Detection of *Cronobacter sakazakii (Enterobacter sakazakii*) in powdered food infants (PIF) and raw milk in Iraq

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Received 11, March, 2014
Accepted 4, May, 2014

Abstract:

This study was conducted to detect *C.sakazakii* PIF and raw milk. Two hundred samples of PIF were taken from the infected hospital infants who used this type of milk and from the local markets in addition to 16 sample of raw milk were collected. The study is the first to report the isolation of *C. sakazakii* and *Enterobacter* spp. from raw milk in Iraq. The distribution of *C.sakazakii* and *Enterobacter* spp. among the presumptive isolates using Vitek-GN2 system gave 1/16(6.25%) isolates of *C.sakazakii* and 4/16 (25%) isolates of *Enterobacter* spp. *Enterobacter* spp. isolates include (*E.cloacae* ssp. *cloacae and E.cloacae* ssp. *dissolvens*, *E.hormaechei*, and *E.ludwigii*) that isolate from raw milk Differences in between percentages of each isolate persence were non-significant (P<0.05). The results of antibioticsusceptibility were determined using Vitek-2GN system; .*sakazakii* isolates showed 100% resistance to cefazolin and cefoxitin, but were highly sensitive to many antibiotics includes (Imipenem, Meropenem, Amikacin, Gentamicin, Tobramycin, Ciprofloxacin, Levofloxacin, Nitrofurantion, Trimethoprim \sulfamethoxazole, Ampicillin, Ampicillin\ sulbactam, Pipercillin \Tazobactam, Ceftazidime, Ceftriaxone, Cefepime Azetreonam and augmentin). The present study did not determine *C.sakazakii* in all the samples of PIF that is available in the local markets.

Key words: *Cronobacter sakazakii*; powdered infant formula (PIF), milk, *Enterobacter* spp. .

Introduction:

Cronobacter sakazakii (*C.sakazakii*) previously known as *Enterobacter sakazakii (E.sakazakii)* is a motile , Gram-negative , non sporing yellow pigmented rod , which belongs to lethal *Enterobacteriaceae* family ; It is an opportunistic human and food- born pathogen [1,2,3]. The organism can be found in broad range of foods including powdered infant formula (PIF) , cheese, meat, vegetables, grain, herbs , spices , tomato , water and households [2,4,5] . Also *C. sakazakii* is ubiquitously found in air, soil, floor drain and dry product processing environment. Due to its virulence *C. sakazakii* cause life threatening infection such as septicemia , and necrotizing enterocolitis (NEC), bacteremia and meningitis in infants [1,6] .The mortality rates of 33 - 80% were reported among infected patients [7]. NEC is the most common gastrointestinal surgical emergency in neonatal populations, which results in a mortality rate of 40–100% in the most severely affected patients [8].Neonatal infections have been reported to be one rise via contact with *C.sakazakii* in the birth canal or through post-birth environmental sources [9]. The organism was resistant to multiple antibiotics and required prolonged treatment with broad spectrum antibiotics [10,11,12]. In recent years, the International Commission on

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Microbiological Specifications for Foods has ranked *C.sakazakii* a "severe hazard for restricted populations." Because of its resistance to certain antibiotics [13].In Iraq, *C.sakazakii* was isolated (5.71%) from 2 commercial samples of PIF from 35 total samples and this isolate produce heat-labile enterotoxin in mouse [14]. Both the source of *Cronobacter* and vehicle of transmission are not always clear. However, powdered infant formula (PIF) has been epidemiologically linked to the cases of infants infection [15], This study has focused on the surveillance of PIF products for the presence of *Cronobacter* compared to raw milk. The aims of the study are detection of *C.sakazakii* from PIF and raw milk from local Iraqi markets and food sample (PIF) from Iraqi infant's hospital patients.

Materials and Methods:

Samples and Isolation

A total of 216 food samples were collected at the following:-

A. Raw milk (16) samples were purchased from local markets, transported in a cool box and transport to the laboratory and testing it on the same day.

B. One hundred samples of PIF were collected from infected hospital infants patients (meningitis and NEC).

C. One hundred samples of PIF were purchased from the local markets.

The samples were taken after recording the labeling information (name of company, commercial name of product, contents, origin, date of expired and production, batch number); then kept at room temperature and in dry condition during the study.

The samples were tested for the presence of *Cronobacter* spp. as described by modified Chap *et al.*, [16]. Briefly, 25 g. of food samples (PIF, raw milk) were added to 225 ml of peptone water and then incubated at 37°C for 18-24 hrs. A 10 ml aliquot was then incubated in 90 ml Enterbacter enrichment broth (EEb) at 37°C for 18-24 hrs. From each enriched sample, 0.1 ml was streaked or spread onto Hicrome *Enterobacter sakazakii* Agar (HESA) .Up to five presumptive *C. sakazakii* colonies that exhibited during culture on HESA were selected for culturing on Trypton soy agar (TSA) at 25°C for 48-72 hrs. For identification use biochemical test, API-20E and Vitek-2 GN system were used (Biomerieux / France).

Antimicrobial susceptibility test.

All the confirmed isolates were tested to antibiotic resistance using Vitek-2 GN system according to production company in AL.Mahmudia Hospital .The tested antibiotics (n=17) includes (Ampicillin, Ampicillin\sulbactam, Pipercillin \Tazobactam, Cefazolin, Cefoxitin, Imipenem, Meropenem, Amikacin, Gentamicin, Tobramycin, Ciprofloxacin, Ceftazidime, Ceftriaxone, Cefepime, Levofloxacin , Nitrofurantion, and Trimethoprim \sulfamethoxazole).

Results and Discussion:

Table (1-1) shows 5 isolates (2.31%) which appears blue-black (n=2) to blue-gray (n=3) colonies on HESA , raised colonies 1-2 mm diameter , with and without halos after 18-24 hr. at 37°C and microscopic examination appears gram negative, straight rods, appeared as single or double chains and motile were considered presumptive *Cronobacter* spp. or presumptive *C.sakazakii* .

Table 1-1: Percentages of presumptive *Cronobacter* spp. isolates and its characteristics on culture media according to the type of sample

Samples (No.)		Characteristics on HESA (No. of presumptive *Cronobacter* spp. Isolates)	% of total samples	Characteristics on TSA (No. of presumptive *Cronobacter* spp. Isolates)	% of total isolate
Food samples (216)	Raw milk (16)	Blue-black (2) blue-gray (3)	2.31	Yellow pigment (1)	100
	PIF (200)	0	0.00	0	0.00
Total	216	5	2.31	1	100

HESA was developed for isolating presumptive colonies of *C. sakazakii* , this media contains two chromogenic substrates (5-bromo-4-chloro-3-indoxyl-α-D-glucopyranoside and 5-bromo-4-chloro-3-indoxyl-β-D-cellobioside) , three sugars (sorbitol, D-arabitol, and adonitol) , a pH indicator, and inhibitors (bile salts, vancomycin, and cefsulodin), which all contribute to its selectivity and differential properties [17].

Biochemical tests

All the tested isolates (n=5) that gave yellow pigmented on TAS give the same result to the biochemical tests. The urease, oxidase, coagulase, indol and H2S production gives negative result, while catalase, citrate and motality test gives positive result.(Table 1-2) .These results showed conformity with the *Cronobacter spp*. or *C.sakazakii* [3,18,19].

Table 1-2: Biochemical tests of the presumptive *Cronobacter spp.* isolates

Test	Results
Urease , Oxidase , Coagulase , Indol , H2S production	Negative
Catalase , Citrate , Motality	Positive

API 20 E test

The tested isolates (n=5) that gave positive results in the biochemical tests appeared different results in API 20E test. One isolate showed conformity with the *C.sakazakii*, while 4 isolates showed conformity with the *Enterobacter cloacae* .API 20E for *C.sakazakii* revealed that ADH, ODC and CIT were positive, ONPG, H_2S, LDC, URE, TDA and IND were negative. VP and GEL were also positive and all sugars GLU, MAN, RHA, SAC MEL, AMY and ARA were positive except SOR which was negative, While the results of API 20E for *Enterobacter cloacae* revealed that ADH, ODC and CIT were positive, ONPG, H_2S, LDC, URE, TDA and IND were negative. VP and GEL were also positive and all sugars GLU, MAN, RHA, SAC, SOR, MEL, AMY and ARA were positive except INO which was negative.

The API 20E biochemical kit has been reported not to be a reliable tool for the confirmation of the identity of *C.sakazakii* [3,20]. Reported that the misidentification of strains by the API 20E biochemical kit was due to its limited biochemical gallery. The latter kit consisted of a wide variety of fermentable carbon sources and was able to correctly identify strain *E. cloacae* rather than as *C. sakazakii*. The correct identification of *C. sakazakii* based on biochemical profile kits has been reported to be problematic [20,21]. The fact that *C.sakazakii* confirmation should be based on more than one confirmation system. Both the API 20E and Biolog Microlog 34.20 systems should be used for confirmation of *C.sakazakii* isolates [17].

Vitek -2 system

Five presumptive isolates (on HESA) were tested by Vitek GN2 system. One

isolate that showed conformity with the *C.sakazakii* in API 20E test showed excellent identification confidence with the *C.sakazakii* organism in Vitek-GN2 with the identification values or probability 99%, while 4 isolate showed excellent identification confidence with the *Enterobacter* spp. that included *E.cloacae* spp. *dissolvens*, *E.cloacae* spp. *cloacae*, *E.hormaechei* and *E.ludwigii* with identification values (94 –99)% . Kim *et al*. [12] identified and conformed 4 isolates of *C.sakazakii* from clinical samples, three of the Vitek GNI biochemical profiles of isolates identified as *C. sakazakii* had identification values of 96 - 99% and that of one strain was 82%. Oonaka *et al*. [22] identified 52 strains of *Enterobacteriaceae* isolated from PIF using Vitek GN2 compact system (Biomerieux) with the identification values of 80 - 99%.From this, the results indicated that Vitek-2 system has the ability to distinguish between *Enterobacter* spp. (*E.cloacae* ssp. *cloacae and E.cloacae* ssp. *dissolvens*, *Enterobacter* spp, *E.hormaechei*, *E.kobei*, *E.ludwigii*) rather than as *C. sakazakii and E. cloacae* only , while API-20E system does not have the ability to distinguish between *E.cloacae* and other *Enterobacter* spp. Because of the phenotypic differences among *Cronobacter* spp., it has been increasingly difficult to confirm the identity of isolates using only one method or one set of *Cronobacter* spp.-specific PCR primers. [3,23]There are also problems with specificity, especially when discriminating between *Cronobacter* spp. and other *Enterobacter* species [24]. Therefore, a combination of confirmation methods might be necessary to completely eliminate false positives and false negatives. The results of this study on PIF differed from the study of Muytjens *et al.* [25] that examined 141 different powdered formulas from 35 countries and isolated *C. sakazakii* at levels ranging from 0.36 to 66 cfu per 100 g from 20 formula samples from 13 countries. Over a24-month surveillance, three *Cronobacter* strains were isolated from 77 powder infant formulas (3.90%) and no *Cronobacter* was detected in liquid milk. [26]

As shown in Table (1-1) from 5presumptive isolates (on HESA) only 1/16 (6.25) from raw milk. Steigerwalt *et al*.[27] have observed that the presence or absence of yellow pigmentation can distinguish between two strains of *Enterobacteriaceae* family (*Enterobacter cloacae* and *C.sakazakii*) when these strains are cultured on TAS. Although Farmer *et al*. [28] reported that not all strains of *C. sakazakii* are yellow-pigmented.

Eventhough *C. sakazakii* has been determined as a dominant contaminant flora in PIF by several researchers up to now, and in Iraq Shareef *et al*.[14]was isolated *C.sakazakii* 2/35(5.71%) from PIF, one of them from sample opened at experimental time and the second from sample opened before 3 days of experimental time by using Blood agar and MacConkey agar with the traditional biochemical test . In the present study *C.sakazakii* was not determined in all samples (n= 200). Table (1-1) ,This result agrees with that mentioned by Block *et al*.[29], who stated that the organism was not isolated from PIF, but it was recovered from prepared formula and from a kitchen blender. The survey conducted by the South Australian Government did not find any pathogens in the 20 samples tested [30].Also it coincides with what Sani and Yen Yi. [31] who reported that *C. sakazakii* was not detected in any of the PIF tested (30 samples) from 8 manufacturers obtained from hypermarkets and a private hospital in Malaysia. And coincide with Güner *et*

al.[32]who did not found *C. sakazakii* in all samples(132 cartons) of PIF from 3 different brands retailed in Turkey . Joosten and Iversen [33]have detected 1.9% (n=104) *C. sakazakii* positive samples.

While PIF may not be commercially sterile, its production is undertaken using rigorous hygienic precautions coupled with monitoring of the process environment and finished product by the manufacturer. These activities assist in reducing the microbial load of PIF [34]. The Codex Alimentarius Commission criteria require that no *C. sakazakii* should be present in 10 g of PIF after primary packaging until the opening of the can for consumption [35] A coordinated survey for *Cronobacter* and related organisms in PIF formula, follow up formula and infant foods was undertaken by 8 laboratories in 7 countries, *C. sakazakii* was isolated from 3/91(3%) follow up formulas [16].

Table 1-3: Distribution of *Enterobacter* spp among the isolates according to type of samples using Vitek-2 system.

Type of samples (No. of isolates)	*E.cloacae* sp. *Cloacae*	*E.cloacae* ssp. *dissolvens*	*E.hormaechei*	*E. ludwigii*	Chi-square value
Raw milk (4)	1(25)	1(25)	1(25)	1(25)	0.043 NS
Total (4)	1(25)	1(25)	1(25)	1(25)	

NS: Not-significant.

As shown in table 1-3 that indicates the presence of the common isolate *Enterobacter spp.* (*E.cloacae* ssp.*cloacae, E.cloacae* ssp. *dissolvens E.hormaechei, E.ludwigii*) from raw milk. This result agrees with Campos *et al.* [36] who has been shown to be of clinical significance by the report of several outbreaks of sepsis in neonatal intensive care units in Brazil and the USA that causes by *Enterobacter spp.* Differences between percentages of each isolate were non-significant($P<0.05$).Species of the *Enterobacter cloacae* complex are widely encountered in nature, but they can act as pathogens. The biochemical and molecular studies on *E. cloacae* has shown genomic heterogeneity, comprising six species: *Enterobacter cloacae*, *Enterobacter asburiae*, *Enterobacter hormaechei*, *Enterobacter kobei*, *Enterobacter ludwigii* and *Enterobacter nimipressuralis*, *E. cloacae* and *E. hormaechei* are the most frequently isolated in human clinical specimens [37].

Antimicrobial susceptibility test

The results of antibiotic susceptibility have been that done using Vitek-2 system *C.sakazakii* isolate was 100% resistant to cefazolin and cefoxitin.*C.sakazakii* isolates are high sensitive to many antibiotics includes (Imipenem, Meropenem, Amikacin, Gentamicin, Tobramycin, Ciproflo-xacin, Levofloxacin, Nitrofurantion, Trimethoprim \sulfamethoxazole, Ampicillin, Ampicillin \sulbactam, Pipercillin \Tazobactam, Ceftazidime, Ceftriaxone, Cefepime Azetreonam and augmentin). The results in the present study coincide with previous studies. Stock and Wiedeman [12] ; Drudy *et al*. [38] that has found *C.sakazakii* isolates locally shown 100% sensitive to ampicillin and gentamycin or ampicillin and chloramphenicol. Shadlia-Matug *et al*.[39] that have found *C.sakazakii*

resistance to cephalothin and sensitive to gentamicin, but not agreement with them that *C.sakazakii* resistance to ampicillin, penicillin. The present results also coincide with Stock and Wiedemann [12] found isolates were susceptible to ampicillin, compound sulphonamides, furazolidone, gentamicin, spectinomycin and streptomycin. In the present study *C.sakazakii* isolates have shown high sensitive to ampicillin, gentamicin this result coincides with pervious study [40,41] .And with Lai, [7] that found *C.sakazakii* were susceptible to Trimethoprim \sulfamethoxazole. The results of this study to not have agreement with Oonaka *et al*. [22] that found *C. sakazakii* were resistant to ampicillin and lincomycin, but they have agreement with them when found sensitive to gentamicin and cephalosporins. Also not agreement with Pitout *et al*.[42]; Girlich *et al*. [10] that they found *C.sakazakii* resistance to ampicillin .And with Kim *et al*., [11] that tested 113 *C. sakazakii* isolates for their antibiotic resistance and 31.8% were resistant to ampicillin and all of the isolates were resistant to at least one antibiotic. The results of the present study coincide with pervious study by Al-Nabulsi *et al*., [43] illustrated the *C. sakazakii* strains they tested were sensitive to gentamycin, kanamycin, ciprofloxacin and amoxicillin.

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التحري عن جرثومة *Cronobacter sakazakii* (*Entrobacter sakazakii*) في الحليب الباودر للرضع والحليب الخام في العراق

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الخلاصة :

أجريت هذه الدراسة للكشف عن *C.sakazakii* من الحليب الباودر للرضع و الحليب الخام . تم جمع 200 عينة من الحليب الباودر من الرضع المصابين المرضى في المستشفى و الأسواق المحلية بألاضافة الى جمع 16 عينة من الحليب الخام . سجلت هذه الدراسة العزل الاول بكتريا *C.sakazakii* و *Enterobacter spp.* من الحليب الخام في العراق. توزعت *C.sakazakii* و *Enterobacter spp* بين العزلات باستخدام نظام فيتيك GN2 - أعطى 16/1 (6.25 ٪) عزلة من *C.sakazakii* و 16/4 (25 ٪) من عزلات *Enterobacter spp*. *Enterobacter spp* المعزولة تشمل *E.cloacae* ssp. *cloacae and E.cloacae* ssp. *dissolvens*, *E.hormaechei*, and *E.ludwigii*). الاختلافات بين النسب المئوية لكل عزل كانت غير معنوية (P < 0.05).أظهرت نتائج المقاومة للمضادات الحيوية باستخدام نظام فيتيك - GN 2العزلات *C.sakazakii* مقاومة 100% cefazolin and cefoxitin ، وجد ان عزلات *C.sakazakii* عالية الحساسية ل كثير من المضادات الحيوية ويشمل (Imipenem, Meropenem, Amikacin, Gentamicin, Tobramycin, Ciprofloxacin, Levofloxacin, Nitrofurantion, Trimethoprim \sulfamethoxazole, Ampicillin, Ampicillin\sulbactam, Pipercillin \Tazobactam, Ceftazidime, Ceftriaxone, Cefepime Azetreonam and augmentin). لم يتم عزل *C.sakazakii* في جميع عينات الحليب الباودر الخاص بالرضع المتوفر في الأسواق المحلية.