

OCCURRENCE OF *CRONOBACTER SPECIES* IN KAREISH AND DOMIATI CHEESES

NAGAH M. SAAD and WALLAA F. AMIN

Dept. of Food Hygiene, Fac. of Vet. Med., Assiut University.

Email: wallaa_800@yahoo.com

ABSTRACT

Received at: 19/6/2014

Accepted: 5/7/2014

A total of 100 samples of Domiati and Kareish cheese were randomly collected and examined for the detection of *Cronobacter species*. Detection was performed using Cronobacter Screening Broth (CSB), Chromogenic Cronobacter Isolation Agar (mDFI) followed by biochemical testing. *Cronobacter species* were isolated from 8% of total samples. *Cronobacter sakazakii* was isolated from both Kareish and Domiati cheeses, while, *Cronobacter malonaticus* and *Cronobacter turicensis* were isolated only from Kareish cheese. The identified isolates of *Cronobacter sakazakii* were further tested by real time PCR. The results indicate that Kareish and Domiati cheeses represent risk for humans and further attention should be paid for manufacturing and handling of such cheeses.

Key words: *Cronobacter species*, Chromogenic Cronobacter Isolation Agar, *Cronobacter sakazakii*, cheese

INTRODUCTION

Cronobacter species are opportunistic Gram-negative pathogens associated with potentially fatal neonatal infections, including meningitis, sepsis and necrotizing enterocolitis (NEC) (Hunter and Bean, 2013). They are considered emerging foodborne pathogens that have drawn attention to the scientific community for the last 50 years. The organism was first characterized in 1929 as yellow pigmented coliform (Farmer *et al.*, 1980).

It was suspected to be the causative agent of septicemia in infants. Also, in the 1960s, it was suspected to be the cause of neonatal meningitis (Urmenyi and Franklin, 1961 & Nazarowec-White and Farber, 1997). By the 1980s, this pathogen was classified as a new species, *Enterobacter sakazakii*, a member of the Enterobacteriaceae family, which was found to cause serious infections as sepsis, necrotic enterocolitis and meningitis in infants and neonates (Farmer *et al.*, 1980).

The bacterium was reclassified into the new genus Cronobacter in 2007 (Iversen *et al.*, 2007). It was validly published in 2008 with 5 species and 3 subspecies (Iversen *et al.*, 2008). Now the genus includes nine species, *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. mytjensii*, *C. dublinois*, *C. universalis* and *C. condimenti* (Joseph *et al.*, 2012).

All *Cronobacter spp.*, except *C. condimenti* have been linked to clinical cases of infection in either adults or infants. The majority of cases occur in adults, most often bacteraemia, in addition to wound

infection, abscesses and ulcers (Healy *et al.*, 2010 and Kucerova *et al.*, 2011).

Due to its ubiquitous nature, the primary reservoir for subsequent contamination of food with cronobacters remained unidentified; however, it can be isolated from a wide variety of foods including milk, cheese and dried foods (Friedemann, 2007).

El-Sharoud *et al.* (2008) isolated the organism from related imitation recombined soft cheese. Moreover, El-Gamal *et al.* (2013) could isolate this pathogen from Domiati cheese.

Owing to the health hazard associated with Cronobacters, this study aimed to search for its occurrence in the most popular soft cheese in Egypt using conventional methods and real time PCR.

MATERIALS and METHODS

Collection of samples:

A total of 100 random samples of Domiati and Kareish cheeses (50 samples each) were collected from different localities in Assiut city then transferred to the laboratory to be examined.

The samples were prepared according to A.P.H.A., 2004.

Isolation of *Cronobacter species* (Iversen *et al.*, 2008)

- Preenrichment of samples

Eleven grams from the prepared cheese sample were transferred to a sterile beaker containing 99 ml of sterile 0.1% peptone water, then were incubated at 37 °C for 18 h.

- Enrichment

0.1 ml of the pre-enriched sample was inoculated into a sterile test tube containing 10 ml Cronobacter Screening Broth (CSB) (Oxoid, CM 1121) and then was incubated at 42 °C for 24 h. Positive result is indicated by color change from purple to yellow.

- Selective plating

A loopful of the incubated broth was then streaked on the surface of Chromogenic Cronobacter Isolation Agar (mDFI) (modified Druggan, Forsythe and Iversen agar) (Oxoid Ltd., Basingstoke, United Kingdom). The plates were incubated at 44 °C for 24 h. Typical colonies appear as blue/green which were transferred to Trypticase Soy Agar (TSA) and incubated at 37 °C for 48 h. Colonies appeared as yellow pigmented.

Identification of the isolated *Cronobacter spp.* was done according to Iversen *et al.*, 2008 by biochemical testing.

- Identification of the isolated *C. sakazakii* using TaqMan real time PCR according to Seo and Brackett, 2005.

DNA extraction:

Strains were cultured on tryptone soy agar (TSA) at 37°C. DNA was extracted from a single colony by using QIAamp DNA mini kit following the manufacturer's instructions.

PCR primers and probe:

Primers and TaqMan probe targeting the *dnaG* gene located internally to the macromolecular synthesis of the (MMS) operon were used to detect *C. sakazakii*.

Primers of MMS operon of Cronobacter:

MMS Forward: gggatattgtccctgaaacag

MMS Reverse: cgagaataagcgcgcatt

Real-time PCR amplification:

Real-time PCR was carried out in an ABI PRISM 7000 sequence real-time PCR system (Applied Biosystems, USA). Thermal cycling conditions were as follows: 50 °C for 2 min., followed by 95 °C for 10 min., 95 °C for 15 sec. (40 or 50 cycles) and 60 °C for 60 sec. The cycle threshold (Ct) was calculated using ABI PRISM 7000 Sequence Detection System Software. Negative values or lack of amplification were considered for Ct values of > 40.

RESULTS

Table 1: Incidence of *Cronobacter isolates* in the examined samples

Samples	No. of examined samples	Positive samples	
		No.	%
Domiaty cheese	50	2	4
Kareish cheese	50	6	12
Total	100	8	8

Table 2: Incidence of different *Cronobacter spp.* in the examined samples

Isolated <i>Cronobacter spp.</i>	Positive samples			
	Domiaty cheese		Kareish cheese	
	No.	%	No.	%
<i>C. sakazakii</i>	2	4	3	6
<i>C. malonaticus</i>	-	-	2	4
<i>C. turicensis</i>	-	-	1	2
Total	2	4	6	12

Table 3: Frequency distribution of different *Cronobacter spp.* in the examined samples using conventional methods

Isolated <i>Cronobacter spp.</i>	Domiati		Kareish	
	No./2	%	No./6	%
<i>C. sakazakii</i>	2	100	3	50
<i>C. malonaticus</i>	-	-	2	33.33
<i>C. turicensis</i>	-	-	1	16.67

Table 4: Frequency percentage of *C. sakazakii* recovered from samples using real time PCR

Samples	No. of tested <i>C. sakazakii</i>	Positive strains by real time PCR	
		No.	%
Domiati	2	-	-
Kareish	3	2	66.67
Total	5	2	40



Photo 1: Results of real time PCR

Each of the amplified strains represented with a curve, curves cutted with a threshold line at a cycle number. When the number of the cycle is low, it indicates that this DNA is the target DNA of *Cronobacter sakazakii*.

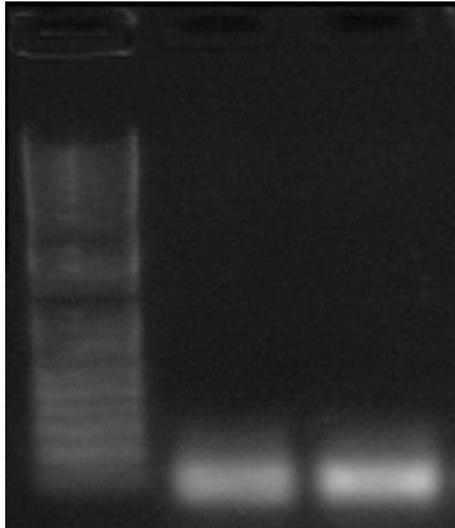


Photo 2: Results of real time PCR by gel electrophoresis using agarose gel stained with ethidium bromide.

DISCUSSION

Cronobacters have been detected in a wide range of environmental sources and from several foods of animal origin. Recent reports have identified *Cronobacter* infections in immunocompromised adults. Therefore, this study focused on the detection of *Cronobacter spp.* in the most widely distributed and consumed types of cheese in Egypt. Results recorded in Table 1 according to the initial screening revealed that 2 (4%) and 6 (12%) of the examined Domiati and Kareish cheese samples were positive for *Cronobacter spp.*, respectively with an overall incidence of 8 (8%) of samples.

Results summarized in Table 2 showed that the isolated strains of *Cronobacter spp.* from Domiati cheese were identified as *C. sakazakii* (4%), while those isolated from Kareish cheese were identified as *C. sakazakii* (6%), *C. malonaticus* (4%) and *C. turicensis* (2%). Table 3 showed the frequency distribution of different *Cronobacter spp.* in the examined samples using the conventional techniques. This association of the pathogen with cheese may be due to that the manufacture of kareish cheese is based on traditional method from raw milk without any regard to the hygienic quality of the product; moreover it is sold in markets without packing. Moreover, Gran *et al.* (2002) concluded that the hygienic aspects of dairy products are linked with transportation, preservation and handling.

Biochemical profiling of *Cronobacter* can be considered a first screening identification method after which the isolates should undergo further diagnostic analysis. So that, the isolates were subjected to Real-time PCR as a second step of identification.

Results recorded in Table 4 indicated that none of the 2 strains isolated from Domiati cheese were confirmed for the identity of *C. sakazakii* by real time PCR. Surprisingly, it is worth mentioning that 2 out of 3 tested strains isolated from kareish cheese were positive for *C. sakazakii* as shown in photos 1 & 2. The higher number of *C. sakazaki* obtained by conventional methods may be due to the close relation between *C. sakazakii* and *C. malonaticus* which cannot be differentiated using the conventional methods and easily differentiated by molecular techniques as real time PCR. From the aforementioned data, it could be concluded that real time PCR was accurate and reliable method for identification of *Cronobacter species*.

El-Sharoud *et al.* (2009) could not detect *C. sakazakii* in some cheese kinds including stored Domiati and Kareish cheese. On the other hand, the current findings were consolidated by Leclercq *et al.* (2002) as they isolated *C. sakazakii* from cheese and El-Sharoud *et al.* (2009) who reported that *C. sakazakii* was isolated from fresh Domiati cheese samples (40%). These results are in parallel with the findings of Gökmen *et al.* (2010) who pointed out that the presence of *C. sakazakii* in white cheese samples was 4% (2/50), while, El-Gamal *et al.* (2013) could isolate this pathogen from Domiati cheese in a percentage of 30%. Also, Zhao *et al.* (2013) detected the organism in the examined cheese samples.

From this study and literatures, this variation in results may be attributed to different processing and analysis techniques, in addition to that there was some sequence variability in the genes of the tested strains (Lehner *et al.*, 2006).

The association of Cronobacters with cheese suggests

that restrict precautions must be taken in its preparation, handling and storage.

REFERENCES

- A.P.H.A. "American Public Health Association" (2004): Standard Methods for the Examination of Dairy Products, 17th Ed., American Public Health Association, Washington, D.C.
- El-Gamal, M.; Dairouty, R.K.; Okada, A.; Salah, S. and El-Shamy, S. (2013): Incidence and interrelation of *Cronobacter sakazakii* and other foodborne bacteria in some milk products and infant formula milks in Cairo and Giza area. World Appl. Sci. J., 26 (9): 1129-1141.
- El-Sharoud, W.; El-Din, M.; Ziada, D.; Ahmed, S. and Klena, J. (2008): Surveillance and genotyping of *Enterobacter sakazakii* suggest its potential transmission from milk powder into imitation recombined soft cheese. J. Applied Microbiol., 105 (2): 559-566.
- El-Sharoud, W.; O'Brien, S.; Negredo, S.; Iversen, C.; Fanning, S. and Healy, B. (2009): Characterization of *Cronobacter* recovered from dried milk and related products. BMC Microbiol., 9: 24.
- Farmer, J.; Asbury, M.; Hickman, F. and Brenner, D. (1980): *Enterobacter sakazakii*: A new species of "Enterobacteriaceae" isolated from clinical specimens. IJSEM, 30 (3): 569-584.
- Friedemann, M. (2007): *Enterobacter sakazakii* in food and beverages (other than infant formula and milk powder). Int. J. Food Microbiol., 116 (1): 1-10.
- Gökmen, M.; Tekinsen, K. and Gürbüz, U. (2010): Presence of *Enterobacter sakazakii* in milk powder, whey powder and white cheese produced in Konya. Kafkas Univ. Vet. Fak. Derg., 16: 163-166.
- Gran, H.; Mutukumira, A.; Wetlesen, A. and Narvhus, J. (2002): Small holder dairy processing in Zimbabwe: the production of fermented milk products with particular emphasis on sanitation and microbiological quality. Food Control, 13 (3): 161-168.
- Healy, B.; Cooney, S.; O'Brien, S.; Iversen, C.; Whyte, P.; Nally, J.; Callanan, J. and Fanning, S. (2010): *Cronobacter* (*Enterobacter sakazakii*): An opportunistic foodborne pathogen. Foodborne Pathogens and Disease, 7(4): 339-350.
- Hunter, C.J. and Bean, J.F. (2013): *Cronobacter*: an emerging opportunistic pathogen associated with neonatal meningitis, sepsis and necrotizing enterocolitis. Journal of Perinatology, 33: 581-585.
- Iversen, C.; Lehner, A.; Mullane, N.; Bidlas, E.; Cleenwreck, I.; Marugg, J.; Fanning, S.; Stephan, R. and Joosten, H. (2007): The taxonomy of *Enterobacter sakazakii*: proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter sakazakii* comb. nov., *Cronobacter sakazakii* subsp. *sakazakii*, comb. nov., *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter mytjensii* sp. nov., *Cronobacter dublinensis* sp. nov. and *Cronobacter* genomospecies 1. BMC Evol. Biol., 7:64.
- Iversen, C.; Mullane, N.; McCardell, B.; Tall, B.; Lehner, A.; Fanning, S.; Stephan, R. and Joosten, H. (2008): *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov., comb. nov., *Cronobacter malonaticus* sp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter mytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter* genomospecies 1, and of three subspecies, *Cronobacter dublinensis* subsp. *dublinensis* subsp. nov., *Cronobacter dublinensis* subsp. *lausannensis* subsp. nov. and *Cronobacter dublinensis* subsp. *lactaridi* subsp. nov. IJSEM, 58 (6), 1442-1447.
- Joseph, S.; Sonbol, H.; Hariri, S.; Desai, P.; McClelland, M. and Forsythe, S. (2012): Diversity of the *Cronobacter* genus as revealed by multilocus sequence typing. J. Clinical Microbiol., 50 (9): 3031-3039.
- Kucerova, E.; Joseph, S. and Forsythe, S. (2011): The *Cronobacter* genus: ubiquity and diversity. Quality Assurance and Safety of Crops & Foods, 3, 104-122.
- Leclercq, A.; Wanegue, C. and Baylac, P. (2002): Comparison of Fecal Coliform Agar and Violet Red Bile Lactose Agar for Fecal Coliform Enumeration in Foods. Appl. Environ. Microbiol., 68 (4): 1631-1638.
- Lehner, A.; Nitzsche, S.; Breeuwer, P.; Diep, B.; Thelen, K. and Stephan, R. (2006): Comparison of two chromogenic media and evaluation of two molecular based identification systems for *Enterobacter sakazakii* detection. BMC Microbiol., 6, 15.
- Nazarowec-White, M. and Farber, J.M. (1997): *Enterobacter sakazakii*: a review. Int. J. Food Microbiol., 34 (2): 103-113.
- Seo, K.H. and Brackett, R.E. (2005): Rapid, specific detection of *Enterobacter sakazakii* in infant formula using a real-time PCR assay. J. Food Prot., 68 : 59-63.
- Urmenyi, A.M.C. and Franklin, A.W. (1961): Neonatal death from pigmented coliform infection. Lancet 1: 313-315.
- Zhao, Y.; Yao, Y.; Xiao, M.; Chen, Y.; Lee, C.; Zhang, L.; Zhang, K.; Yang, S. and Gu M. (2013): Rapid detection of *Cronobacter sakazakii* in dairy food by biofunctionalized magnetic nanoparticle based on nuclear magnetic resonance. Food Control, 34 (2): 436-443.

تواجد الكرونوباكتر فى الجبن القريش والدمياطى

نجاح محمد سعد ، ولاء فاروق أمين

Email: wallaa_800@yahoo.com

تم جمع ١٠٠ عينة من الجبن القريش والدمياطى لعزل عترات الكرونوباكتر. تم العزل على المستنبت الخاص بالكرونوباكتر ثم تم التعرف على كل ميكروب بالتجارب البيوكيميائية وعلى ذلك وجد بنسبة ٨% من العينات الكلية. عزل كرونوباكتر ساكازاكي فى نوعى الجبن بينما عزل كرونوباكتر مالونيتيكاس وكرونوباكتر توريسنسيس من الجبن القريش فقط. العترات المعزولة من كرونوباكتر ساكازاكي خضعت للفحص باستخدام تفاعل البلمرة المتسلسل. اشارت النتائج الى ان الجبن القريش والدمياطى يمثلان خطرا على الانسان ويجب الانتباه اكثر لتصنيع ومعاملة هذه الاجبان.