SANITAS MAGISTERIUM

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Received: 23.08.2019

https://dergipark.org.tr/ijhadec

Published: 07.01.2020

January 2020 •

CRONOBACTER SAKAZAKII: AN EMERGING PATHOGEN IN FOOD SAFETY

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Abstract

Cronobacter sakazakii is a pathogen that causes fatal infections like necrotizing enterocolitis, and meningitis, especially in preterm and microsomic infants. Even though traces of the C. sakazakii has been detected in various food samples and other environmental sources, only contaminated powder infant formulas have been epidemiologically associated with infections caused by C. sakazakii. However, exposure to environmental and foodborne C. sakazakii species, pose a serious human health risk. Nevertheless, to be able to point out the importance of this issue and its widespread impact, there is a need for a significant number of studies about the detection of C. sakazakii species by rapid and sensitive molecular methods which are not available in our country for now.

The molecular examination of the virulence of each of the steps of food, such as production, harvesting, storage, processing, commercialization, and privatization, is important for the protection of consumer health and prevention of diseases caused by Cronobacteria. Molecular identification methods are one of the most important innovations for developing technology in the field of microbiology. The addition of new molecular identification techniques day by day is of great benefit to scientific studies carried out for different purposes.

Thus, this review was conducted to draw attention to public health risks that may be caused by the pathogenicity and virulence factors of the C. sakazakii.

Keywords:

Cronobacter sakazakii, Molecular Identification, Food Safety, Pathogen, Public Health

Citation: : Demirci Ü., (2020)Cronobacter Sakazakii: An Emerging Pathogen In Food Safety, International Health Administration and Education (Sanitas Magisterium), 6(1), 54-63.

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Introduction

Cronobacter sakazakii may lead fatal infections in human body with a mortality rate of 40-80%. Some of these severe infections are necrotizing enterocolitis, bacteremia, and meningitis, in newborns and children in the first three years of their life, and adults with a suppressed immune system. (Heperkan et al., 2017; Demirci, et al., 2018).

Worldwide known, very few superintendence data are available for C. sakazakii-associated ailments because of the lack of attainable information on superintendence systems, which are insufficient to investigate susceptibility factors. The World Health Organization has detected coarsely 120 singly documented contingencies of Cronobacter spp. infections in infants and young children up to three years of age (Ivy R. A. et al., 2013). Registering the information of foodborne pathogenic isolated samples is vital to enhance the number of the identification techniques, treatment strategies, and preventive medical intervention. Foodborne disease superintendence and/or outbreak data classification encompassing C. sakazakii infections have been established in most countries and the data collected through by these systems suggest that mortality results more likely to occur for the new borns so that there is a greater risk factor for infants (FAO/WHO).

A distinct multiplicity of pollutants, which can emerge throughout various phases of fabrication and retention, may be exhibit in formulas and baby foods, bulk of the researches have recounted the existence of C. sakazakii in baby formula, follow-up baby food, growing-up formula, children's formula, semolina, milk powder, starch, and rice flour (Shaker et al., 2007) (Cava Gümüş et al., 2017) (Demirci et al., 2018); dairy powders (Heperkan et al., 2017); dairy products, sahlab, and dust samples (El-Sharoud et al., 2009) herbs and spices (Jaradat et al., 2009); dried herbs and vegetables (Hunter et al., 2013) and frozen food, seafood, spices, and ready-to-eat snacks (Ogihara et al., 2014).

Alternative to breast milk, formulas can be considered as the most important part of a baby's nutrition in the early years of life. Dehumidified formulas have been considered as one of the best fattening place for bacterial growth of C. sakazakii. The appearance of C. sakazakii in infant formula is being considered as a crucial public health problem due to its virulence factors which can cause fatal infections such as enterocolitis and meningitis. In the field of storage, transport, and production, especially by the manufacturers, various measures have to be taken to minimize bacterial contamination of powder products and training is required to reduce the risk of constant contamination of infant food to the caregivers of infants. New born formulas, cereals, dried foods which frequently consumed by infants and children, must be free of C. sakazakii pursuant to national and international authorities. In addition, C. sakazakii contamination has repeatedly been detected in factories processing baby foods and ingredients used for making baby foods (Kim et al., 2010). Dust particles in the air of such a facility may act as a vector of C. sakazakii dissemination. The higher levels of C. sakazakii are mostly observed in dust filters, vacuum cleaners, bagging and packaging areas (Zimmermann et al., 2017)

The Pathogen-Cronobacter sakazakii

C. sakazakii, is a bacterium which belongs to the Enterobacteriaceae family, gram-negative, facultative anaerobic, rod-shaped, does not create sports, moving with peritric flagella. It produces bright or dull colonies on solid medium and colony types are depending on the medium varies (Ivsen et al., 2003). C. sakazakii can grow in a wide temperature range between 6-47 ° C. Ivers and colleagues (2004) found in their research that six strains developed at 37-43 °C and one capsulized strain could grow at 47°C.

Even though, C. sakazakii is resistant to high temperatures, it is stated that standard pasteurization conditions applied in powdered baby food production are sufficient to inactivate many C. sakazakii strains that are resistant to high temperatures (Ivsen et al., 2004; Shaker et al., 2007; Al-Nabulsi et al., 2011; Jaradat et al., 2014). Moreover, it is also reported that the micronutrient adding process into infant food after pasteurization or irrigation might create secondary contamination sources for these microorganisms (Tocay et al., 2008).

C. sakazakii is a microorganism that exhibits significant resistance to low pH levels. In research has been made by Dancer and colleagues in 2009, the growing up ability of C. sakazakii strains in low pH conditions was examined. As a result, it has been observed that, while all strains were able to grow in pH 4.5, at pH 4.3, 4.1 and 3.9 the growing up percentages were 98.6, 95.8 and 79.2% respectively. In another research, it was found that a decrease in the number of living cells in the pH greater than 5 hours in 37 °C was less than 1 logarithmic unit for 10 strains, and in PH 3.0, this decrease was at the level of 4log kob/ml for all strains (Edelson & Mammel et al., 2006). Furthermore, in another study, C. sakazakii has been isolated from different food samples in pH 2.5 and researchers have stated that microorganisms could not continue their liveliness at that pH level (Fakruddin et al., 2014). However, even though the gastric juice pH levels are in between 2-3 (Freeman, et al., 1978), in newborns, especially in premature babies, stomach acid is not fully developed, constitutes a risk.

Pollution of baby food with biological pollutants can affect the health of a newborn or a baby. In addition, since newborns/infants ' immune system and other defense mechanisms are not as advanced as an adult, these pollutions may cause serious health problems and even death. C. sakazakii is a well-known type of Enterobacter that can contaminate dry baby food and baby formula and cause serious poisoning and infections. Various studies made for many years about the isolation of the C. sakazakii have proven that the cases of neonatal meningitis or necrotizing enterocolitis were associated with the ingestion of powdered infant formulas contaminated with C. sakazakii. Furthermore, in other cases, the organism has been isolated from kitchen utensils, like mixers to blend formulas and potential cross-contaminants of baby food. In such cases even though the samples have not been isolated directly from the infant formulas, a relationship is assumed as there may be a risk of cross-contamination from kitchen tools. Recent publications have shown that this microorganism can be found in a wide range of food, water, and environment, including home and hospitals. (Erkekoglu et. al., 2009)

C. sakazakii were found in many foods like meat, vegetables, cheese, seeds, herbs, spices (Ivsen & Forsythe, 2004) despite the fact that food-borne C. sakazakii infections are not common (Lampel & Chen, 2009). Although, the presence of the bacterium in powdered baby food is more important because of the development of the immune system in newborns (Ivsen & Forsythe, 2004) and the high mortality rates observed in infected infants with C. sakazakii (40-80%) (Lampel & Chen, 2009). Although the origin and transmission of infections caused by C. sakazakii in newborn infants are not fully known, it has been suggested that powdered infant food is the main cause of neonatal meningitis (Nazaretroc &White & Farber, 1997, 1999).

According to a prospective review study, has been made for years from 1960 to 1999 of newborn, infant and young children who infected with C. sakazakii, it has been found that 21 of the patients had meningitis, 7 of the patients had bacteremia, 1 of the patients had urinary tract infection, 1 of the patients had diarrhea, and 1 of the patients had dermoid cyst (Lai et. al., 2001). Besides, it has been shown that bacteremia caused by C. sakazakii can occur in all age groups (Bowen et. al., 2008). Burdette and Santos examined a child patients patient who was showing signs of sepsis for 6 days, in 2000 and they have reported C. sakazakii was detected in child's blood, urine, cerebrospinal fluid, and purulent fluid. In another research, samples collected from different facilities evaluated and the presence of the C. sakazakii has been identified in different levels. In eight of the nine factories producing powdered baby food, five of the 16 houses, as well as all the environmental resources in the factories along the line of production C. sakazakii detected (Kandhai et al., 2004). Furthermore, at the same research, presence of C. sakazakii has been detected, in 152 samples of which they obtained from the floor of three milk powder production plants, dried scraps and vacuum cleaning bags (Kandhai et al., 2004). According to another study made by Reich and colleagues at 2010, it has been reported that in the samples they have collected from a company producing powdered baby food, there were 28%, 5.3% and 8% Cronobacter spp. Respectively, in vacuum cleaning points, filling machines and filling band.

Infectious Dose of Cronobacter sakazakii in Food Samples

There is insufficient epidemiological data on the dose of C. sakazakii infection, although Neisseria meningitis, E. coli O157: H7 and Listeria as with monocytogenes, it is stated that the dose of the oral infection can be considered as 1000 units of cells (Parra & Flores et al., 2015). In addition, it has been described that this dose may vary depending on the microorganism's environmental stress conditions,

the host's healthy immune system, and the food content (Iversen & Forsythe, 2003) In a study conducted by Iversen and Forsythe in 2003, 0.36 kob/100g of C. sakazakii containing powder formula used for a meal of the baby food and it has been reported that the prepared food should be kept at 9 days 8 C or 17.9 hours room temperature in order to reach the minimum infection dose. During the calculation, it has been assumed that the microorganisms did not die with the temperature of the water used during the preparation of the food and did not multiply in the stomach (Erkekoğlu, et al., 2009)

Pathogenesis and Virulence Factors

C. sakazakii is a recently differentiated species of the Cronobacter genus. Nevertheless it's virulence marks remain weakly researched (Ye Y. et. al., 2015). Furthermore an enhanced comprehension of this bacterium has begun to define the virulence factors and pathogenic potential of C. sakazakii (Eshwar A. K. et al., 2016). These improvements have been received by developed DNA-based techniques (Eshwar A. K. et al., 2016). Innovational reports have ascertained many virulence factors in C. sakazakii like seven O-serogroups and eleven proteolytic enzymes demonstrating potential linked to virulence. (Erkekoğlu, et. al., 2009). Amid the virulence-related proteins, outer membrane proteins (OmpA and OmpX) take part in the colonization of the gastrointestinal tract and may have impacts in aiding the organism permeate the blood-brain barrier (Ye Y. et al., 2015). The two presumptive virulence factors, Zinc-metalloprotease (zpx) and Cronobacter plasminogen activator (Cpa), hold a major part in vivo pathogenesis. The factor zpx provokes cell distortion and rounding of cells, whilst Cpa providing impedance to the bactericidal activity of serum, triggers plasminogen, and deactivates alpha2-antiplasmin. Consequently, their characterization is essential to allocate pathogenic from nonpathogenic strains. Pursuant to a investigation made in 2015 by Singh et al., the characteristics of popular virulence factors of C. sakazakii driven to genes demonstrated in the Table 1.

Table: 1

Virulance Factors of Cronobacter sakazakii

Factors	Genes	Potential role
Outer membrane proteins (OMPs)	*OmpX-OmpA*	Engaged with the basolateral attack of enterocyte-like human epithelial cells
Enterotoxin	Not known yet	Warmth stable poison explained by the pathogen
Outer membrane protease	*cpa*	Gives opposition against bactericidal action of serum; initiates plasminogen and inactivates α2-AP
Iron acquisition system	*iuc*	Encodes an iron-take-up framework interceded by the dynamic siderophore that assumes a job in iron vehicle and guideline
Efflux system	*ibeB*	Encodes copper and silver obstruction cation efflux framework encouraging intrusion of cerebrum

		microvascular endothelial cells (BMEC)
Proteolytic enzymes	*zpx*	Cause cell twisting and adjusting of cells
Lipopoysaccaride	*Chromosomal encoded genes*	Disturb epithelial tight intersections

Taxonomy

Species in the Enterobacter family representing a heterogeneous and large group have been identified increasingly pathogenic in recent years. In the Enterobacter family, 14 species have been accepted as taxonomical. Among them, C. sakazakii, Enterobacter Gergovia with 9 species in the Enterobacter cloacae complex defined as the main types of infections that cause hospital infections in humans (Dauga & Breeuwer, 2008). In 1929, Pangalos reported a coliform bacteria forming yellow pigment as the cause of septicemia in a baby (Gurtler et al., 2005). C. sakazakii has been reported as the agent of meningitis for the first time by Urmeyni and Franklin.

There are two major grades that have been differentiated in prokaryotic taxonomy, one characterized by the application of phenotypic studies and other one characterized by a focus on genotypic characteristics. In mycobacterial taxonomy, the first technique has been used until 1980s and the second technique which is based on genotypic characteristics has discovered in the 20th century. Nevertheless, even though initial genotypic researches corroborated the authenticity of formerly defined phenotype-based taxonomy, lately, with the progression of the higher technology, the decomposition of some other species and the ex-novo delineation of others resulted. The reasoning of genotypic taxonomy is associated with the determination of eminently protected regions within the genome, comprising hypervariable sequences with specific deletion, placement, or modification of single nucleotides. The 16S rRNA coding gene has existed for many years and is still the primary target of molecular taxonomic studies and plays a small role in some other genomic regions. Even though the function of genetics in the eventual advancement of mycobacterial taxonomy has come to the forefront, the chemotaxonomic research has also promoted vastly to this field. This approach has led to precise results with mycobacteria thanks to the presence of lipids containing unique molecules, such as mycolic acids in the cell wall. As a result of these two developments, in recent years there has been an increase in the number of mycobacterial species identified. It has been shown that DNA profiling techniques for instance ribotiplication and fragment length polymorphisms (AFLP) differ at species and sub-species levels and may cater estimable supplemental data for external studies (Clermont O, et. al., 2001).

AFLP method has been examined in plant and microbiological studies. AFLP describes the molecular ecology of miscellaneous features and this technique can be used to govern the relevance of the species (Iversen C, et. al., 2006). Moreover, in one study, organs of the same genomic strain cluster were determined by AFLP analysis and it was proposed that the unborn genomic delineation of bacterial species could be based on this approximation (Mougel B, et. al., 2002) Furthermore, in another research, separate molecular techniques such as F-AFLP, automatic ribotiplexing, full-length 16S rRNA gene sequencing, and DNA-DNA hybridization were used to clarify the taxonomic relationship of 210 strains identified as C. sakazakii. Consequently, 210 C. sakazakii strains were subjoined to the BioGroup characterize by Iversen and his colleagues with 16 BioGroup defined by Farmer and his colleagues. Hereby, this research, while all C.sakazakii strains have more than 62% pattern resemblance, further C. sakazakii strains have less than 62% pattern similarity In four groups, 1-r, 2-R, 3- R and 4-R, and 16S rRNA groups were used to identify separate sakazakii groups corresponding to 1- 4 with a greater similarity than 70%. DNA-DNA hybridization was implemented with two strains. E sakazakii type strain, ATCC 29544T, demonstrated ATCC 12868, and 70% of DNA homology of the identical category which accounts they belong propounding the consanguinity of DNA 70% which is usually presumed to be the border for species. Despite the fact that the value may seem low, the resemblance between the 16S rRNA gene rotations for these two strains is 99.6% and is thoroughly clustered using F-AFLP analysis and ribotiplication that supports the similar kind

of relationship. Beforehand, DNA hybridization of 13 E. sakazakii strains resulted in an ideal renaturation temperature of 59.5°C at a rate of 72-95% relative binding. Biogroups 5, 9, and 14 isolates were classified as a subgroup of Group 1 in the ribotype analysis and representatives of this subgroup formed a consistent cluster in the F-AFLP analysis. Whereas for the other strains in Group 1, there is no other connection among biogroup and subcluster. Biogroups called 5, 9, and 14 strains demonstrated in 16S rRNA gene sequences (Iversen et al., 2007)

Detection and Typing Methods

Conventional Bacteriological Culture

Early determination method developed for Cronobacter species was described by Muytjens et al in 1988. According to this method, the US Food and Drug Administration (US FDA) proposed a technique for isolating and sequencing C. sakazakii from powdered formula in 2002. In 2006, the International Organization for Standardization (ISO 2009) and the International Federation of Dairy Products drafted a standartizied protocol known as ISO/TS 22964 for the detection of Cronobacter species from milk-based powder formula (Anonymous 2006a, B). More recently, the US-FDA method has been revised for the detection technique to combine both a PCR analysis and two newly developed chromogenic Agars (Chen et al. 2009; Chen 2011). In these three protocols, pre-enrichment of PIF (Powdered Infant Formula) samples tested and the duration varies from a maximum nocturnal period (18 to 24 hours) to a minimum period of 6 hours, followed by selective enrichment and isolating using selective Agars. Typical colonies are confirmed using a selective agar and/or an appropriate real-time PCR test, and final identification is made, either based on biochemical or molecular characterization. An enrichment stage is available in the revised US-FDA protocol, followed by a molecular method later used for rapid confirmation. In this approach, two days are eliminated from the detection procedure compared to the original protocol. Muytjens et al. (1984) to the α-glycosidase enzyme token as defined by (Iversen et al., 2004b) and to the β-selobiosidase activity present in all Cronobacter strains (Restaino et al., 2006) based on Leuschner-Bew Agari (Leuscher and Bew, 2004), Druggan-Fosythe-Iversen Agari (Iversen et al., 2004b), Oh-Kang agar (Oh and Kang 2004), ESPM Agar (Restaino et al., 2006) and Hijrome Cronobacter spp. selective medium for Cronobacter has been developed, including Agar (Sigma-Aldrich, Switzerland). Also, for the isolation of Cronobacter from foods (Druggan & Iversen 2009; Forsythe 2009), purple-red gall Agar (VRBA), Macconkey Agar, and desoxycholate Agar, selective for gram-negative bacteria, are favorable. Nevertheless notwithstanding the approachability of selective Agars, some have been proven to insufficiently prop the escalation of every bacteria strain (Iversen and Forsythe 2007) and other correlated categories of bacteria just as Enterobacter Helveticus, Enterobacter triturates, and Enterobacter turicensis, which can be found in the same ecological family. Thence, accomplishments were needed in the pattern of selective media to isolate and identify Cronobacter. O'brien et al. (2009) suggested the pattern of a one-step pre-enrichment and enrichment protocol in which a chromogenic medium is used. In this identification strategy, the specific medium advanced [referred to as Cronobacter enrichment medium] conducted to the preparation of a short two-day culture method for the detection of Cronobacter species in powdered infant formula. Mullane et al. (2006) utilized a cationic magnetic bead capture technique to boost Cronobacter detection sensitivity from PIF. The precision and authenticity of commercially available Cronobacter identification kits have been questioned by false-negative and false-positive reports (Restaino et al., 2006; Iversen and Forsythe, 2007). Nonetheless, Gen III is the only Identification Kit accessible (Healy, 2010) that contains the pioneer to six species covered.

Molecular-based Detection Protocols

Molecular detection techniques have always been perceived as suitable mechanisms for a enhanced perception of an organism's epidemiology. Typically, these analyses are aforethought to eliminate and aimed exceptional genes present in the pathogen. Plenty of the analysis placement produced with present technology are based on real-time PCR, and some are built for more detailed detection of Cronobacter (Malorny and Wagner, 2005; Seo and Brackett, 2005; Drudy et al., 2006; Nair and Venkitanarayanan, 2006; Kothary et al., 2007; Zhou et al., 2008). Protocols created to detect Cronobacter ease the definition of all seven known species within the genus Cronobacter using a mismatch-PCR-based approach. Utilizations of these molecular, based processes can support conventional culture-based approaches. Even so, using the last process indicated, C. malonaticus and C. sakazakii were not distinguishable by this approach, and therefore a second PCR requirement was

formed to correctly identify each of these species. A study by Yan et al in 2011 found that a PCR and sequence-based biological token validation study was required to detect and identify Cronobacter spp. The researchers aim to shed light on virulence markers that may be useful as biological markers to distinguish Cronobacter spp and Salmonella spp from other food-derived pathogens. Although these assumed markers have been identified, further verification experiments are already being conducted. Molecular subtyping has been recognized as an approach that can be applied to elucidate the nature of these bacteria that colonize a particular ecological niche. The study demonstrated a basis for the improvement of effective interventions to reduce Cronobacter in the PIF production environment. Then, an another approximation developed using Multi-Focus variable-numbered short-sequence Repeat analysis (MLVA), a second-generation subtyping method, was applied to perform subtyping of the archive of different Cronobacter isolates, both genotypic and phenotypic. Yet, a standardized PFGE process is taking a final build, and international laboratory networks for detecting foodborne infections have been validated by PulseNet The list of molecular detection protocols for C. sakazakii defined by Yan et al. in 2011, demonstrated in the Table 2.

Table: 2

Detection Protocols for Cronobacter in Powdered Infant Formula

Procedure	FDA (Original)	ISO/TS 22964	FDA (revised)
Preenrichment	Make1:10 (w/v)sample indistilled water, hatched medium-term at 36oC	Make 1 : 10 (w/v) of test in BPW, brooded at 37°C for 18 ± 2h	Make 1 : 10 (w/v) of test in BPW, hatched at 36°C for 6 h
Selective enrichment	Move 10 ml pre improvement to 90 ml EE soup, brooded medium-term at 360C	Move 100 µl pre-enrichment to 10 ml mLST/vancomycin medium, hatched at 44°C for 24 ±2 h	
Selection/ isolation	Make a disconnection streak and spread plate from every EE stock onto VRBG agar, hatched medium-term at 36°C	Streak from the refined mLST/vancomycin medium one loopful on the chromogenic agar in Petri dishes, brooded at 44°C for 24 ± 2h	Axis 40 ml examples, 3000 g, 10 min and resuspend pellet in 200 µl PBS; Spread 100 µl onto chromogenic media, brooded medium-term at 36°C
Confirmation	Pick five hypothetical positive provinces and streak onto TSA, hatched medium-term at 25°C	Select five run of the mill settlements and streak on TSA agar, brooded at 25°C for 48 ± 4 h	Pick two regular states from each chromogenic media affirmed with real-time PCR, Programming interface 20E, Fast ID 32 E

Identification	Yellow provinces are affirmed with the API 20E test kit	Select one yellow province from every TSA plate for biochemical portrayal	
Detection time (hrs)	120	144	72

Conclusion

Cronobacter species are an of late ordered class, and there are insufficient examines have been made to completely comprehend this one of a kind gathering of creatures. Precise recognition and legitimate ID of nourishments and clinical situations are most significant hotspots for information accumulation. Consequently, atomic procedures are regularly simpler and quicker when contrasted with ordinary microbiological (phenotype) based techniques. But endeavors have been made to refresh databases for accessible spotting frameworks, upgrades can be made in the recognizable proof and separation of Cronobacter species. The requirement for specific instruments and administrator preparing discourages a few analysts to pursue these conventions well ordered. By and by quick demonstrative conventions are urgent in the clinical use. The execution of practices, for example, proteomics, similar genome examination, transcriptomics and bioinformatics is important to reveal complex contacts between the pathogen and host. Also, there is next to no that researchers think about the danger variables and pathogenicity of Cronobacter species in vivo, which is the most significant part for restorative treatments. Another methodology and vision to the movement and pathogenesis of maladies related with Cronobacter species is required. Especially use of the in vitro cell-based examinations joined with creature model investigations ought to be researched further. Hence, transmission and conveyance of new innovations, conventions, and sanitation issues to industry, specialists, scientists, and purchasers ought to contemplate.

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