates gave Blue / green pigmented colonies figure (2), also this represents (40%) from 105 different samples collection.

Studies have demonstrated that 100% of *Enterobacter sakazakii*are positive for α-glucosidase while 100% of other Enterobacter species are negative for this enzyme (14). On the basis of these observations, the chromogenic substrate 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside (X-α-glucoside) has been proposed to differentiate *Enterobacter sakazakii* from other members of the Enterobacteriaceae family (15). The enzyme α-glucosidasehydrolyzes the X-α .

Figure (2): Colonies C.*sakazakii* culture on ESIA at 37ºC for72hrs

Genomic DNA Extraction:

Genomic DNA was extracted from *C.sakazakii* isolates. Genomic DNA was extracted from 42 isolates that was confirmed as bands by gel electrophoresis. The results of DNA extraction showed sharp bands of

chromosomal DNA (Figure 3). DNA concentration and purity were measured by Nanodrop spectrophotometer, all the isolates had DNA concentration between 50-100 ng/µl and purity of the DNA were = 1.8 ~ .

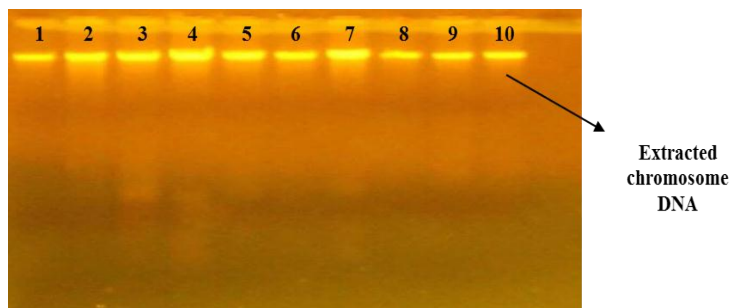

Figure (3): Agarosegel electrophoresis of extracted genomic DNA of *C. sakazakii* isolates using 1% agarose gel at 7volt/cm for 20 minutes. Lane 1-10: Extracted genomic DNA.

Molecular identification of *Cronoobacter Sakazakii* by detection of *16S rRNA* gene:

The PCR results (Figure 4) showed that *16S rRNA* gene (282bp) exists in all 42 *C.sakazakii* which identified by the previous identification methods All isolates taken in the study were thus *C.sakazakii* results PCR consistent with the results of previous tests, and this confirmed the accuracy of tests and methods used for identification this genus.

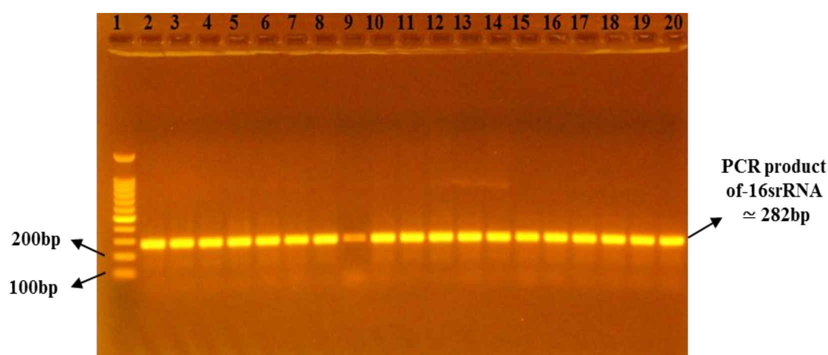

Figure (4): Agarose gel electrophoresis of PCR amplified products for 16S rRNA gene of *C.sakazakii*~282 isolates using 1.5% agarose gel at 7volt /cm for 1 hour. Lane (1): 100 bp DNA ladder, Lane (2-19): PCR products of 16S rRNA gene .

***Thermotolerance* gene expression of *C. sakazakii* isolates by quantitative real-time PCR**:

The RT- PCR was adopted to detect the gene expression of *thermotolerance* gene for *C.sakazakii* isolates, the samples were analyzed and standardized against the gene expression of housekeeping *rpoD* gene. The relative changes in the mRNA expression levels were determined using comparative threshold cycle (CT) method ($2^{-\Delta\Delta Ct}$) between the *C.sakazakii* isolates that have been grown at two different temperature include 37ºC and 58ºC for 24 hours differentiated by *C. sakazakii* bacteria In dried infant formula.

Thermal Tolerance which arrives. To 58 ° C for 4.0 minutes(16). Amplification and detection of *thr* gene of *C.sakazakii* was carried out using

SYBR Green qRT-PCR method. The positive results showed amplification at CT (threshold cycle) value were in range 8.1 – 30.8 for housekeeping *rpoD* gene and 25.7-29.8-24.99 for *C.sakazakii*, respectively, the melting temperature (Tm) values obtained for isolates were in range 72-79ºC for house keeping *rpoD* gene and *C.sakazakii* were in range of 84.5-86.5ºC As shown in the table (7) .

Normalization of delta delta Ct method (17). Delta deltaCt (ΔΔCt) method is the simplest one, as it is a direct comparison of Ct values between the target gene and the reference gene. Relative quantification involves the choice of a calibrator sample. The calibrator sample can be the untreated sample,optimum temperature 37 ºC, or any sample want to compare the unknown to. Firstly, the ΔCt between the target gene and the reference gene is calculated for each sample (for the unknown samples and also for the calibrator sample) as shown in the following equation:

ΔCt = Ct target gene - Ct reference gene.

Then the difference between the ΔCt of the unknown and the ΔCt of the calibrator is calculated, giving the ΔΔCt value, as the following equation:

ΔΔCt = (Ct target - Ct reference) sample - (Ct target-Ct reference) calibrator.

The normalized target amount in the sample is then equal to $2^{-\Delta\Delta Ct}$and this value can be used to compare gene expression levels of *Thr* gene in the samples of *C.sakazakii,* the samples were analyzed and standardized against the gene expression of housekeeping *rpoD* gene. The relative changes in mRNA expression levels were determined using comparative threshold cycle (CT) method ($2^{-\Delta\Delta Ct}$) between the *C.sakazakii* isolates that grown at two different temperatures including 37ºC and 58ºC for 24 hours.

Calculation of the data means that Ct for reach difference in folding the expression between genes used various equation such as:

Ratio= $2^{\Delta Ct}$ = R=$2^{[CT\ target-\ CT\ control]}$

Ratio of folding differences = $2^{-\Delta\Delta Ct} = 2^{-[\Delta Ct\ target - \Delta Ct\ control]}$

Table (7): Fold of *thermotolerance* gene expression of *C.sakazakii* primer depending on ΔΔCt method

Temperature of incubation	Sample	Ct of reference *rpoD*	Ct of target *Thr* gene (p.a)	ΔCt	ΔΔCt	Relative quantification (Folds)*	Mean of *thr* gene folds
58	1	16.6	25.7	9.1	-15.9	60.337	457.932
	2	25.6	26.3	0.7	-16.8	110.598	
	3	30.8	29.5	-1.3	-19.6	780.834	
	4	29.3	28.2	-1.1	-19.7	849.959	
37	1	8.1	29.8	21.7	0.0	1.0	1
	2	10.4	27.1	17.4	0.0	1.0	
	3	9.4	27.6	18.2	0.0	1.0	
	4	8.1	26.7	18.6	0.0	1.0	

p. a.: primer of*C.sakazakii
***Fold = $2^{-\Delta\Delta Ct}$**

The results of table (7) showed that there are significant differences between the *C.sakazakii* that incubated at different incubation temperatures, The mean value of thermotolerance gene folding was 450.432 fold in group of isolates that incubate at 58°Cof the incubation comparing with strains

incubated at 37°C, while the minimum mean of folds was at 37°C of the incubation which reached to 1 , in all the samples, the raise of mean of folds for*C. sakazakii* iat 58°C of incubation could be due to the ability of this bacteria to grow better at high temperature, also can be grow but low when the incubation temperature decreased. Expression of the *thermotolerance* gene was not equal between two groups samples incubated at different temperature (58°C and 37°C).This is important in reflecting the original mRNAs present in the samples. It is evident from these results that the temperature is associated with the highest copy number of mRNAs reflecting its higher expression.

Conclusions:

The following conclusions are drawn:

The current study has shown that *Cronobacter sakazakii* is an opportunistic food borne pathogen isolated from powdered infant formula and infants milk found in local markets from some global origins . *Cronobacter sakazakii* has the ability to grow at different temperatures ranging 58°C.Molecular- techniques based (PCR) are more sensitive, more specific and more rapid for identification of *Cronobacter spp*. compared to biochemical identification kits. The mean value of thermotolerant gene folding was 450.432 fold in group of isolates that incubate at 58°Cof the incubation comparing with strains incubated at 37°C, while the minimum mean of folds was at 37°C of the incubation which reached to 1, in all the samples, the raise of mean of folds for *C. sakazakii* iat 58°C of incubation could be due to the ability of this bacteria to grow better at high temperature, also can be grow but low when the incubation temperature decreased. Expression of the thermotolerance gene was not equal between two groups samples incubated at different temperature (58°C and 37°C).This is important in reflecting the original mRNAs present in the samples. It is evident from these results that the temperature is associated with the highest copy number of mRNAs reflecting its higher expression.

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