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Prevalence, identification and molecular characterization of *Cronobacter sakazakii* isolated from retail meat products

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A B S T R A C T

Cronobacter sakazakii is an opportunistic foodborne pathogen predominantly in neonates with reported incidences in adults especially immunocompromised and the elderly. The current study was carried out to determine the prevalence of these organisms on meat products sold at Mansoura city, Egypt; besides its ability to compete with *E. coli* O157:H7 and *Salmonella* on their media. Suspected colonies were phenotypically-identified using API 20E system, additional biochemical tests, besides production of yellow-pigmented colonies; and genotypically-identified using species-specific PCR assays to detect the gene responsible for α-glucosidase activity, 16S rRNA gene and internal transcriber spacer sequence between 16S and 23S rRNA. Out of the 93 tested isolates, 14 isolates were confirmed as *C. sakazakii* with contamination rates of 16% (8/50) and 15% (6/40) of ground beef and beef burger samples, respectively and overall contamination rate of 15.6% (14/90) of all tested samples. The isolates were distributed among 7 different biogroups. These results added new epidemiological evidence about the widespread occurrence of these pathogens on tested meat products and such occurrence is an indicator for potential contamination with pathogens and so a health risk for consumers. Furthermore, *C. sakazakii* is an important competitor to *E. coli* O157:H7 and *Salmonella* on their selective media.

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1. Introduction

Cronobacter (C.) sakazakii is an emerging opportunistic pathogen associated mainly with neonatal infections and causing rare but life-threatening meningitis, necrotizing enterocolitis, and septicemia with high fatality rates ranged from 40 to 80% (Lai, 2001; Nazarowec-White & Farber, 1997). Although, the most documented cases were in infants, infections have been also reported in all age groups, especially those with immunocompromising conditions and the elderly (Healy et al., 2010).

Cronobacter, which is rod-shaped, motile and facultatively-anaerobic bacteria of the family Enterobacteriaceae, was firstly identified as a unique species by Farmer, Asbury, Hickman, and Brenner (1980). Originally, the genus *Cronobacter* consists of six species, namely *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dublinensis*, and *C. genomospecies* 1 (Iversen et al., 2008). Since then, all species were classified as pathogens by the WHO (FAO/WHO, 2008). Additionally, *C. condimenti* was identified as the seventh species recently by Joseph et al. (2012), and *C. universalis* replaced *C. genomospecies* 1.

Cronobacter species are ubiquitous organisms and are regularly recovered from the human gastrointestinal tract and thought like as members of the normal fecal flora (Gosney, Martin, Wright, & Gallagher, 2006). The source and vehicle of transmission of *C. sakazakii* is unknown. However, powdered infant formula (PIF) has been implicated as a vehicle for infection in numerous cases (WHO, 2004), these organisms have been isolated from wide assortment of foods and beverages including meat and meat products, milk, cheese, fermented bread, vegetables, grains, herbs and spices (Friedemann, 2007; Gassem, 1999; Iversen & Forsythe, 2003; Kandhai, Reij, Gorris, Guillaume-Gentil, & van Schothorst, 2004; Kimura et al., 1999; Muytjens et al., 1983). Moreover, different environments such as households, livestock facilities, food manufacturing operations, in particular PIF production facilities were also included (Bar-Oz, Preminger, Peleg, Block, & Arad, 2001). Recently, Vineeth et al. (2014) emphasized that these bacteria may

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contaminate the cattle environment and so may contaminate their meat.

Cronobacter sakazakii exhibit many of the special features including unusual resistance to heat, dry and acid stress growth conditions compared to other Enterobacteriaceae species (Nazarowec-White & Farber, 1997), in addition to their abilities to grow on all media used for isolation of enteric organisms such as eosin methylene blue, MacConkey, and deoxycholate agars (Iversen & Forsythe, 2003). To date, the methods used for isolation and identification of *C. sakazakii* rely on detection of α-glucosidase reaction and production of yellow pigment as presumptive differentiating phenotypic characteristics (FDA, 2002). However, several molecular means, including PCR assays that targeted the gene responsible for α-glucosidase activity (gluA), 16S rRNA gene sequencing and internal transcriber spacer (ITS) sequence between 16S and 23S rRNA, have been shown to be more reliable method for detection of *Cronobacter* species (Lehner, Tasara, & Stephan, 2004). It is well-established that unlike all bacterial species of the family Enterobacteriaceae, *Cronobacter* species are positive for α-glucosidase enzyme (Cawthorn, Botha, & Witthuhn, 2008; Muytjens, van der Ros-van de Repe, & van Druten, 1984), which considered the base for development of numerous chromogenic media proposed for isolation and identification of these organisms. Moreover, the 16S rRNA gene revealed that there is a considerable heterogeneity between *C. sakazakii* and other Enterobacteriaceae at the three hypervariable regions V1, V2, and V3 (Hassan et al., 2007). Furthermore, the ITS sequence is one of the most popular targets for bacterial identification due to the variation in its sequences among bacterial species (East, Allaway, & Collins, 1992).

Despite the worldwide studies conducted to determine the prevalence of *C. sakazakii*, the epidemiology of these organisms still incomplete and poorly described. Moreover, there had been few studies on the presence of these bacteria in meat and meat products especially in developing countries as Egypt. Therefore, the objective of this study was to evaluate the prevalence of *C. sakazakii* in meat products sold at Mansoura city, Egypt which may be a severe hazard for specified groups of populations, in addition to their ability to compete with *E. coli* O157:H7 and *Salmonella* during their isolation on specific media.

2. Materials and methods

2.1. Bacterial strains

A total of 93 different bacterial isolates in this study were subjected to both phenotypic and genotypic characterization in order to detect *C. sakazakii* species. The tested isolates were distributed as follow: 48 non sorbitol fermenting (non *E. coli*) colonies picked from sorbitol MacConkey agar (Oxoid CM0813) supplemented with cefixime (final concentration 0.05 μl/ml) and potassium tellurite (final concentration 5 μl/ml) (Oxoid SR0172E) (CT-SMAC) according to the typical method intended for isolation and identification of *E. coli* O157:H7 recommended by the International Organization for Standardization (ISO, 2001), in addition to 45 pink (non *Salmonella*) colonies taken from xylose-lysine-desoxycholate (XLD) agar (Oxoid CM0469) (ISO, 2002, updated in 2007). The isolated strains were taken from randomly selected and microbiologically-tested 90 meat product samples (50 ground beef and 40 beef burger), purchased from different supermarkets distributed at Mansoura city, Egypt; as a part of previous investigations (Mohammed, Sallam, Eldaly, Ahdy, & Tamura, 2014; Sallam, Mohammed, Ahdy, & Tamura, 2013; Sallam, Mohammed, Hassan, & Tamura, 2014).

The microbiological steps besides phenotypic characterization of isolates were conducted in Food Hygiene and Control Department, Faculty of Veterinary Medicine, Mansoura University, Egypt, while the genotypic characterization was performed in the Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Sapporo, Japan.

2.2. Phenotypic identification of isolates

All the collected colonies were individually purified and overnight cultured on tryptone soya agar (TSA) (Oxoid, CM0131) plates then subjected for identification. All isolates were examined for biochemical properties using API 20E system kits (bioMérieux, France), according to the manufacturer's instructions and the API 20E results were interpreted using the API 20E v4.1 computer programs. Additionally biochemical tests, including indole production, oxidase production, Voges-Proskauer, methyl red, malonate utilization, reduction of nitrate to nitrite, acid production from dulcitol, and gas production from D-glucose, were conducted for complete identification of isolates according to the protocols mentioned by Harrigan (1998). Likewise, isolates were tested for motility in test tubes containing semisolid agar medium, wherein pure culture were stab-inoculated, then incubated at 37 °C for 3 days. The presumptive *C. sakazakii* isolates, biochemically-identified, were morphologically-tested for the production of non-water soluble yellow pigment. The purified isolates were individually streaked onto TSA plates and incubated at 25 °C for 48–72 h (FDA, 2002).

The reference strain (*C. sakazakii* RIMD0377001) used as positive control was kindly provided by Prof. Takeshi Honda, Department of Bacterial Infections, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan.

2.3. Genotypic identification of presumptive *C. sakazakii*

Species-specific PCRs were carried out to confirm the phenotypically-identified isolates using four different primer sets specific for *C. sakazakii* (Table 1).

2.3.1. Preparation of bacterial genomic DNA

From individually purified isolates on TSA plates, single colonies were transferred to 5-ml nutrient broth (Oxoid CM0001), and incubated overnight at 35 °C. The DNA was isolated according to the method described by Wang and Levin (2006). Briefly, cells were harvested from 1 ml of culture by centrifugation at 13000 rpm for 2 min then washed twice with distilled water and resuspended in 200 μl of distilled water then boiled in water bath for 10 min to lyse the cells, after that, cell debris was pelleted by centrifugation at 13000 rpm for 2 min and subsequently, the supernatant was transferred into a new microcentrifuge tube and stored at −20 °C for use as template DNA for PCRs intended to characterize the isolates.

Table 1
Primers used in this study.

Primer name	Primer sequence (5′ − 3′)	Target	Amplicon size (bp)	Reference
EsAGf EsAGr	TGAAAGCAATCGACAAGAAG ACTCATTACCCCTCCTGATG	gluA[a]	1680	Lehner et al. (2006).
Es-F Es-R	GAGTTTGATCCTGGCTCAG AAGGAGGTGATCCAGCC	16S rRNA	1533	This study
Saka 1a Saka 2b	ACAGGGAGCAGCTTGCTGC TCCCGCATCTCTGCAGGA	16S rRNA	952	Hassan et al. (2007).
SG-F SG-R	GGGTTGTCTGCGAAAGCGAA GTCTTCGTGCTGCGAGTTTG	ITS[b]	282	Liu et al. (2006).

[a] Gene responsible for α-glucosidase activity.
[b] Internal transcriber spacer (ITS) sequence between 16S and 23S rRNA.

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