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REVIEW

Cronobacter spp., foodborne pathogens threatening neonates and infants

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Abstract *Cronobacter* spp. (formerly *Enterobacter sakazakii*) are special foodborne pathogens. *Cronobacter* infection can cause necrotizing enterocolitis, sepsis and meningitis in all age groups, especially neonates and infants, with a high fatality of up to 80%, although the infection is rare. Outbreaks of *Cronobacter* infection are epidemiologically proven to be associated with contaminated powdered infant formula (PIF). *Cronobacter* spp. can resist dry environments and survive for a long period in food with low water activity. Therefore, *Cronobacter* spp. have become serious pathogens of neonates and infants, as well as in the dairy industry. In this review, we present the taxonomy, pathogenesis, resistance, detection and control of *Cronobacter* spp.

Keywords *Cronobacter* spp., desiccation resistance, pathogen control, pathogen detection, powdered infant formula

1 Introduction

Cronobacter spp. (formerly *Enterobacter sakazakii*) are Gram-negative foodborne pathogens that can cause necrotizing enterocolitis, sepsis and meningitis in all age groups, especially neonates and infants[1,2]. Fatality resulting from infections in neonates and infants is up to 80%[3,4]. Thus, the International Commission for Microbiological Specifications for Foods listed *Cronobacter* spp. as "severe hazard for restricted populations, life threatening or substantial chronic sequelae or long duration"[5]. In 2004 and 2006, FAO-WHO listed *Cronobacter* spp. and *Salmonella* spp. as Category A pathogens after reviewing the organisms found in powdered infant formula (PIF) and epidemiologically linked with neonatal infections[6].

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2 Taxonomy of *Cronobacter*

In 1961, two doctors reported the first cases of *Cronobacter* spp. infection in the UK[7]. In 1965, *Cronobacter* spp. were called "yellow-pigmented *Enterobacter cloacae*" because of their similar characteristics to *Enterobacter cloacae* and their yellow pigment production[8]. The differences between *Cronobacter* spp. and other Enterobacteriaceae were revealed with the development of technology. DNA-DNA hybridization distinguished *Cronobacter* spp. from *Enterobacter cloacae* (difference between *Cronobacter* and *Enterobacter cloacae* was over 50%) and *Cronobacter* spp. were named as *Enterobacter sakazakii* in 1980. 16S rDNA and hsp60 sequencing justified *Cronobacter* being erected as a new genus[9]. Currently there are seven described *Cronobacter* spp.: *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. universalis*, *C. muytjensii*, *C. condimenti* and *C. dublinensis* (with three subspecies *dublinensis*, *lactaridi* and *lausannensis*)[10].

Analysis of 16S rRNA gene sequence is widely used in phylogenetic studies of bacteria. It can identify strains to genus, and often to species[11]. However, 16S rRNA gene sequence analysis is not accurate enough to distinguish *C. sakazakii* from *C. malonaticus*[9]. Multilocus sequence typing is a better option for *Cronobacter* species identification. It characterizes bacterial isolates by multiple housekeeping gene sequence analysis (usually seven genes). A multilocus sequence typing scheme has been established for *Cronobacter* based on the genes *atpD, fusA, glnS, gltB, gyrB, infB* and *ppsA*. All seven species of *Cronobacter* spp. can be identified and distinguished[12] and the results also show that *C. sakazakii* strain ST4 has caused the major meningitis cases investigated[6]. The

protocol and related data are publicly accessible online (PubMLST website: Cronobacter MLST Database), providing profiles and details of 2233 strains (accessed: 28 October 2017).

3 Disease caused by *Cronobacter* infection

All *Cronobacter* spp. are associated with human infections except for *C. condiment*[10]. In the US, the infection rate of *Cronobacter* is about 1:100000[13]. The US Centers for Disease Control and Prevention (CDC) usually reports four to six infection cases every year (data source: CDC website/CDC features/Disease and Conditions/Learn about *Cronobacter* infection, accessed: 10 April 2017). However, the CDC also said they did not have a full count. Developing countries reported fewer infection cases, perhaps due to the absence of well-established microbial analysis systems[14]. Therefore, the infection incidence is probably underestimated. Nevertheless, it is certain that *Cronobacter* infection is rare. However, the high mortality, serious neurological sequelae and susceptible population of the infection still make it a serious health threat. *Cronobacter* infection is also the most costly food-associated infection in the US due to loss of life and complicated treatment after acute infection, with an estimated cost of 1 million USD per case[15].

Cronobacter usually causes meningitis, septicemia bacteremia, and necrotizing enterocolitis[3]. Meningitis is the most common symptom of *Cronobacter* infection, accounting for about 42% of all cases. Neonate and infant patients may have symptoms of fever, irritability and high-pitched crying. Meningitis may further develop into vasculitis, cerebritis, ventriculitis, hydrocephalus and brain abscesses at a surprising rate[16]. The death rate is about 50%[17] and progression to death is usually rapid in infant patients. Survivors often suffer from serious neurological sequelae such as quadriplegia and mental retardation[14,18]. Necrotising enterocolitis is another important symptom of *Cronobacter* infection. Necrotising enterocolitis induced by *Cronobacter* spp. has a mortality rate of 19.0%[19]. Factors such as an incomplete immune system, hypothermia and hypoxia contribute to the necrotising enterocolitis development[20]. Table 1 shows *Cronobacter* spp. infection cases over the past 10 years.

4 Pathogenesis of *Cronobacter*

Cronobacter spp. generally infect the human body through the digestive system. Intestinal epithelium invasion is the first step of *Cronobacter* infection. Then, neonates and infants may suffer from necrotizing enterocolitis. After that, *Cronobacter* may enter and multiply in the blood stream to survive and proliferate in blood macrophages. Thus, *Cronobacter* spp. can spread throughout the body via blood circulation and patients develop symptoms of bacteremia. Meningitis occurs after *Cronobacter* spp. invade brain endothelium and cross the blood brain barrier. In conclusion, attachment, invasion and host cell injury by *Cronobacter* infection are the main pathogenic route, and this involves various virulence factors.

Protein ompA is an outer membrane protein of *Cronobacter* spp. It is important in *Cronobacter* invasion of cells. *Cronobacter* strains lacking gene *ompA* show reduced invasion, 87% less in INT-407 cells[26] and 83% less in human brain microvascular endothelial cells[27]. Protein ompA can also promote the attachment of *Cronobacter* spp. to INT-407 epithelial cells but cannot improve its attachment to human brain microvascular endothelial cells[17]. The attachment mechanism is likely host cell-type specific.

Lipopolysaccharide is a major cell wall component of Gram-negative bacteria. It is stable even at 100°C and can persist in PIF for a long time after spray drying. It helps *Cronobacter* spp. translocate through the body and cross the blood-brain barrier to cause meningitis[28,29]. Lipopolysaccharide can also cause inflammatory response and inhibit tissue repair[30,31]. This contributes to the development of necrotising enterocolitis and meningitis.

The Type VI secretion system was found recently. About 25% of Gram-negative bacteria including *Cronobacter* have this system[32] and it has important roles in adhesion, virulence, invasion and proliferation of *Cronobacter* in cells. This system also produces proteinaceous toxins that are potential virulence factors[17]. Five related genes were identified from the *Cronobacter* genome sequence and their contributory roles in meningitis are under investigation[33–36].

Other factors such as flagella, zinc metalloprotease, *pmrA* gene and *Cronobacter* plasminogen activator also participate in the invasion of *Cronobacter* spp.[37–40].

Table 1 Reported cases of *Cronobacter* spp. infection in neonates and infants since 2006

Year	Location	No. of infections/(death)	Age	Symptoms	Source
2006	India	1/(0)	2 months	Septicemia	NS[21]
2007	India	2/(1)	NS	Respiratory disease	PIF [21]
2008	USA	2/(1)	NS	Severe brain injury and hydrocephalus	NS[22]
2009	India	1/(0)	NS	Urinary tract infection	NS[23]
2013	USA	1/(0)	33 days	Bacteremia	PIF (possible)[24]
2014	USA	1/(0)	18 days	Meningitis and neurological sequelae	Non-fortified breast milk[25]

Note: NS, not specified.

However, further studies are still needed to confirm their function.

Studies on *Cronobacter* spp. have increased exponentially in recent decades. However, the precise pathogenic mechanism of *Cronobacter* spp. is still unclear. A thorough understanding this mechanism should provide better strategies to treat and control infection. Consequently, the fatality of *Cronobacter* infection could be reduced and patients with neurological sequelae will have better quality of life.

5 Source of *Cronobacter*

Cronobacter spp. are ubiquitous in the nature. They have been isolated from PIF, herbs, fruits, water, meat and food processing equipment[41,42]. Table 2 shows *Cronobacter* spp. isolated from different sources over the past 5 years. Of all sources, PIF is epidemiologically linked with neonates and infants infections[21,55,67–69]. Although water activity of PIF is low and so numbers of *Cronobacter* spp. are low, they can proliferate with inappropriate storage and reconstitution.

6 Desiccation resistance of *Cronobacter*

Contaminated PIF is the critical infection source of *Cronobacter* spp. PIF has a low water activity (a_w: 0.2–0.5) and most common pathogens cannot survive for a long time in such a dry environment, however, *Cronobacter* is an exception. Edelson-Mammel et al.[70] studied the long-term survival of *Cronobacter* spp. in milk powder. The initial inoculum dosage was about 10^6 CFU·mL^{-1} and there were still 300 CFU·mL^{-1} in the milk powder after 687 days' storage. Furthermore, Barron and Forsythe[71] prepared milk powder samples inoculated with Enterobacteriaceae including *Cronobacter* spp. These samples were kept at room temperature for 30 months. All non-*Cronobacter* strains had died by 15 months but some *Cronobacter* strains survived for 30 months. Fei et al.[72] studied the desiccation resistance of six *C. sakazakii* strains and two *C. malonaticus* strains isolated from PIF and processing environments. The results showed that *C. sakazakii* ST4, ST8 and ST12 had the greatest survival after 1 year. Among these strains, *C. sakazakii* ST4 has caused the majority of *Cronobacter* meningitis cases. Therefore, the pathogenesis of *Cronobacter* spp. is likely

Table 2 Presence of *Cronobacter* spp. in foodstuff and the environment

Time	Source	Country	Reference
2012	Food powers	Republic of Korea	Chon et al.[43]
2012	Kitchen equipments	America	Kilonzo-Nthenge et al.[44]
2012	Dry goods	Indonesia	Gitapratiwi et al.[45]
2012	Food powers and agricultural products	Republic of Korea	Lee et al.[46]
2012	Environment and food samples	Czech Republic	Putthana[47]
2013	Spices and herbs	Syria	Belal et al.[48]
2013	Powdered infant formula and dairy products	Egypt	El-Gamal et al.[49]
2014	Dry goods	Japan	Ogihara et al.[50]
2014	Powdered infant formula	China	Pan et al.[51]
2014	Dairy products	Italy	Casalinuovo et al.[52]
2014	Vegetables	China	Chen et al.[53]
2014	Milk powder	China	Xu et al.[54]
2014	Infant formula foods, cereals, vegetables, fruits	China	Li et al.[55]
2015	Foods, herbs and environment samples	India	Singh[56]
2015	Weaning food and water samples	China	Cui et al.[57]
2015	Instant foods	China	Xu et al.[58]
2015	Rice flour	China	Huang et al.[59]
2015	PIF and infant formula production factory	China	Fei et al.[60]
2015	Pasta	Germany	Akineden et al.[61]
2016	PIF/Baby food	Egypt	Abdel-Galil et al.[62]
2016	Baby milk products	China	Li et al.[63]
2016	Food of plant origin and environmental samples from farms and supermarkets	Czech Republic	Vojkovska et al.[64]
2017	Retail foods	Brazil	Brandão et al.[65]
2017	Baby foods and baby food ingredients	Turkey	Heperkan et al.[66]

to be associated with their resistance to drying. Their desiccation resistance also improves the resistance of *Cronobacter* to other environment stress such as ionizing radiation[73], heat[74] and antimicrobial substances[75–78].

The mechanism of desiccation resistance in *Cronobacter* spp. is still under investigation. Trehalose is a disaccharide formed by two α-glucose units through an α,α-1,1-glucoside bond. It participates in the responses of many organisms to environment stresses, such as *Escherichia coli*[79], *Gossypium*[80] and *Saccharomyces cerevisiae*[81]. *Cronobacter* spp. mainly produce trehalose in the stationary phase after a drying treatment[82]. The resistance of *Cronobacter* spp. to drying is also improved when trehalose is added in the medium[83]. Transposon mutagenesis has been applied to analyze gene expression in *Cronobacter* spp. under osmotic and drying stress[84]. The osmotic and desiccation resistance mechanisms of *Cronobacter* spp. are quite different. Genes *dnaK* and *dnaJ*, encoding two molecular chaperones are responsible for resisting drying. *RpoS* (RNA polymerase sigma factor) that is a central regulator of stress response also regulates the process of desiccation resistance because *C. sakazakii* strains deficient in *rpoS* proved to be more sensitive to drying[85]. However, none of these genes is related to trehalose and the role of trehalose in the desiccation resistance of *Cronobacter* spp. needs further examination. Recently, a comparative proteomic analysis of *C. sakazakii* by iTRAQ was carried out. Results showed that expression level of genes involved in unnecessary survival functions, such as virulence, adhesion and invasion, decreased, while expression level of genes involved in trehalose and betaine uptake such as ABC transporter and secretion system increased during the response of *C. sakazakii* to desiccation[86]. As with the pathogenic mechanism of *Cronobacter* spp., current studies have not completely described the mechanism of desiccation resistance but it seems that multiple metabolic pathways are necessary. Further studies concerning desiccation resistance of *Cronobacter* spp. are encouraged, to figure out better hazard control strategies in the dairy industry and consuming process.

7 Detection of *Cronobacter*

Culture methods were first used to isolate and identify *Cronobacter* spp. The core of these methods was to determine the special physiologic properties of *Cronobacter* spp. They can produce α-glucosidase and this distinguishes them from other Enterobacteriaceae. α-Glucosidase can hydrolyze 4-nitrophenyl-α-D-glucopyranoside[87], 4-methylumbelliferyl-α-D-glucopyranoside[88] and other substrate to produce a color change. In conjunction with other characteristics such as yellow pigment production and insensitivity to vancomycin, *Cronobacter* spp. can be isolated from food samples and distinguished from other pathogens. The International Organization for Standardization then adopted this detection method as ISO 22964:2017 Microbiology of the food chain. The evaluation process for *Cronobacter* detection is shown in Fig. 1. Culture methods are important for *Cronobacter* detection. However, they are time consuming and these steps are complex. Therefore, simpler and more rapid detection methods have been developed.

Immunological methods have been developed for *Cronobacter* detection and enzyme linked immunosorbent assay (ELISA) has been widely used[86,87]. Nevertheless, ELISA still takes a long time. Therefore, a colloidal gold test strip for *Cronobacter* detection have been developed to shorten the time and simplify the steps[89]. It is easy to use and can be adapted to various situations. To make antibody preparation easier and faster, a single chain variable fragment specific to *Cronobacter* spp. was also identified and produced in a prokaryotic expression system[90]. Compared with polyclonal and monoclonal antibodies, the preparation time and cost of single chain variable fragment is reduced. Immunological methods can also be combined with better detection methods for *Cronobacter* spp., such as immunomagnetic separation-polymerase chain reaction (IMS-PCR)[91].

Nucleic acid amplification methods are more widely used in the detection of *Cronobacter* spp. Mining of specific gene targets is important in nucleic acid amplification detection methods. Here, we summarize these specific target genes used for the detection of *Cronobacter* spp. and the PCR and real-time PCR detection methods targeting them (Table 3). Isothermal amplification detection methods have also been also developed. These methods can be run at a stationary temperature and thermal cycling equipment is unnecessary, making isothermal amplification detection easier to carry out. Most isothermal amplification detection methods also show higher amplification efficiency than PCR methods[108] and isothermal amplification is a promising alternative to PCR. Two isothermal amplification detection methods have been developed for *Cronobacter*, loop-mediated isothermal amplification (LAMP)[109] and helicase-dependent isothermal DNA amplification (HDA)[110]. They are simpler, without the need for PCR amplification, and the sensitivity of the LAMP assay is 1000 and 100 times higher than regular PCR and real time quantitative PCR, respectively[109]. In addition to these main detection methods mentioned above, other valuable additions to the methods for *Cronobacter* detection have been developed, such as MALDI-TOF MS[111] and a label-free aptasensing platform[112].

However, due to low *Cronobacter* numbers in contaminated PIF[113] and the inhibiting effect of food components, enrichment is still necessary in order to increase cell concentrations to the detection limit. To improve detection efficiency, methods that enrich detection targets, such as immunomagnetic separation[91] and probe-magnetic separation[114], should be developed.

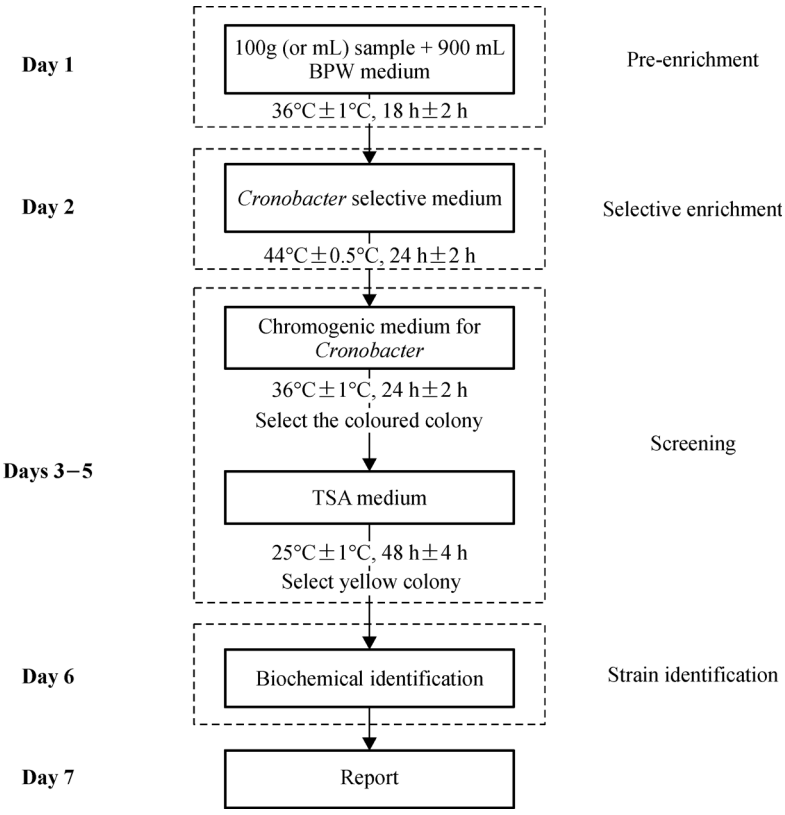

Fig. 1 The process for detection of *Cronobacter* ISO 22964:2017. BPW, buffered peptone water; TSA, tryptic soy agar.

8 Control of *Cronobacter* in PIF

Cronobacter spp. are ubiquitous in nature and they cannot be completely removed from production environments[115]. Hence, contamination in PIF can occur easily and PIF is the most common vehicle for *Cronobacter* infection. However, according to national standards, they should not be detectable in PIF given the severe consequences of infection. Therefore, it is important to develop inactivation methods for PIF. The organoleptic, nutritional and functional properties of PIF change under heat treatment[116], and drying increases the heat resistance of *Cronobacter*[74]. Therefore, there has been a focus on non-thermal inactivation methods for *Cronobacter* spp.

Hydrostatic pressure processing is a non-thermal sterilization method for food products[117]. Inactivation levels of five to seven $\log_{10}$ cycles in reconstituted PIF were observed with treatment at 100 to 600 MPa[118]. Gamma irradiation is another useful sterilization for PIF. *C. sakazakii* and concentration of 8 to 9 $\log_{10}$ CFU·g^{-1} in a dehydrated infant formula could be eliminated with irradiation at 5.0 kGy[119,120]. Ultrasound waves also inactivate *Cronobacter* spp. However, this method has a limited lethal effect compared with hydrostatic pressure and gamma irradiation. Researchers have tested the addition of antimicrobials to PIF to control *Cronobacter* spp., including lactoferrin, nisin[121], vanillin[75] and polyphenol-rich cocoa powder[122]. These all had good inhibitory effect on *Cronobacter* spp., although the safety of such antimicrobials needs to be fully assessed prior to

Table 3 Gene targets for *Cronobacter* detection

Target gene	Genus/Species	Reference
*dna*G	Genus	Seo et al.[92]
gluB	Genus	Lehner et al.[93]
$tRNA_{Glu}$	Genus	Hassan et al.[94]
23S rDNA	Genus	Derzelle et al.[95]
16S rDNA	Genus	Hassan et al.[94]
wehC, wehI	Species	Mullane et al.[96]
zpx	Genus	Jaradat et al.[97]
wzx	Genus	Jarvis et al.[98]
*gyr*B	Genus	Huang et al.[99]
*grx*B	Genus	Dong et al.[100]
*omp*A	Genus	Zimmermann et al.[101]
MMS	Genus	Tan et al.[102]
*cgc*A	Species	Hu et al.[103]
*rpo*B	Genus	Li et al.[104]
ITS	Genus	Liu et al.[105]
PapC	Species	Chen et al.[106]
*glu*B	Species	YE et al.[107]

being recommended or approved for industrial application.

The CDC, WHO and the US Food and Drug Administration have all provided guidelines to avoid contamination and infection of *Cronobacter* spp.: (1) PIF should be prepared in a clean location with sterilize bottles and plastic nipples; (2) The temperature of water for reconstitution must exceed 70°C; (3) The reconstituted PIF must be stored under 4°C and use within 24 h; (4) Once a feeding has started, the reconstituted PIF should be finished within 2 h[14].

9 Conclusions

To promptly identify contaminated food and control infection, reliable, sensitive and rapid detection methods for *Cronobacter* are necessary and need further development. In addition, we still know little about the transmission and pathogenesis of *Cronobacter* spp. Infection still occurs, albeit at low prevalence, though with often fatal consequences in neonates and infants. Control methods other than the current heating and drying during processing should be considered as alternatives or supplements, given the heating and desiccation resistance of *Cronobacter*. To thoroughly control the *Cronobacter* contamination of PIF, increased multidisciplinary effort is needed, including studies of infection surveillance systems, virulence factor identification, regulatory mechanism of resistant genes, efficient non-thermal inactivation methods and reliable prevention strategies.

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