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Research article

Isolation and identification of *Cronobacter* spp. obtained from different food samples in Iraq

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ABSTRACT

The study aims to isolate and identify *Cronobacter spp* **isolated** from different food in Iraq. The identification of *Cronobacter* was depending on biochemical profile, VITEK2 system and polymerase chain reaction (PCR) technique. The antibiotic susceptibility for six recovered isolates was done also. 120 food samples were tested. *Cronobacter spp* was identified in 4 (3.33%) food samples by VITEK2 system. The internal transcribed spacer (ITS) regions were detected in 11 (9.16%) isolates of *Cronobacter spp.* Six isolates of *Cronobacter spp* were tested for antibiotic susceptibility (8 antibiotics) using disc diffusion method. The results showed that 5 isolates (83.3%) were resistant to ampicillin and cephalothin, and 2 isolates (33.3%) were resistant to kanamycin, streptomycin and tetracycline. While, 5 isolates (83.3%) were sensitive to chloramphenicol, 4 isolates (66.7%) were sensitive to tetracycline, and 3 isolates (50%) were sensitive to gentamicin and nalidixic acid.

Keywords: Antibiotic susceptibility, Biochemical test, Cronobacter spp, food samples, ITS regions, PCR.

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INTRODUCTION

Cronobacter spp. are Gram negative rod-shaped bacteria, belong to the family enterobacteriaceae. They are motile by peritrichous flagella, non-spore forming, and facultative anaerobes. *Cronobacter* spp. (formerly *enterobacter sakazakii*) are opportunistic food borne pathogens associated with lethal infections in neonates and infants, particularly those that they are premature or immune-compromised [1]. *Cronobacter* spp. have been implicated as causative agents of meningitis [2], septicemia and necrotizing enterocolitis [3] in infants and neonates. *E. sakazakii* previously referred to as (yellow pigmented *E. cloacae*) was defined as a new species in 1980 [4]. The difference between *Cronobacter* spp. and *E. cloacae* was based on biochemical reactions, the ability of *Cronobacter* spp. colonies to produce yellow pigments, and by DNA–DNA hybridization. *Cronobacter* spp. were classified in to six species according to Iversen *et al*. (2005), *C. sakazakii*; *C. malonaticus*; *C. muytjensii*; *C. dublinensis*; *C. turicensis* and *Cronobacter* [5]. Although *Cronobacter* spp. are linked to infant form-

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ula, they have also been isolated from clinical specimens, environmental samples and from a wide range of foods including cheese, meat, milk, vegetables, grains, spices and herbs [6,7]. Traditional culture methods for identifying *Cronobacter* spp. were laborious and time-consuming and required steps of enrichment and biological tests that requires 6 to 7 days. PCR assay is considered rapid, sensitive, specific and more reliable method for early detection of pathogenic bacteria, including *Cronobacter* spp. [8]. *Cronobacter* spp. constantly have been reported as remarkably resistant to osmotic stress and dryness and moderately thermo tolerant as some encapsulated *Cronobacter* spp. were still recoverable from desiccated infant formula after storage for up to 2.5 years [9]. The composition of dry foods and infant formula combined with their low (water activity) ***aw*** significantly affected the survival of *Cronobacter* spp. in these foods [10]. Infant formula has been identified as one route of transmission for infection in infants. However, the primary reservoirs for subsequent food contamination of foods with *Cronobacter* spp. remain undefined due to the ubiqu-itous nature of these bacteria. Recently, infections in adults have been reported, especially amongst the elderly and immunocompromised patients [11,12] with the indication that *Cronobacter* spp. infect both infants and vulnerable adults it is important that a wide variety of foods be evaluated. Al-joubori (2014) Isolated and Identified *C. sakazakii* and *Enterobacter* spp. from clinical samples of meningitis, necrotizing enterocolitis and infant food in Baghdad. In this study the author could not isolate *Cronobacter* spp. from infant food but she isolated it from clinical samples, and adopted only the biochemical methods in identification process [13].

The study aims to isolate, detect and identify *Cronobacter* spp. from different Iraqi food samples depending on biochemical tests and PCR assay and determine the antibiotic susceptibility patterns of six recovered isolates.

MATERIALS AND METHODS

Samples Collection

Hundred and twenty different food samples were collected during 2014 from different local markets across Iraqi capital (Baghdad). The samples composed of six categories 20 samples of each, powdered milk, spices and dried food, cereals and flour, meats and meat products, dairy products and vegetables. This work was done according to approval of academic committee of the department of biology, College of science, university of Baghdad, Baghdad, Iraq.

Isolation, detection and identification of Cronobacter spp

Food and drug administration (FDA) method with modification was used for isolation of *Cronobacter spp*. from different food samples. Ten grams of each food samples were mixed thoroughly with 90 ml of pre-warmed sterile distilled water at 45ºC, and incubated for 15-20 min in a water bath at same temperature (this is called pre-enrichment step) (in case of meat and vegetable samples, blender was used to obtain homogenized suspension). Ten milliliters of each suspension were resuspended in 90 ml of enterobacteriaceae enrichment broth (EE, HiMedia, India) and incubated overnight at 37ºC (This for enrichment step). A loopful of each culture broth was streaked onto violet red bile glucose agar (VRBGA, Oxoid, England) and another 0.1 ml of the same culture was spread on VRBGA plates and incubated for 24 h at 37ºC (This is called the selection step). The purple or pink colonies were picked and streaked on tryptic soy agar (TSA, Oxoid, England) and incubated for 48 h at 37ºC to look for the characteristic yellow pigmented colonies of *Cronobacter spp*. The yellowish colonies on TSA were picked and subjected to further characterization using chromogenic medium, biochemical tests (VITEK2 system) and PCR analysis.

Chromogenic medium, enterobacter sakazakii isolation agar (ESIA, HiMedia, India) was used to detect the α-glucosidase activity. ESIA agar containing 5- bromo – 4 Chloro – 3- indolyl – α – D – glucopyranoside (X- α Glc) , which upon hydrolysis of this substrate, typical colonies for *Cronobacter spp*. give blue / green colonies typical for *Cronobacter spp.*, after incubation at 37 ºC for 24 h.

Biochemical Identification by VITEK2 system -

Presumptive identification of yellow colonies was performed by VITEK2 system (BioMerieux, France) biochemical profiling tests according to manufacturer's instructions.

Molecular Confirmation using PCR

DNA was isolated using wizard Genomic DNA purification kit (Promega, USA), and PCR was performed with master cycler gradient (Eppendorf, Germany). Two pairs of *Cronobater spp*. specific primers were used in this study. Primer SG-F/SG-R and SI-F/SI-R, originally described by Liu *et al*. (2006), they designated for the sequences between 16S rDNA and 23S rDNA (internal transcribed spacer, ITS). PCR mixture was set up in a total volume of 25 µl including 12.5 µl of PCR green master mix (Promega, USA), 1 µl of each pairs of primers (10 Picomole/µl) and 1 µl of template DNA (100 ng/µl) have been used. The rest volume was completed with sterile deionized distilled water (Bioneer, Korea). Negative control was contained all material except template DNA, so instead of that distilled water was added. PCR conditions were hot start for 5 min at 95°C followed by 35 cycles of 1 min at 95°C; 57°C for 1 min; 72°C for 1 min and a final extension of 5 min at 72°C. PCR products were then analyzed using 2% (w/v) agarose gel electrophoresis in 1X Tris-Borate-EDTA (TBE) buffer at a constant voltage of 90 V for 1 h, then visualized under UV light to confirm the presence of the amplified DNA [14].

Antibiotic Susceptibility Test

Antibiotic susceptibility test was performed using the modified Kirby-Bauer disc diffusion method. Six *C. sakazakii* food isolates were tested for susceptibility to ampicillin (10 µg), cephalothin (30 µg), chloramphenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), streptomycin (10 µg) and tetracycline (30 µg). Antibiotic discs were obtained from (Bioanalyse, Turkey).

RESULTS AND DISCUSSION

In this study *Cronobacter spp.* was isolated from different food samples. It is difficult to confirm the identity of isolates using only one method or one set of *Cronobacter spp.* specific PCR primers [15] because of phenotypic differences among *Cronobacter spp*., thus many techniques were used in this study such as chromogenic, biochemical and molecular techniques for isolation, detection and identification of *Cronobacter spp.* from different food samples.

One hundred and twenty food samples were tested for the presence of *Cronobacter spp.*, 11 samples were found positive which formed yellow colonies on TSA agar after 48 h of incubation at 37ºC and this confirmed by chromogenic media (ESIA), they were positive for α –glucosidase activity on X α –Glc that gave blue /green colonies after incubation at 37ºC for 24 h.

All positive isolates (11 isolates) were identified using biochemical profiling tests (VITEK2 system). however, when the isolates subjected to VITEK2 biochemical profiling, only 4 isolates (36.3%) which it is represent (3.33%) of total food samples were identified as *Cronobacter spp.* (table 1), thus VITEK2 biochemical profiling system can be considered as a presumptive identification method for *Cronobacter spp.*

Table 1. Percentage of presence of *Cronobater spp.* in different food samples according to VITEK2 system

Food samples	Number of samples analyzed	No. of *Cronobacter* isolates	% of total samples
Powdered milk	20	0	0
Spices and Dried food	20	1	5
Cereals and Flour	20	0	0
Meats and meat products	20	2	10
Dairy products	20	0	0
Vegetables	20	1	5
Total	120	4	3.33

Generally, phenotype analysis may provide unreliable identification as long as it is based on unstable expression of the markers [16], highlighting the need for other methods of confirmation such as molecular analysis.

PCR was performed by using two pairs of *Cronobater spp.* specific primers as described above and all (11 isolates) samples (9.16%) of total food samples were shown a positive results with a correct-sized amplification product of 282 and 251 bp according to the primers SG and SI, respectively (**fig. 1 and fig. 2**).

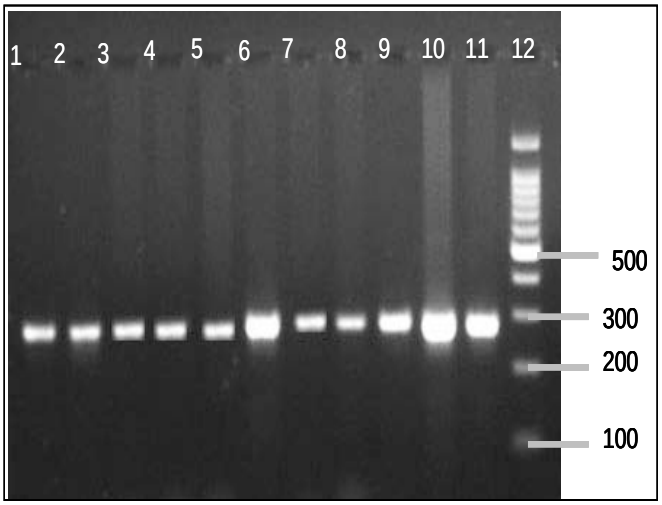

Fig 1. Agarose gel electrophoresis (2%) of amplified ITS region (282bp) of *Cronobacter spp*. Lanes 1-11, *Cronobacter spp*. Lane 12, 100 bp DNA ladder.

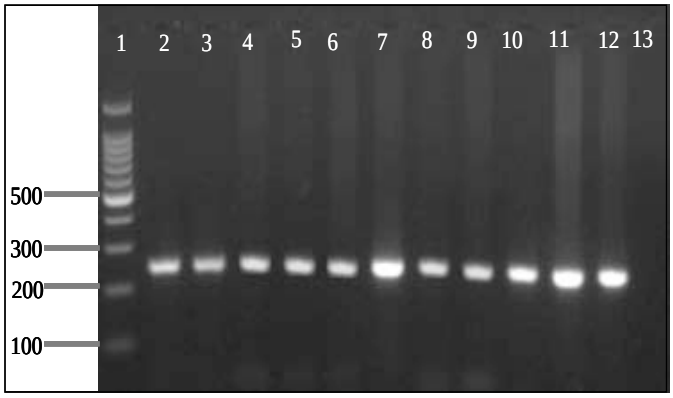

Fig 2. Agarose gel electrophoresis (2%) of amplified ITS region (251bp) of *Cronobacter spp*. Lane1, 100 bp DNA ladder. Lanes 2-12, *Cronobacter spp*. Lane 13, negative control (all PCR mixture except DNA template).

Table 2 shows the confirmation of *Cronobacter spp.* isolates by chromogenic, biochemical testing VITEK2 and two sets of *Cronobacter spp.* specific primers (SG and SI). The antibiotic susceptibility test for 6 *Cronobater spp.* isolates indicated that five isolates (83.3%) were resistant to ampicillin and cephalothin, and two isolates (33.3%) were resistant to kanamycin, streptomycin and tetracycline, whereas five isolates (83.3%) were sensitive to chloramphenicol, four isolates (66.7%) were sensitive to tetracycline and three isolates (50%) were sensitive to gentamicin and nalidixic acid (**table 3**). The

results were interpreted according to guidelines recommended by the clinical laboratory standard institute (CLSI) [17].

Table 2. Confirmed isolates of *Cronobater spp* by chromogenic, biochemical testing VITEK2 and two sets of *Cronobater spp.* specific primers (SG and SI).

		PCR primers			
ID	Source	ESIA	VITEK2	SG	SI
CA1	Corn	+	-	+	+
OA2	Olive	+	+	+	+
TA3	Thyme	+	+	+	+
LA4	Lettuce	+	-	+	+
HA5	Hamburger	+	+	+	+
HA6	Hamburger	+	+	+	+
PMT1	Powdered milk	+	-	+	+
ST2	Spice	+	-	+	+
PMT3	Powdered milk	+	-	+	+
LT4	Lettuce	+	-	+	+
CFT5	Canned fish	+	-	+	+

Eleven strains of *Cronobater spp.* were isolated in this study from different foods. All strains were analyzed using chromogenic, biochemical profiling and molecular techniques. VITEK2 system was not enough technology for identification of the isolates since it detect only 4 isolates out of 11, thus his biochemical profiling system needs other tests such as chromogenic medium ESIA that gave 11 isolates that characteristics for *Cronobacter spp.*, which appeared as blue /green colonies.

Table 3. Antibiotic susceptibility of *Cronobacter spp.* of food isolates. S, sensitive; R, resistance; I, intermediate.

Antibiotics	Concentration µg/disc	Isolates %		
		S	R	I
Ampicillin	10	16.7	83.3	-
Cephalothin	30	16.7	83.3	-
Chloramphenicol	30	83.3	-	16.7
Gentamicin	10	50	-	50
Kanamycin	30	16.7	33.3	50
Nalidixic acid	30	50	-	50
Streptomycin	10	-	33.3	66.7
Tetracycline	30	66.7	33.3	-

Fanjat *et al*. (2007) revealed that 28 isolates of *E. sakazakii* were correctly identified to the species level by VITEK2 compact GN since these finding was fully comparable to the results that obtained through the reference method 16S rRNA sequencing [18]. Jaradat *et al*. (2009) showed that none of the chromogenic media was 100% reliable for confirming the identity of *Cronobacter spp.* isolates because these media gave false positive and false negative results [7]. PCR technique is rapid, sensitive, specific and more reliable method for early detection of *Cronobacter spp.*, two pairs of primers SG and SI have been used in this study, which detected 11 *Cronobacter* strains. Jaradat *et al*. (2009) and Belal *et al*. (20013) were used the above primers for identification of *Cronobacter spp.* [7,19]. In this study, *Cronobacter spp.* isolated from a wide range of local food samples, while Al-joubori [13] reported that local powdered infant formula (PIF) was not contain *C. sakazakii* because the study was based on biochemical identification only.

Antibiotic resistance in Gram negative bacteria has been reported previously and it is believed that the mechanism of resistance lies within the expression of plasmid and /or transposon. Drudy *et al*. (2006) showed that *E. sakazakii* like other enterobacteriaceae members had acquired resistance by inactivating broad spectrum beta lactam antibiotics due to the production of beta lactamases [20]. Other mechanisms include: decreased cell permeability, active efflux, modification of drug receptor site, synthesis of resistant methabolic pathway, and acquisition of plasmids and transposons [21], these could be the reasons for strong resistance against ampicillin and cephalothin (83.3%). Streptomycin is an aminoglycoside antibiotic, the mode of action is to bind to the 30S ribosomal subunit of bacteria, a possible reason behind this observed resistance could be an alteration to the binding site protein of the 30S subunit. Resistance patterns observed in *Cronobacter spp.* food isolates may indicate increasing a public health risks. The present study confirmed that the multiple methods of detection of *Cronobacter spp* in food is the best.

Conflict of interest

The authors declare that they have no conflict of interests.

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