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Cronobacter sakazakii, a New Threat: Characteristic, Molecular Epidemiology and Virulence Factors

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MH and AA designed the study and wrote the protocol. Author AM wrote the first draft of the manuscript. Authors GK and MGM managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Cronobacter spp. are opportunistic pathogenic bacteria that can cause different diseases through consumption of contaminated food. *Cronobacter sakazakii* is the most important species found in Powdered Infant Formula (PIF) and baby food. PIF is the most significant source of *C. sakazakii* which provides an appropriate environment for its growth and survival. Infant formula is heated during its production, although this amount of heating is not enough for sterilization and disinfection. *C. sakazakii* is considered a threat in all age groups. It may cause different illnesses such as meningitis, bacteraemia, sepsis and necrotizing enterocolitis in children. It may also cause infections in adults such as pneumonia septicemia, osteomyelitis, splenic abscesses, and wound infections. The mortality rate is high (40-80%) and the survivors suffer from severe neurological complications. Poor hygienic preparation of infant food at home or at hospitals has been reported as one of the

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main reasons of *C. sakazakii* infection. The gold standard based on FDA-recommended methods (Food and Drug Administration) for the isolation and identification of *C. sakazakii* obtained from powdered infant formula, is both time consuming and labor intensive.Since the rapid detection and diagnosis of this bacterium is important, new molecular methods are being used today. These methods have meaningful and significant advantages as compared to traditional methods on account of speed, sensitivity, specificity and accuracy. The aim of this study is to investigate the characteristics of *C. sakazakii* and its molecular identification and virulence genes.

Keywords: Powdered infant formula; foodborne disease; epidemiology; virulence genes; polymerase chain reaction; Cronobacter sakazakii.

1. *Cronobacter sakazakii* CHARACTERISTIC

1.1 Chemical Characteristics

The molecular identification of *Cronobacter* has classified this bacterium into seven groups: *C.universalis, C. condimenti, C. sakazakii, C. malonaticus, C. dublinensis, C. muytjensii, C. turicensis*, and among which, *C. malonaticus*, *C. turicensis*, and *C. sakazakii* are the most common species isolated from contaminated newborns while *C. pulveris*, *C. zurichensis* and *C. helveticus* are the reclassified kinds which have been identified lately [1]. *C. sakazakii* is a food-borne pathogen belonging to the family of *Enterobacteriacea* with characteristics such as being facultative anaerobe, gram-negative, motile with a peritrichous flagella and rapid growth on laboratory media [1]. Before 1980, *E. sakazakii* with yellow-pigmented cloacae belonged to *Enterobacteriacea* family [2]. This bacterium has a similar biochemical profile as *E. cloacae*, but unlike *E. cloacae,* it is always d-sorbitol negative and extracellular deoxyribonuclease positive and which could produce yellow-pigmented colonies [3,4]. This organism could grow on agar plate with two forms of colonies: glossy or matte, which depends on bacterial strain and its growth environment. It can grow on MacConkey agar with "Blue-Green" colonies, because it can produce α-glucosidase enzyme [5]. This bacterium can also grow on Eosin Methylene Blue (EMB) and deoxycholate agar. It can be identified with a typical non-diffusible yellow pigment colonies on Tryptic Soy Agar (TSA) at 25℃ [3,5]. Voges-Proskauer test is positive for this bacterium, while citrate assimilation, B-glucosidase (ONPG) and methyl red test reactions are negative [6]. The bacterium is indole positive, oxidase negative, catalase positive and citrate positive, MR-VP and nitrate reduction negative, and it is able to ferment glucose with the production of acid and gas, lactose rhamnose, xylose, trehalose, arabinose, cellubiose and melibiose. It can also de-carboxylate arginine, hydrolyseesculin and liquefy gelatin, but it cannot ferment dulcimer and malonate [7].

C. sakazakii is able to produce a delayed extracellular DNAase reaction against toluidine blue agar at 36℃ after 7 days. It is α-glucosidase positive that can be recognized by 4-nitrophenyl-a-d-glucopyranoside after 4 h at 36 ℃. Researchers have found two major differences between *C. sakazakii* and other *Enterobacter* species; one of them is α-glucosidase activity which was shown in all *C. sakazakii* strains, but it was not found in any of the *Enterobacter* strains; therefore the absence of phosphoamidase enzyme was unique in *C. sakazakii* isolates [7].

The organism produces d-lactic acid and it is mucate negative. Most of the isolates produce esterase enzyme, this indicates another difference between *C. sakazakii* and *E. cloacae* [8] along with not fermentation of sorbitol. It can produce a novel hetero polysaccharide comprising 29-32% glucuronic acid, 23-30% d-glucose, 19-24% d-galactose, 13-22% d-fucose and 0-8% d-mannose [2,3,6,9,10]. In 2002, the International Commission on Microbiological Specifications for Foods (ICMSF) has ranked *C. sakazakii* as " dangerous and life threatening with substantial chronic side effects "[3,11]. Afterwards, it has a similar ranking with some familiar food and water-borne pathogens such as *Listeria monocytogenes*, *Clostridium botulinum* types A and B and *Cryptosporidium parvum* [3].

2. FOODS AND ENVIRONMENTAL SOURCES OF *C. sakazakii*

C. sakazakii is reported to be commonly isolated from different environments and food sources [35,36]. It has also been isolated from the surfaces of the equipments and food production

environments [37]. Reports showed that the cells of the bacterium in biofilms could grow on silicon or latex and polycarbonate, and to a lesser extent on stainless steel material [14] like aluminum and intestinal tube, because this cell form increase the resistance of bacteria to chemical agents. The death and destruction of the cells in biofilms are influenced by relative humidity [20]. The sources of the bacteria and the devices which transmit it to something new are not constantly clear. However, the pathogen has been found in different food resources, its cooperation with powdered infant formula (PIF) is of the greatest concern [1,38]. The important food and environmental sources are described in Table 1.

2.1 Newborn Formula (Important Source)

Due to being the main source of this pathogen, many researchers have focused on newborn formula [45]. Liquid feeds, dried milk powders and powered newborn formula are heated during their production, although this amount of heat is not enough for sterilization and disinfection [18]. From another point of view, the newborn's gut flora is not complete and the stomach acid secretion is also low; therefore, children could easily get infected. The growth of multiple pathogens is supported by PIF efficiently and it could be contaminated [64]. It was shown that PIF contains *C. sakazakii* and has been epidemiological connected to several clinical cases [27].

It is also reported that the contamination prevalence of powdered milk can be ranged from 6.6% in Brazil to 29% in China [65]. Although, the great majority of the cases worldwide are reported from five countries: USA, UK, France, Belgium and the Philippines [51], *C. sakazakii* is considered as food safety risk which is harmful to human health, specifically in minimally processed foods and also in new foods processed by alternative technologies [11].

Table 1. Foods and environmental sources of *C. sakazakii*

Meat	
Meat, sausage, minced beef, fish, shrimp, pork (dry, raw, cured), poultry	[20, 27, 39, 40, 42, 43, 44]
Grain and seeds	
Biscuits, bread, dry ingredients (almonds, coconut powder, pistachio, lentils), pea soup powder, grains, flour or meal (corn, rice, soy, wheat), cereal (adult and newborn), almond, nuts and seeds, seed sprouts, sorghum seeds , ground rice , rice seeds , sesame seeds, semolina samples, lentils, Bean	[3, 14, 42, 45, 46, 47, 48, 49, 50]
Food	
Soup, pasta, egg	[14, 51, 52, 53]
Beverages	
Khamir (fermented sorghum bread), fermented bread, sobia (fermented beverage), tempé (fermented soybean)	[3, 45, 54]
Vegetables and fruit	
Vegetables (mixed salad), Laver (red algae), herbs, Mexican fruit flies, tomato, courgettes, lettuce, potato, sponge mix	[27, 39, 48, 51, 55, 56, 57, 58]
Environment	
Soil, milk powder factories, milk stored in a milk bank, chocolate factories, households, hospitals, doctor’s stethoscope, food preparation equipment: spoons, blender	[3, 27, 45, 48]
Water	
central and local drinking water supplies	[28]
Milk	
UHT milk, newborn formula, powdered milk, cheese,	[3, 39, 45, 59]
Animals	
gut of stable fly larvae, rats, animal sources	[45]
Clinical sources	
cerebrospinal fluid (CSF), blood, bone marrow, sputum, urine, inflamed appendix, intestinal and respiratory tracts, eye, ear, wounds, feces	[27, 42, 47, 60, 61, 62, 63]

3. RESISTANT CHARACTERISTICS OF *C. sakazakii*

3.1 Resistance to the Environment

C. sakazakii is sensitive to heat, it can tolerate high desiccation and osmotic stress and could also survive during milk powder processing [12, 13]. Furthermore, the formation of biofilm of *C. sakazakii* assists the organism to survive in milk powder and on the surface of processing utilities [14,16], it also enables the organism to resist multiple stress conditions including water and nutrient shortages and biocides presence [17]. *C. sakazakii* can grow over an extended temperature range (21℃) with the maximum temperature of 41-45℃ and minimum temperature of 5.5- 8.0℃. Generation time of this bacterium is 40 min at 23℃, 4.18-5.52 h at 10℃ and 75 min at 25℃ in rebuiltinfant milk powder (IFM) [18]. The fastest rate is almost 10 h in IFM at 10℃, therefore, it grow with slower rate, under cool conditions [19]. Skladal et al. (1993) have reported milk acidification and *C. sakazakii* growth in UHT milk cartons inoculated with 10–15 *C. sakazakii* cells/500 ml and incubated at 30℃. The increase in milk acidity was due to d-lactate production. Studies on pasteurized milk stored at 30℃ showed that the bacteria could survive due to the appropriate temperature for the growth of this bacterium and milk acidification because of D-lactate production. Beuchat et al. showed that the infected agent survived better in dried formula and cereal at low aw (0.25-0.30) than at high a_w (0.69-0.82) and at 4℃ compared to 30℃. *C. sakazakii* grows in formulas and cereals reconstituted with milk or water at 12-30℃. They have also discovered that the composition of formulas or cereals does not noticeably affect this rate of development as well. *C. sakazakii* can grow on fresh cut apple, watermelon, lettuce, cantaloupe, carrot, cabbage, cucumber and tomato at 25℃ and on some other products at 12℃. The bacteria could live longer and better in PIF at a_w 0.25-0.30 than in PIF at a_w 0.43-0.50 at two different temperatures; 21℃ and 30℃ [20]. The minimum and maximum PH for the bacteria is 3.89 and 5-9 respectively [21]. Strains of *C. sakazakii* cannot survive at very low pH (2.5) [5].

High hydrostatic pressure has been reported as a pasteurization treatment which does not involve heat progress. Under experimental circumstances, 7-$\log_{10}$ cycle reduction was obtained at 350-400 MPa for 10-15 minutes at ambient 25℃ [22]. The lowest resistance and the highest growth of the bacteria in hydrostatic pressure occur at 30-37℃ with a PH of 4.The highest resistance is also observed at low a_w (a_w = 0.98), because the coating of bacteria is more stable [11]. Strains of *C. sakazakii* could grow at 10% NaCl and 5% bile salt concentration [5].

3.2 Resistance to Antimicrobial

The efficiency and productivity of disinfectants are usually employed in both clinical and food environments to promote clean surfaces and to avert *Cronobacter* spp. [23]. Both antibiotic sensitivity and bacterial resistance have been managed after a couple of deadly reported infections among the newborns [24-26] which are shown in Table 2.

4. CHARACTERISTICS OF THE DISEASE

C. sakazakii is an opportunistic and life-threatening pathogen [66]. It has been known to be implicated in the development of bacterial infections in newborns since 1961 [67]. This pathogen has a 40-80% mortality rate among the infected newborns and seems to be specifically associated with strains of *C. sakazakii* sequence type-4 (ST4) [5,36,67]. It is considered an emerging opportunistic pathogen, responsible for cases of neonatal infections including necrotizing enterocolitis (NEC), meningitis, bacteraemia and septicemia [18]. NEC is characterized by inflammation and intestinal tissue damages and is one of the most common reasons of gastrointestinal disorders in neonates [1, 66], due to the use of contaminated newborn milk formula [18]. High rates of fatal outcome and neurological complications of neonatal *Cronobacter* infections such as diarrhea, urinary tract infections, splenic abscesses, osteomyelitis, pneumonia, wound infections, urinary tract infections and conjunctivitis [29,68,69] occur in neonates with a lower frequency [70]. Meningitis symptoms include ventriculitis, brain abscess, hydrocephalus and cyst formation as well as necrotizing enterocolitis characterized by intestinal necrosis and pneumatics intestinal; pulmonary, urinary and blood stream infections [5]. The survivors would probably suffer from severe neurological complications [66,67].

It may cause infections in all age groups [18] including newborns, children, and immuno-compromised adults and people with underlyin medical conditions [20]; although, premature

Table 2. Antibiotic susceptibility of *C. sakazakii* in different studies

Susceptibility			
1.	Aminoglycosides [27]	17.	Cefoperazone [31]
2.	Antifolates [27]	18.	Ceftriaxone [31]
3.	Ampicillin [27]	19.	Ceftazidime [10]
4.	Aminoglycosides [28]	20.	Carbapenems [27, 29]
5.	Ampicillin-gentamicin [27,29]	21.	Imipenem [10]
6.	Ampicillin-chloramphenicol [27, 29]	22.	Gatifloxacin [31]
7.	Aztreonam [10]	23.	Gentamicin [11, 30, 32]
8.	β-lactams [27]	24.	Kanamycin [11]
9.	Carbenicillin [11]	25.	Levofloxacin [10, 31]
10.	Cephalothin [11]	26.	Norfloxacin [10]
11.	Cephalosporins [29, 30]	27.	Nalidixic acid [11]
12.	Chloramphenicol [27,29,31]	28.	Ofloxacin [10]
13.	Colistin [11]	29.	Quinolones [27]
14.	Cefoxitin [31]	30.	Sulfadiazine [11]
15.	Ciprofloxacin [31]	31.	Streptomycin [10, 11]
16.	Cefotaxime [10]	32.	Tetracyclines [11, 27]
		33.	Trimethoprim-sulfamethoxazole [10, 29]
Resistant			
1.	Ampicillin[10, 11, 30, 32]	14.	Novobiocin[34]
2.	Amoxicillin-clavulanic acid [31]	15.	Nitrofurantoin[5]
3.	Chloramphenicol [11]	16.	Neomycin[1]
4.	Clindamycin [27]	17.	Penicillin[11]
5.	Cephalothin[1, 11, 32]	18.	Penicillin G[5, 32]
6.	Cefazolin [29]	19.	Rifampicin[10, 27, 31]
7.	Cefotaxamine[33]	20.	Streptogramins[27]
8.	Doxycycline [5]	21.	Streptomycin [31]
9.	Erythromycin [34]	22.	Sulfamethoxazole[6, 11]
10.	Fusidic acid [27]	23.	Tetracycline [11, 31]
11.	Fosfomycin[27]	24.	Trimethoprim[1]
12.	Lincomycin[27, 30]	25.	Vancomycin[5]
13.	Macrolides[30]		

newborns (earlier than 36 weeks) particularly newborns younger than 28 days old, underweight newborns <2.5 kg, newborns with immunodeficiency, newborns whose mothers are HIV-positive and newborns hospitalized in intensive care units are at higher infection risk due to formula feeding [5,18]. Nowadays, based on different cases worldwide,50% of the infected adults suffering from a severe course of illness, are less than 60 years old [71].

5. EPIDEMIOLOGY

One type of neonatal meningitis was first emerged in England in 1958 and led to the death of 2 children. Most cases were reported in five countries including: United States of America (USA), United Kingdom (UK), France, Belgium and The Philippines, the published neonatal *Cronobacter* infections are listed in Table 3 [72].

6. VIRULENCE FACTORS

The seriousness of the illness is linked with a high rate of death (up to 80%) in newborns and neonates infected by *C. sakazakii*, therefore, further studies on virulence and survival factors of this gastrointestinal infecting agent are required.

Intestinal bacterial colonization is considered a multifactorial process which requires additional experimental examination in order to achieve a better perception of the generation and development of a disease in pathogenesis of *C. sakazakii* bacteria in GI tract; although some selective genes could activate in the virulence of *Cronobacter* bacteria [101,103]. Researchers discovered an association between virulence genes and their abilities to adhere and invade Caco-2 cells [104]. First step after attacking abdominal tract, is the binding of the bacteria to tissue cells. Several researches have been conducted in order to identify this infectious

agent, they have shown that fibronectin, a glycoprotein which shapes a proportion outside a cell matrix of eukaryotic tissue, plays an important role in host cell adhesion and attachment, growth, differentiation and movement [105,106]. Studies have revealed that fibronectin binding is the most crucial spot of the attachment of bacteria to digestive tract cells [107]. A fibronectin-binding protein with a key role of being attached to this bacterium, is outer membrane protein A (*OmpA*) [105,108]. After joining or binding to the host tissue, the bacterial invasion occurs in 60 minutes [1]. The attack mechanism of *C. sakazakii* has not been determined yet, but various factors such as the host and bacterial membrane proteins may affect this progress. In healthy people, the tight-junctions between gut epithelia cells have a significant function to inhibit the entrance of the bacteria into epithelial cells. Hence, it is not unexpected that the attack by *C. sakazakii* disrupts the function of tight junctions by lipopolysaccharides (LPS) in the bacterial membrane [43,109]. Although, Kim et al. (2010) revealed that another outer membrane protein, *OmpX*, can also have a key role in attacking Caco-2 epithelial cells and Hep-2 cells by *C. sakazakii* [110,111].

C. sakazakii is a well-known special microorganism that can pass through the barrier between brain tissues and circulating blood which can damage human *Endothelial microvascular* brain cells (HBMEC) [112]. *OmpA* protein has also been involved in the invasion of HBMEC. Nair et al. [108] revealed that the deficiency of this protein leads to a decrease of HBMEC invasion by 83%. While both *OmpA* and *OmpX* seem to be able to enter the blood-brain barrier causing brain cell necrosis with an unknown mechanism; although it is probable that the response of the host's immune system is involved here [36]. Moreover, *C. sakazakii* dominates *zpx*, a gene which encrypts a zinc containing metalloprotease and it is also responsible for the lysis of collagen and could probably enable the bacteria to cross the blood-brain barrier [113]. Another important virulence factor is the ability of some strains of *C. sakazakii* to survive in macrophages up to 48 hours. The flagella of *Cronobacter* spp. is crucial for biofilm formation and cell attachment [15]. Flagellum plays an important role in the activation of pro-inflammatory cytokines in macrophages [103]. Flagellation genes: *flgA, flgB, flgC, flgD, flgF, flgG and fliE*, are all engaged in flagellar basal body biosynthesis in the membrane [114]. Bacterial toxin is another virulence factor which can tolerate higher temperatures (90℃ for 30 mins), with a highpoisonous potential (LD50=56 pg) [104,115]. Survival in macrophages is different among the strains, which is related to the presence of putative *sod* genes, encoding a superoxide dismutase [116].

Disturbance of epithelium cells stimulates the release of different pro and anti-inflammatory chemokines and cytokines such as changing growth factor β (TGF-β) and Nitric Oxide (NO) [117]. Another virulence factor is the ferric decipher transport framework which has been detected in *C. sakazakii* and *C. malonaticus* clinical cases, which revealed that iron procurement framework is fundamental for selectivity of *Cronobacter* organisms [118]. The other virulence factor of *C. sakazakii* is capsular material, which settles in plant and causes the production of a new hetero polysaccharide. This capsular material could make the organism easily attach to plant surfaces [10]. Another destructive factor is O-antigen (O-polysaccharide) that is composed of various oligosaccharides and is a lipopolysaccharide factor in the external layer of Gram-negative microorganisms. There are 17 O-sero groups for *Cronobacter spp.*, among which, 7 are identified as *C. sakazakii* [119].

7. MOLECULAR TYPING METHODS

Certain types of *Cronobacter* are considered as opportunistic pathogens, however their demodulation in drain items as per ISO/TS 22964 may take up to six days [120]. Therefore molecular methods are used as quick and trustworthy tools to study bacterial genomic diversity and to track sources of infection [6, 82,121]. For a precise characterization of *Cronobacter* species in PIF and its associated environments, various molecular based protocols have been improved, which contain direct target gene detection (Table 4) and subtype methods [100,122].

7.1 Polymerase Chain Reaction (PCR)

Molecular methods for example, polymerase chain response (PCR) could provide intense apparatuses to fast, particular, and delicate identification of sustenance borne pathogens and are respected to be solid contrasting options to conventional bacteriological strategies. This method is involved in internal amplification controls [123].

Table 3. Published neonatal *Cronobacter* infections (1958-2013)

Year	Location	Cases	Outcome	Infection	Source	Reference
1958	England	2	2	Meningitis	Unknown	[73]
1958	Denmark	1	1	Meningitis	Unknown	[74]
1958	Georgia	1	0	Bacteremia	Unknown	[75]
1958	Oklahoma	1	1	Meningitis, sepsis	Unknown	[76]
1958	Indiana	1	0	Meningitis	Unknown	[77]
1961	England	2	2	Meningitis	Unknown	[73]
1965	Denmark	1	Unknown	Meningitis	Unknown	[74]
1979	Georgia	1	0	Bacteremia	Unknown	[75]
1977-1981	Denmark	8	6	Meningitis	IFM	[78]
1977-1981	Czechoslovakia	NS	NS	NS	NS	[8]
1981	Greece	1	Unknown	Meningitis, sepsis	Unknown	[76]
1981	Indiana	1	0	Meningitis	Unknown	[77]
1983	Denmark	8	6	Meningitis	PIF	[78]
1984	greece	11	4	Colonisation	Unknown	[26]
1984	Missouri	1	0	Meningitis	Unknown	[79]
1984	England	2	0	Meningitis	Unknown	[80]
1985		1	0	Meningitis	Unknown	[79]
1986-1987	Iceland	3	2	Meningitis	IFM	[81, 82]
1987	Greece	11	5	Meningitis, sepsis	Unknown	[26]
1981–1988		2	NS	Meningitis	Unknown	[84]
1988	England	2	0	Meningitis	Unknown	[80]
1988	Tennessee	4	0	Sepsis, bloody, diarrhea	IFM, blender	[25, 82]
1988	Maryland	1	0	Bacteremia	IFM	[85]
1988	Ohio	1	0	Meningitis	NS	[86]
1989	Iceland	3	1	Meningitis	PIF	[81]
1989	Spanish	2	2	Appendicitis, conjunctivitis	NS	[83]
1989	Portugal	4	0	Meningitis	Unknown	[84]
1989	Tennessee	1	0	Sepsis, diarrhea	PIF	[25]
1990	Georgia	4	NS	Septicemia	PIF	[82]
1990	Baltimore	1	0	Bacteremia	PIF	[85]
1991	Ohio	NS	NS	Meningitis	NS	[86]
1994	Hong Kong	1	NS	Necrotising enterocolitis	Unknown	[87]

Year	Location	Cases	Outcome	Infection	Source	Reference
1994	Erlangen-Nümberg	1	0	Meningitis	Unknown	[88]
1996	Canada	1	0	Wound infection	Unknown	[89]
1998	Belgium	12	0	Enterocolitis	IFM	[24]
1999-2000	Israel	NS	NS	NS	IFM, blender	[90]
1999-2000	Israel	2	0	Bacteremia,meningitis	IFM, blender	[91]
1999-2000	USA	1	0	Bacteremia	NS	[29]
2000	USA	1	0	Meningitis	NS	[92]
2001	Tenessee	11	1	Meningitis, enterocolitis	IFM	[93]
2001	Belgium	12	0	Enterocolitis	PIF	[24]
2001	Israel	2	0	Bacteremia,meningitis	PIF	[91]
2001	USA	1	0	Bacteremia	NS	[29]
2002	Israel	NS	NS	NS	PIF	[90]
2002	Tenessee	11	1	Meningitis, enterocolitis	PIF	[93]
2004	France	2	NS	NS	NS	[69]
2007	India	2	1	Respiratory distress,sepsis	PIF	[94]
2009	India	1	0	Urinary tract infection	NS	[95]
2009-2010	Tanzania	12	NS	septisemi	artificial feeding(milk powder)	[96]
2010	Argentina	1	unknown	septicemia	unknown	[97]
2010	Mexico	2	0	gastroenteritis	R-PIF, PIF, and fecal matter	[98]
2010	Mexico	2	NS	NS	NS	[99]
2007 - 2013	Czech Republic	31	NS	NS	NS	[100]

•NS Not specified in papers, not stated

Table 4. Molecular detection methods and related genes in *Cronobacter sakazakii*

Target gene	Metod	Reference
GLUB	PCR-RFLP*	[37]
	PCR	[147]
gluA		[148]
(α-glucosidase gene)		[10]
		[149]
	PFGE	[150]
gyrB	species-specific PCR	[151]
(DNA gyrase subunit B)		
fusA	Sequencing	[122]
	MLST	[152]
	MLVA	[152]
Weh C	PCR	[141]
Weh I	PCR	[141]
wzx	PCR	[142]
hsp60	PFGE	[96]
Zpx	PCR	[113]
(zinc-containing metalloprotease)		[153]
dnaG	real-time PCR	[154]
		[152]
	PCR	[154]
	PFGE	[150]
Dps	real-time PCR	[155]
LuxS	real-time PCR	[155]
palE	PCR	[123]
(Isomaltulose, 6-O-α-glucopyranosyl-D-fructose)		
C. sakazakii-specific CDS	PCR	[66]
Cpa	PCR	[156]
(Cronobacter plasminogen activation)		
cgcA	mPCR	[157]
RpoB	PCR	[158]
		[96]
		[159]
(RNA polymerase beta-subunit gene)		[98]
		[160]
		[161]
		[100]
		[17].
		[31].
RpoB	mPCR	[157]
	PFGE	[96]
		[98]
		[31]
	mLST	[161]
	mLVA	[161]
ompA	real-time PCR	[162]
(Outer membrane protein A)		[120]
		[37]
		[162]
	PCR	[160]
		[161]
		[10]
		[31]
	duplex PCR	[139]

Target gene	Metod	Reference
	mPCR	[31]
	mLST	[161]
	mLVA	[161]
16S rRNA	PCR	[38]
		[64]
		[163]
		[159]
		[59]
		[164]
	real-time PCR	[149]
	PFGE	[163]
16S rDNA	real-time PCR	[42]
		[114]
	PCR	[14]
		[96]
	PFGE	[96]
23S rRNA	PCR	[64]
		[42]
23S rDNA	PCR	[165]
16S–23S rDNA	PCR	[42]
	duplex PCR	[139]
t-RNA-glu (transfer (t)RNAGlu)	PCR	[166]
t-RNA-glu–23S RNA	real-time PCR	[165]
bacterial DNA	PMA-mPCR-IAC**	[167]
	MLSA	[168]
recN		
	rep-PCR	[168]
O-antigen serotype	MLST	[161]
(O-polysaccharide)	MLSA	[161]

**Restriction fragment length polymorphism*
***Internal amplification control (IAC)/multiplex PCR (mPCR)/ propidium mono azide (PMA)*

7.2 Real-Time PCR

Real-time PCR is highly sensitive and allows quantification of rare transcripts and small changes in gene expression. It is also easy to perform, provides the necessary accuracy and produces reliable rapid quantification results. many studies different have used real time pcr teqnique targeting genes successfully (Table 4) [123].

7.3 Multiplex PCR (mPCR)

Unlike normal PCR, multiplex PCR (mPCR) can perceive at least two pathogens in a solitary examination and it is definitely swifter, less demanding, and less expensive to be run. However, it cannot distinguish between living and dead bacteria [37]. The DNA from dead microscopic organisms can fill in as a format amid PCR enhancement bringing about false positive outcomes. A promising procedure to defeat this downside is the utilization of nuclei corrosive intercalating colors, for example, propidiummonoazide (PMA), as a specimen pre-treatment preceding mPCR. PMA is a DNA-intercalating color which can selectively enter the dead cells with compromised membrane integrity and make a cross link with the DNA utilizing its azide groups upon light-exposure [124]. This adjustment brings about the elimination of DNA amplification from dead cells during PCR. Consolidating the quick and affectability of mPCR, this methodology has turned into a normal option strategy for the qualification amongst live and dead cells over the most recent couple of years [125-127].

7.4 Repetitive Element Sequence-Based PCR (Rep-PCR)

Repetitive element sequence-based PCR (rep-PCR) can be called a typing procedure with the ability to activate the generation of DNA fingerprinting which can separate and distinguish bacterial strains [128].

7.5 PCR-RFLP (PCR - Restriction Fragment Length Polymorphism)

An alternate, conventional and standard RFLP system is PCR-RFLP, which includes PCR amplification of particular DNA sequences in the organism and designating the PCR amplicons with a restriction enzyme to make a DNA banding pattern [129]. For PCR-RFLP to be successful, the amplified gene used for RFLP typing should have conserved regions of DNA that flank variable sequences to allow differences in the digestion of the amplicon [130].

7.6 Pulsed-Field Gel Electrophoresis (PFGE)

PFGE is a form of RFLP typing in which the bacterial genome is digested with an uncommon cutting enzyme, which creates fewer DNA fragments of different sizes that can be separated using particular electrophoresis techniques. Differences in the restriction profiles are used to carry out genetic comparisons among isolates [131].

Pulsed-field gel electrophoresis with two restriction enzymes (Xba1 and Spe1) is an approved procedure for tracking isolates across the food chain; this is an appropriate approach for epidemiological studies [6]. Likewise, PFGE is well established and widely applied as a gold-standard method for molecular typing of bacteria including *Cronobacter* spp [132,133].

This procedure is restricted, although, as not all strains can be typed, non-identical strains can give the same PFGE profile, and the procedure does not identify the relationship between strains [134].

7.7 MLST (Multi Locus Sequence Typing)

On a bigger scale, multi locus sequence typing (MLST) was developed for *Cronobacter* species, which concentrates on single nucleotide polymorphisms associated with seven housekeeping genes (including *atpD, fusA, glnS, gltB, gyrB, infB*, and *pps*) and identifies the related alleles (Joseph, Cetinkaya et al. 2012). This convention has been utilized to portray a portion of the assorted qualities related to the genus (Hariri, Joseph et al. 2013). Both PFGE and MLST have been broadly used to study the genomic diversity of *Cronobacter* isolated from manufacturing facilities, business PIF, follow-up equation and also clinical isolates [122,135,136].

7.8 Multilocus Sequence Analysis (MLSA)

To obtain a higher resolution of the phylogenetic relationships of species within a genus or genera within a family, multi locus sequence analysis (MLSA) is used widely. In MLSA studies, partial sequences of genes coding for proteins with conserved functions ('housekeeping genes') are used to generate phylogenetic trees and subsequently deduce phylogenies. Although MLSA is not only suggested as a phylogenetic tool to support and clarify the resolution of bacterial species with a higher resolutionas in 16S rRNA gene-based studies, it has been discussed as a substitution for DNA-DNA hybridization (DDH) in species delineation. However, despite the fact that MLSA has become an accepted and widely used method in prokaryotic taxonomy, no common generally accepted recommendations have been devised to date for either the whole area of microbial taxonomy or for taxa-specific applications of individual MLSA schemes [137].

7.9 Multiple-Locus Variable Number Tandem Repeat Analysis (MLVA)

Multiple-Locus Variable number couple repeated investigation (MLVA) is a technique utilized to actualize sub-atomic writing of specific microorganisms. It utilizes the naturally occurring variation in the number of tandem repeated DNA sequences found in many different loci in the genome of a variety of organisms [138].

7.10 Duplex Polymerase Chain Response (PCR) in Mix with Slender Electrophoresis–Laser Incited Fluorescence (CE–LIF) Locator

Duplex PCR incorporated into narrow electrophoresis-laser-prompted fluorescence sensor is another method to identify the species in food products. Also it is suitable to detect food borne pathogenic bacteria that may be present in small or large amounts in the samples. low or high numbers of food borne pathogenic bacteria in samples. This procedure could make the whole recognition procedure of *Cronobacter* spp. faster, less expensive, more sensitive and environmentally friendly [139].

7.11 Molecular O-Antigen Typing

O-antigen is a constituent of LPS (Lipopolysaccharide) structure which is arranged on the external surface of Gram-negative microbes and is in charge of serological assorted qualities. As a Gram-negative pathogen, the cell surface of *Cronobacter* is ensured with a thick region of LPS (endotoxin) with a lipid center, the lethal moiety framing 8% of the aggregate LPS weight [140]. It was also shown that different strains of *Cronobacter* have the LPS with different structures, and they exhibit refinement in pathogenicity which rely upon contrasts to structure (linearversus spread) and synthesis of LPS.

It has been described as a useful method to depict Gram-negative bacteria, O-antigen typing and studies describing the nature of the O-antigen associated with *Cronobacter* spp. [141, 142]. Mullane, O'Gaora et al. [141] initially developed a molecular serotyping method, based on amplification of the rfb encoding locus (in Gram-negative enteric bacteria and this is located between *halF* and *gnd*) followed by MboII digestion.

7.12 Fluorescence in Situ Hybridization

Fluorescence in situ hybridization is a technique to detect the bacteria. It is based upon thebinding of specific probes to nucleic acid target regions. This method is used in PIF when there are mixed bacterial populations and in lower concentration to other bacteria(Almeida, Azevedo et al. [143] which recently described the application of a novel peptide nucleic acid probe in detecting *Cronobacter spp*. in PIF. Studies demonstrated that these methods can distinguish *Cronobacter* spp. They have also observed that peptide nuclei corrosive fluorescence in situ hybridization facilitated the detection of *Cronobacter* spp.

8. NUMEROUS LOCUS VARIABLE-NUMBER COUPLE REHASH INVESTIGATION

Recently, other molecular subtyping techniques have been developed and compared with PFGE for the analysis of Cronobacter strains. Variable-number tandem-repeat motifs are short repeat sequences dispersed throughout bacterial genomes that are highly polymorphic. These regions have been used for subtyping bacteria [143-146].

9. PREVENTION & CONTROL MEASURES

There is a high bacterial resistance to low pH, high temperature, dryness and osmotic stress, therefore, baby food industry tried to minimize bacterial food safety hazards in their products. Safety measures in PIF are as follows: bioactive peptides, probiotics and prebiotics, protectants or optional procedures, organic acids, HHP, gamma irradiation, supercritical carbon dioxide, head lettuce by utilizing a combination and a union of ultrasound and sodium hypochlorite, presentation to microwaves or to a union of UV and close infrared radiation warming [51]. Contamination can occur in post-pasteurization stages or at the time of product preparation or before using the product, therefore, these steps can be considered as critical control points. The factors which increase the infection are: the patient sensitivity, resilience to temperature, dosage of irresistible, development rate and the destructiveness of the microorganism. In general, there must not be any sign of the microorganism in 10 grams of the tested product taken from 30 samples [18]. The combined steps to decrease the risks of *C. sakazakii* infection are given in Table 5 [18,20,51,169].

Table 5. Combined steps to reduce risks of *C. sakazakii* infection

During production
Preparation of powdered formula for aseptic technical skills by trained personnel.
Supervision of raw substances, specifically before using components which are needed to heat up.
Reducting the level of bacterial contamination in the early stages of production to reduce or prevent the presence of bacteria in the next steps
Increasing the frequency of inspections in food production environments and end products
Recognize potential pollution resources and enhance them.
Adjusting correct instructions regarding high water temperature, but not higher than 80 ℃, for solubility (> 70 ℃). Too high temperature damages the nutritional features of the product.
Enforce appropriate control measures which can assess potential hazards, identify critical control points (CCP), monitor non-conformities and necessary corrective actions, and register results.

At home
Use sterilized containers
Products should be kept in refrigerator right after the usage at 2-3 ℃ and use the formula within 2 h of preparation.
Prepare enough food and do not prepare for more than 1 or 2 meals.
Select the proper newborn formula which is sold in liquid form, especially when your baby is a newborn one or very young. The liquid formulations of infant formula have been made to be sterilized and should not contain *Cronobacter* spp.
In hospitals
Producing guidelines regarding preparation, protection and control of the products and the provision of these guidelines by trained personnel.
Sterilize all utensils used for preparation with thermal treatment (washing in dishwashers) or with autoclave.
Whenever possible use disposable tool and throw them away after usage.
Use qualified and specialized personnel (dieticians).

10. CONCLUSION

C. sakazakii is considered as an opportunistic pathogen and it is classified as category B which means it could cause infections in all age groups, specifically the newborns, with resistance pattern against many antibiotics. Food and Drug Administration (FDA) procedures ,in order to identify and isolate these bacteria from PIF, are time consuming and labor intensive ,so new molecular procedures are being used instead. Molecular methodologies based on nucleic acid recognition such as PCR and PFGE have been successfully improved during the last few years, especially *rpoB* and *ompA* which were more applicable among various researches. Consequently, all these new advances still need further improvements, such as reproducibility and cost advantage proportion issues, before they are considered as standard ones.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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