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Cronobacter Species in Powdered Infant Formula and Their Detection Methods

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Abstract *Cronobacter* species have been associated with disease outbreaks and sporadic infections, particularly in premature and immunocompromised infants. *Cronobacter* species can cause foodborne infections such as neonatal meningitis, septicaemia and necrotising enterocolitis. Accordingly, there is an urgent need to control and monitor the *Cronobacter* species in food, especially in powdered infant formula (PIF) and other baby foods. Therefore, in this review, the isolation and prevalence of *Cronobacter* species in infant food including PIF and the recent advance of detection methods are discussed for the better understanding on the current research status of *Cronobacter* species.

Keywords *Cronobacter* species, powdered infant formula, isolation, prevalence, detection

Introduction

Powdered infant formula (PIF), an industrially produced substitute for mother's milk in the absence of breastfeeding, is a worldwide major source of nutrition for infants (Guo et al., 1998; Cama-Moncunill et al., 2016). Conventional bovine milk-based PIF is generally formulated using a specific combination of proteins, fats, carbohydrates, vitamins, and minerals. There is always being a high chance that PIF may be contaminated with pathogens which could lead a serious illnesses in infants (Cahill et al., 2008). Infants and young children are vulnerable for foodborne illness owing to their weak immune systems to fight against the infections. The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) (FAO, 2004) have discussed the microbial and microbial toxin-related contamination in PIF. Among the microorganism, *Cronobacter* species has a clear evidence of infection and illness in infants, including severe diseases with developmental sequelae and death. Therefore, monitoring and controlling *Cronobacter* species is particularly important to attest the safety of PIF. In view of the above, present work is a summary related

to researches of *Cronobacter* species in PIF, including pathogenicity, outbreaks, clinical cases, prevalence, as well as the detection and monitoring methods.

Pathogenicity of *Cronobacter* species in PIF

Cronobacter species are opportunistic pathogens, which cannot cause infectious disease when ingested by healthy adult but pose a very high risk to neonates and immuno-compromised individuals (Healy et al., 2010; Strydom et al., 2012). These organisms can affect the human central nervous system, and survivors often suffer from severe neurological impairments following infection, including hydrocephalus, quadriplegia, and developmental delays (Gallagher and Ball, 1991; Lai, 2001; Gurther et al., 2007; Strydom et al., 2012). *Cronobacter* species have caused diseases in all age groups, most importantly in infants, and pre-term, underweight, or immune-compromised infants at particularly high risk (FAO/WHO, 2004).

Cronobacter species have been isolated not only from plant food and products such as cereals, fruits and vegetables, legume, herbs, and spices, but also from the animal foods such as milk, meat, fish, and products made from these foods (Lee et al., 2012). Despite the reservoir and mode of transmission of *Cronobacter* species remain unknown, they are believed to be the principal sources of food contamination. Soil, water and vegetables, whereas rodents and flies may serve as a secondary route of contamination (Iversen and Forsythe, 2003). PIF is the only food source that has been epidemiologically linked to outbreaks of *Cronobacter* species (FAO/WHO, 2004).

Outbreaks and reported cases of *Cronobacter* species related with PIF

There are many reported cases of *Cronobacter* species since the first two documented cases of infection occurred in 1958 at St. Albans, England (Urmenyi and Franklin, 1961). Outbreaks and cases of *Cronobacter* species until 2005 had been well summarized by many researchers as well as shown in Table 1 (Gurtler et al., 2007; Bowen and Barden, 2006; Iversen and Forsythe, 2003). Pagotto and Farber (2009) reported a 17 days infant with born weight of 2.9 kg developed a *Cronobacter* infection leading to meningitis after fed infant formula powder that had been left at her doorstep. Centers for Disease Control and Prevention (CDC) (2009) reported 2 unrelated cases of infection of *Cronobacter* species in New Mexico, USA. A female infant was born in September 2008 at full term by vaginal delivery without complications and had been fed PIF exclusively since birth. *Cronobacter* species was positive in Cerebrospinal fluid cultures but was negative in blood cultures. After treatment in the hospital, the infant was discharged on February 6, 2009, with severe brain injury, hypertonicity resulting from central nervous system damage, and hydrocephalus. A male infant was born in April 2008 at 40 weeks' gestation by vaginal delivery without complications and breastfed exclusively until age 6 months and then began transitioning to PIF and age-appropriate foods. A *Cronobacter* organism was found in the blood culture obtained during autopsy. The two infants lived approximately 200 miles apart in different counties of mostly rural southeastern New Mexico. Flores et al. (2011) reported two cases of acute gastroenteritis occurred in 5-month-old infants hospitalized in a mother-and-child hospital. *C. sakazakii* was recovered from the PIF, rehydrated infant formula powder fed to infants, and their fecal samples and no deaths were observed in those cases. CDC (2012) reported 4 infants (children under 12 months of age) infected with *Cronobacter* bacteria resided in four states: Florida, Illinois, Missouri, and Oklahoma. One infant was born in slightly premature, and all four infants became infected during the first five weeks of age. Three infants developed meningitis (inflammation of the membranes around the brain and spine), and one had a bloodstream infection and 2 infants died. Broge and Lee (2013) reported a female infant born at 37 weeks via vaginal delivery to a 22-year-old mother. On day of life 33, the infant began

Table 1. Outbreaks and cases of infections

Location	Year	Cease/Death	References
St. Albans, England	1958	2/2	Urmenyi and Franklin, 1961
Denmark	1965	1/None	Joker et al., 1965
Macon, GA, USA	1979	1/Not mentioned	Monroe and Tift, 1979
Indianapolis, IN, USA	1981	1/None	Kleiman et al., 1981
The Netherlands	1983	8/None	Muytjens et al., 1983; Muytjens, 1985; Muytjens and Kollee, 1990
Athens, Greece	1985	1/Not mentioned	Arseni et al., 1985
Reykjavik, Iceland	1986-1987	3/1	Biering et al., 1989; Clark et al., 1990
Boston, MA, and New Orleans, LA, USA	1987	2/Not mentioned	Willis and Robinson, 1988
Memphis, TN, USA	1988	4/Not mentioned	Simmons et al., 1989
Baltimore, MD, USA	1990	1/Not mentioned	Noriega et al., 1990
Cincinnati, OH, USA	1990	1/Not mentioned	Gallagher and Ball, 1991
Israel	1993, 1995, 1997, 1998	4/Not mentioned	Block et al., 2002
Boston, MA, USA	1995/1996	5/4	Lai, 2001
Belgium	1998	12/2	Van Acker et al., 2001
Winston Salem, NC, USA	2000	1/None	Burdette and Santos, 2000
Israel	1997-2000	5/None	Block et al., 2002; Bar-Oz B et al., 2001
Knoxville, TN, USA	2001	10/1	Himelright et al., 2002; Weir, 2002
Tennessee, USA	2002	1/1	CDC, 2002
Wisconsin, USA	2002	1/Not mentioned	CDC unpublished data, Bowen and Barden, 2006
USA	2003	6/Not mentioned	CDC unpublished data; Bowen and Barden, 2006
France	2004	2/Not mentioned	Coignard et al., 2004; Bowen and Barden, 2006
USA	2004	2/Not mentioned	CDC unpublished data; Bowen and Barden, 2006
USA	2005	2/Not mentioned	CDC unpublished data; Bowen and Barden, 2006
Canada	2007	2/Not mentioned	Pagotto and Farber, 2009
New Mexico, USA	2008	2/None	CDC, 2009
Queretaro, Mexico	2010	2/None	Flores et al., 2011
Florida, Illinois, Missouri, and Oklahoma, USA	2011	4/2	CDC, 2012
Not mentioned	2012	1/None	Broge and Lee, 2013
China	2017	2/1	Cui et al., 2017

sleeping more than usual and was not feeding well. Her mother recorded an axillary temperature of 38.3°C, prompting a visit to her pediatrician. *Cronobacter* species was positive from blood cultures. The infant was discharged after treatment. Recently, Cui et al. (2017) reported two cases of *Cronobacter* species infection of infants in China. The first case was of a female infant born after 36 weeks of gestation that developed fever and muscle spasms in her extremities and fed poorly after 11 days of birth. *Cronobacter* was isolated from the cerebral spinal fluid using selective medium. The second case was an abandoned female infant with no detailed birth record. She was fed with PIF and developed anemia, diarrhea, moderate dehydration, and severe malnutrition after an operation when she was 10 days old. The infant died due to circulatory and respiratory failure after 4 days in hospital. *Cronobacter* was isolated and identified from the blood sample of infected infant.

Prevalence of *Cronobacter* species in food

Cronobacter species can be isolated from a range of environments, as well as clinical, food, and beverage sources such as water, vegetables, cheese, meat and ready-to-eat foods. Most of the documented outbreaks of *Cronobacter* species have been associated with consumption of contaminated PIF. Therefore, isolations of *Cronobacter* species from infant food and dairy food in recent years are summarized in Table 2. According to Jung and Park (2006), 20% of infant formula powder was contaminated with *Cronobacter* in the Republic of Korea. A study carried out by Shaker et al. (2007) showed that *Cronobacter* species have been isolated from 17% of PIF, all the food samples were purchased from retail stores across Jordan. Fu et al. (2011) also reported that 4% (3/77) of analyzed PIF were positive for the *Cronobacter* species. Lee et al. (2012) also reported that *Cronobacter* species were isolated from 18.6% of detected food samples. Li et al. (2014) analyzed 195 food samples, including cereals, cereal products, PIF, infant food formula, herbs, spices, vegetables, and fruits collected from the local supermarkets in Nanjing, China for the presence of *Cronobacter* species. PIF and infant food formula were negative while 14.1% (12/85) cereal and cereal products and 4.5% (1/22) spices were positive for *Cronobacter* species. Pei et al. (2016) collected 2,282 samples of PIF and follow-up formula from the retail markets in China from January, 2012 to December, 2012 to determine *Cronobacter* species contamination in PIF and follow-up formula in China. *Cronobacter* species have been isolated from 1.1% (25/2,282) collected samples, and the positive rates in infant formula powder and follow-up formula were 0.90% (10/1,011) and 1.18% (15/1,271). Pan et al. (2014) also surveyed 399 samples of PIF and follow-up formula purchased from retail stores and supermarkets in Shijiazhuang, China. The positive rates from PIF and follow-up formula were 11.5% (19/165) and 12.8% (30/234), respectively, including 48 *C. sakazakii* and 1 *C. malonicus*. These researchers reported the analysis and isolates of *Cronobacter* species in PIF, despite the source of contamination have not mentioned. Yao et al. (2016) isolated *Cronobacter* species from 12% (16/133) of indigenous infant flours sold in public health care centers with in Abidjan, Cote d'Ivoire.

Physiological and Biochemical Characteristics of *Cronobacter* Species

Cronobacter species are Gram-negative, facultative anaerobic, oxidase-negative, catalase-positive, and rod-shaped bacteria belonging to the family Enterobacteriaceae (Strydom et al., 2012). Most of the *Cronobacter* species are motile, reduce nitrate, use citrate, hydrolyze esculin and arginine, and are positive for L-ornithine decarboxylation (Strydom et al., 2012). Besides this, they frequently produced acid from D-glucose, D-sucrose, D-raffinose, D-melibiose, D-cellobiose, D-mannitol, D-mannose, L-rhamnose, L-arabinose, D-trehalose, galacturonate, and D-maltose (Strydom et al., 2012). Most of the

Table 2. Prevalence of *Cronobacter* in powdered infant formula

Year	Location	Positive rate (%)	References
2006	Republic of Korea	20	Jung and Park, 2006
2006	Jordan	18	Shaker et al., 2007
2011	China	4	Fu et al., 2011
2012	China	1.1	Pei et al., 2016
2012	Republic of Korea	18.6	Lee et al., 2012
2014	China	0	Li et al., 2014
2014	China	11.5	Pan et al., 2014
2016	Abidjan, Cote d'Ivoire	12	Yao et al., 2016

Cronobacter species are also positive for acetoin production (Voges–Proskauer test) and negative for the methyl red test, indicating 2,3-butanediol fermentation rather than mixed acid fermentation (Strydom et al., 2012). Urmenyi and Franklin (1961) reported a case of neonatal infection caused by a pigmented coliform in 1961, which was the first published report of *C. sakazakii* (Beuchat et al., 2009). In 1980, the “yellow-pigmented *Enterobacter cloacae*” was reclassified as a new species of *Enterobacter sakazakii* (Farmer et al., 1980). This organism was first defined as a new genus in 2007, when Iversen et al. (2008) proposed reclassification of *Enterobacter sakazakii* within a novel genus of *Cronobacter*, containing *C. sakazakii*, *C. malonaticus*, *C. turicensis*, and *C. muytjensii* (Iversen et al., 2007; Iversen et al., 2008). After, five new *Cronobacter* species, *Cronobacter condiment*, *Cronobacter universalis*, *Cronobacter zurichensis*, *Cronobacter helveticus*, and *Cronobacter pulveris* were added to this group (Brady, 2013). In 2014, Stephan et al., reclassified *C. zurichensis*, *C. pulveris*, and *C. helveticus* as *Franconibacter helveticus*, *Franconibacter pulveris*, and *Siccibacter turicensis* (Stephan et al., 2014). Currently, the genus *Cronobacter* contains seven species, *Cronobacter sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dublinensis*, *C. condimenti*, and *C. universalis*.

Detection Methods for *Cronobacter* Species

Culture based detection methods

Culture based detection is traditional and most widely used method for detecting foodborne pathogens. The culture methods most often used for the detection of *Cronobacter* species are based on the United States Food and Drug Administration (USFDA) method for isolation and enumeration of PIF (Druggan and Iversen, 2009; Strydom et al., 2012). In 2002, the USFDA devised a most probable number method for isolation and identification of *Cronobacter* species in PIF. However, this method requires food samples to be pre-enriched, and 5-7 days are required to identify organisms and confirm the results (USFDA, 2002). For this method, 1 g, 10 g, and 100 g of infant formula powder are rehydrated in sterile distilled water and incubated at 36°C overnight to resuscitate stressed cells, after which 10 mL of sample are taken from the pre-enrichment broth and added into 90 mL of Enterobacteriaceae Enrichment broth for incubating at 36°C overnight. Next, the enrichment broth is spread or streaked onto violet red bile glucose agar (VRBGA), after which the colonies selected by VRBGA are streaked onto tryptone soya agar (TSA). Following cultivation, samples are tested using an API 20E kit for identification.

In 2012, the USFDA released a new method for isolation and enumeration of *Cronobacter* from formula samples and confirmation of *Cronobacter* using PCR screening and selective media (USFDA, 2012). In this method, formula samples are incubated in buffered peptone water (BPW) at a 1:10 ratio and 36°C for 24±2 h without shaking. At 6-8 h, four aliquots of 40 mL enrichment culture of formula sample are removed from the culture and centrifuged at 3,000×g for 10 min to collect the *Cronobacter*. The collected bacterial cells are then suspended in 200 µL of phosphate buffered saline. Two of these samples are used for real-time PCR and the other two are plated onto two Druggan-Forsythe-Iversen (DFI) chromogenic agar plates and two R & F *E. sakazakii* chromogenic agar plates. If the 6-8 h culture are confirmed as positive by real-time PCR and RAPID ID 32E, no further analysis is necessary, otherwise, the 18-24 h culture is processed and analyzed (USFDA, 2012).

The International Organization for Standardization (ISO, 2006) also released a method for the detection of *Cronobacter* species in milk and milk products. In this method, sample was incubated in BPW at a 1:10 proportion at 37°C for 18±1 h without shaking (ISO, 2006). Next, 0.1 mL of culture was inoculated into 10 mL of Modified Laurylsulfate-Tryptose broth with vancomycin and incubated for 24±2 h at 44±0.5°C, followed by streaking onto selective chromogenic agar and incubating for 24±2 h at 44±1°C. Five colonies selected from selective chromogenic agar were then streaked onto TSA plates and incubated for 24±2 h at 25±1°C, after which a colony from each TSA plate was tested by biochemical identification (ISO,

2006). The ISO method also requires 5 days to complete all tests.

In addition to the FDA and ISO methods, several selective media have been developed for the isolation and detection of *Cronobacter* species. Iversen et al. (2008) developed a differential medium, *Cronobacter* screening broth (CSB), to complement agars based on hydrolysis of chromogenic α -glucopyranoside substrates. In this method, spiked samples are incubated for 24 h at 37°C, then diluted to 1:10 in CSB and incubated for 24 h at 42°C. Each sample is then streaked onto TSA that is subsequently incubated at 37°C for 24 h to detect presumptively positive *Cronobacter* colonies. The presumptively positive *Cronobacter* colonies are distinguished using the methyl red and Voges-Proskauer tests. Kim and Rhee (2011) also reported a selective and differential medium for the isolation of *Cronobacter* species, which contained salicin as a differential agent. In this new medium, *Cronobacter* species generates typical colonies with characteristic violet-colored centers surrounded by a transparent to opalescent border, and the growth of other microorganisms are inhibited or produce visually distinguishable colonies. Oh and Kang (2004) developed a fluorogenic selective and differential medium for isolation of *Cronobacter* species using 4-methylumbelliferyl-D-glucoside as a selective marker. For this technique, *C. sakazakii* mixed with cocktail is plated onto OK medium and incubated, resulting in distinct fluorescent colonies when exposed to long-wavelength UV light. The fluorescent colonies are then confirmed using the API 20E biochemical system.

PCR based detection methods

PCR is the most well-known and established nucleic acid amplification technique for detection of pathogenic microorganisms (DeCory et al., 2005; Stoop et al., 2009; Zhao et al., 2013). In this method, double-stranded DNA is denatured into single strands, and specific primers or single-stranded oligonucleotides anneal to these DNA strands, followed by extension of the primers complementary to the single stranded DNA with a thermostable DNA polymerase. These steps are repeated, resulting in doubling of the initial number of target sequences with each cycle. The quantity of the products of amplification can be visualized as a band on an ethidium-bromide-stained electrophoresis gel. Identification based on PCR amplification of target genes by sequencing is considered to be a reliable technique when properly developed and validated for a certain species. With the distinct advantages of rapidity, specificity, sensitivity, and fewer samples over culture-based methods, many PCR assays for the detection and validation of foodborne bacteria and viruses in food have been developed and applied. In addition to PCR, a number of gene-specific hybridization probes have been designed and used for the detection of toxin genes in foodborne pathogens (DeCory et al., 2005; Ikeda et al., 2007; Xu et al., 2014a; Zhao et al., 2014). A new method combining a real-time PCR based assay with selective agar has been also developed that can efficiently reduce the detection time to 2 days (Lampel and Chen, 2009). Stoop et al. (2009) reported a different *rpoB* based PCR system that enabled identification to the species level of strains previously confirmed to belong to the genus *Cronobacter* by using designed primer pairs based on the *rpoB* sequences of six *Cronobacter* species type stains. Zimmermann et al. (2014) developed a fast and sensitive PCR based detection system for *Cronobacter* species that consisted of enrichment, DNA-isolation and detection by real-time PCR using the outer membrane protein gene *ompA* as a target. Zhou et al. (2008) reported an immobilization and detection method of *C. sakazakii* from PIF. In this method, *C. sakazakii* cells were immobilized with zirconium hydroxide and separated from the sample by centrifugation. The immobilized cells were then suspended in sterile nutrient broth and subjected to DNA extraction for duplex PCR detection. Ruan et al. (2013) reported a method of detecting *Cronobacter* species by duplex PCR in combination with a capillary electrophoresis-laser induced fluorescence detector. The specific gene sequences of the 16S-23S rDNA internal transcribed spacer and the outer membrane protein A (OmpA) of *Cronobacter* species were amplified by duplex PCR. The PCR products were separated and determined sensitively by

capillary electrophoresis-laser-induced fluorescence within 12 min. Wang et al. (2012) reported a TaqMan real-time PCR assay incorporating an internal amplification control for the detection of *Cronobacter* species. In this study, the sensitive and accurate TaqMan real-time PCR assay was improved by the introduction of internal amplification control to control false-negative results that may be caused by malfunction of the thermal cycler, incorrect PCR mixture, inhibitory substances, etc.

Immunological detection methods

Methods based on antigen-antibody binding are widely used to identify foodborne pathogens. An indirect non-competitive enzyme-linked immunosorbent assay (INC-ELISA) (Song and Kim, 2013) and a sandwich ELISA (Park et al., 2012) were reported for the detection of *C. muytjensii* in formula powder. These methods were sensitive and efficient for the detection of *C. muytjensii* using anti-*C. muytjensii* IgG. Similarly, Xu et al. (2014b) reported two novel analytical methods, sandwich ELISA and indirect enzyme-linked ELISA, based on polyclonal and monoclonal antibodies for rapid detection of *Cronobacter sakazakii*. Song et al. (2015) reported a fluorescence-based liposome immunoassay for detection of *C. muytjensii* that used developed anti-*C. muytjensii* IgG targeted liposome. In this study, samples or cultures were coated onto 96 well plates and anti-*C. muytjensii* IgG targeted liposome was added and incubated. The immunoliposome bounded to the cell was lysed and measured at an excitation wavelength of 550 nm and an emission wavelength of 585 nm using the microplate reader. Blazkova et al. (2011) reported an immunochromatographic strip test for detection of members of the genus *Cronobacter*. Following the shortened bacteria cultivation and isolation of DNA, a specific gene sequence targeting 16S rRNA from *Cronobacter* species was amplified by PCR using 5-end labeled specific primers. The PCR product, amplicon labeled with digoxigenin on one side and biotin on the other side, was added directly to a immunochromatographic strip test composed of nitrocellulose membrane with bound antibody against digoxigenin in the test line. The visualization was mediated by colloidal carbon conjugated to neutravidin, and the appearance of a grey/black line was indicative of the presence of a specific amplicon. The color intensity of the test line in the pathogen-positive assay was visually distinguishable from that of negative samples within 10 min. Zhao et al. (2010) reported a method of cross-priming amplification under isothermal conditions combined with immuno-blotting analysis. Briefly, a set of specific displacement primers, cross primers and testing primers were designed based on six specific sequences in the *Cronobacter* species 16S-23S rRNA internal transcribed spacer. The specific amplification and hybridization steps were processed simultaneously under isothermal conditions at 63°C for 60 min.

Biosensor based detection methods

Tools for on-site detection of *Cronobacter* species are also receiving a great deal of attention in recent years (Vigayalakshmi, 2010). Several rapid methods to detect *Cronobacter* species have been reported. Chen et al. (2014) reported an immunochromatographic strip for rapid detection of *Cronobacter* species in powdered infant formula in combination with silica-coated magnetic nanoparticles separation and a 16S rRNA probe. In this method, silica-coated magnetic nanoparticles were used to separate nucleic acid from *Cronobacter* cell lysate and eliminate the interference of food matrices and two 5'-end labeled probes that were complementary to the 16S ribosomal DNA of *Cronobacter* were used to hybridize with the nucleic acid. The hybrid product, labeled with digoxigenin on one side and biotin on the other side, was submitted directly to the immunochromatographic strip test which anti-digoxigenin monoclonal antibody was immobilized on nitrocellulose membrane in the test line. Visualization was achieved using gold nanoparticles conjugated to streptavidin, and double red bands appearing in both the test and control line indicated the presence of *Cronobacter* in the sample. Xu et al. (2014a) reported a method of probe-magnetic separation PCR for detection of *Cronobacter* species. In this method, a specific probe for *Cronobacter* species

was designed, verified and conjugated on magnetic-beads. The targeted gene captured by the probe-magneticbead was separated from tested samples by magnetic force. Next, the target gene captured by probe-magneticbeads, and the total number of target sequences in each dilution was detected with primer pair CsspF₁ and CsspR₁ by using real-time qPCR. This system is able to detect as few as 10³ CFU/mL of *Cronobacter* species in artificially contaminated PIF in less than 4 h, without prior lengthy pre-enrichment culturing of samples. Shukla et al. (2016) developed an immunoliposome-based immunomagnetic concentration and separation assay for the rapid detection of *C. sakazakii*. In their method, *C. sakazakii* cells were separated and concentrated by magnetic nanoparticles (diameter 30 nm) which coated with anti-*C. sakazakii* IgG, followed by reaction with immunoliposomes which were modified with anti-*C. sakazakii* IgG. After removing the free immunoliposomes by washing, the immunomagnetic nanoparticle-*C. sakazakii*-immunoliposome complexes was mixed with n-octyl- β -D-glucopyranoside solution and measured an excitation wavelength of 550 nm and an emission wavelength of 585 nm using a microplate reader. The detection limit of this method was around 10³ CFU/mL in both pure culture and in PIF. Mullane et al. (2006) developed a method using cationic-magnetic-beads to capture *C. sakazakii*, followed by identification after plating the captured *C. sakazakii* onto DFI chromogenic agar, which can detect 1-5 CFU/500 g of PIF in less than 24 h. Zhao et al. (2013) reported an immunoassay based on nuclear magnetic resonance for detection of *Cronobacter* species in dairy samples with biofunctionalized magnetic nanoparticles. In this method, the amino-modified silica-coated Fe₃O₄ was conjugated with anti-*C. sakazakii*-antibody. *C. sakazakii* bound to the antibody-modified nanoparticles could be separated and measured based on their nuclear magnetic resonance using magnetic resonance relaxometry.

Other methods

Mass spectrometry based on intact cell matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) protein profiling is a rapid and reliable tool for identification of microorganisms that is now widely used, even in clinical laboratories (Koubek et al., 2012; Krasny et al., 2014). Javurkova et al. (2012) tested MALDI-TOF mass spectrometry for the detection of *Cronobacter* species and compared the results with those obtained by the classical cultivation microbiological method. Javurkova's method is specific and only requires 46 h compared with conventional method. Zhu et al. (2012) reported a method combining impedance technology and a rRNA lateral flow assay. In this method, 100 g of spiked or commercial PIF samples were diluted in 900 mL BPW and incubated at 30°C for 16 h, after which 0.1 mL of pre-enriched samples was transferred into measuring wells filled with 10 mg/L vancomycin. The impedance was then measured at 42°C in a BacTrac 4300 Microbiology Analyzer. The medium impedance (M-value) detects changes in the impedance caused by variations in the conductivity of the growth medium during the culture period. As soon as the threshold value (5% for the M-value) was achieved, samples were subjected to further confirmation using the RiboFlow™ *Cronobacter* kit. This method was found to have 85% sensitivity and 100% specificity and could complete detection within 29 h.

Research overlook and future aspects

Many researches are dedicating to develop faster and more reliable detection method for *Cronobacter* species in PIF. They combined conventional detection method with other newly developed methods. For examples, Lampel and Chen (2009), Mullane et al. (2006), and Oh and Kang (2004) mingled selective media with real-time PCR, cationic-magnetic-beads and fluorescent. The incorporation of new technique with selective media made the detection time shorten from 5-7 days to 1 or 2 days. Similarly, the PCR works with other techniques such as immobilization, electrophoresis-laser induced fluorescence detector (Table 3). Immunological methods are also introduced with other methods such as liposome, strip and cross-priming

Table 3. Detection methods for *Cronobacter* species

Techniques	Specificity	Detection time	Detection limits	References
Culture based detection methods				
Selective media	<i>Cronobacter</i> species	5-7 days	1 CFU/ 100 g	USFDA, 2002; ISO, 2006; Iversen et al., 2008; Kim and Rhee, 2011;
Selective media and fluorescent	<i>Cronobacter</i> species	24 h	Not mentioned	Oh and Kang, 2004
PCR based detection methods				
Real-time PCR, selective agar	<i>Cronobacter</i> species	Within 2 days	1 CFU/ 100 g	USFDA, 2012; Lampel and Chen, 2009
Conventional PCR	<i>Cronobacter</i> species	1 h	Not mentioned	Stoop et al., 2009
PCR-based detection	<i>Cronobacter</i> species	Less than 24 h	1 CFU/ 100 g	Zimmermann et al., 2014
Duplex PCR, immobilization	<i>Cronobacter</i> species	2-3 h	3 CFU/g	Zhou et al., 2008
Duplex PCR, electrophoresis-laser induced fluorescence detector	<i>Cronobacter</i> species	2-3 h	1.6 CFU/mL	Ruan et al., 2013
TaqMan real-time PCR	<i>Cronobacter</i> species	1 day	1 CFU/mL	Wang et al., 2012
Immunological detection methods				
INC-ELISA	<i>Cronobacter mytjensii</i>	36 h	1 cell/25 g	Song and Kim, 2013
Sandwich ELISA	<i>Cronobacter mytjensii</i>	Within 2 days	6.3×10 ⁴ CFU/mL without enrichment	Park et al., 2012
Sandwich ELISA and indirect enzyme-linked ELISA	<i>Cronobacter sakazakii</i>	Within 2 days	10 ⁴ CFU/mL, 1 CFU/ mL with enrichment	Xu et al., 2014b
Fluorescence-based liposome immunoassay	<i>Cronobacter mytjensii</i>	13 h	6.3×10 ⁴ CFU/mL	Song et al., 2015
Immunochromatographic strip	genus <i>Cronobacter</i>	16 h	10 cell/ 10 g	Blazkova et al., 2011
Immuno-blotting analysis, cross-priming amplification	<i>Cronobacter</i> species	60-70 min with pre-enrichment	10 ⁰ CFU/100 g	Zhao et al., 2010
Biosensor based detection methods				
Silica-coated magnetic nanoparticles, immunochromatographic strip	<i>Cronobacter</i> species	15 min	10 ⁶ CFU/mL	Chen et al., 2014
Immunomagnetic nanoparticles, immunuliposome	<i>Cronobacter sakazakii</i>	2 h and 30 min	3.3×10 ³ CFU/mL	Shukla et al., 2016
Cationic-magnetic-beads, selective media	<i>Cronobacter</i> species	24 h	1-5 CFU/500 g	Mullane et al., 2006
Probe-magnetic-bead, real-time qPCR	<i>Cronobacter</i> species	Less than 4 h	10 ³ CFU/mL in artificially contaminated PIF	Xu et al., 2014a
Biofunctionalized magnetic nanoparticles, nuclear magnetic resonance	<i>Cronobacter</i> species	2 h	10 cells/10 g	Zhao et al., 2013
Other detection methods				
MALDI-TOF MS	<i>Cronobacter</i> species	46 h	Less than 10 cells/100 g	Javurkova et al., 2012
RNA hybridisation assay, lateral flow	<i>Cronobacter</i> species	29 h	10 ⁰ CFU/mL	Zhu et al., 2012

amplification. The antibodies against *Cronobacter* species are widely cooperated with other techniques, especially biosensor, to develop more rapid and convenient detection. At the same time, new techniques, such as MALDI-TOF mass spectrometry and RNA hybridisation assay, give more ideas and inspiration for research on *Cronobacter* detection.

As an important opportunistic foodborne pathogen, *Cronobacter* species is receiving increased attention from researchers and food regulatory authorities. *Cronobacter* species are found in PIF, and there are many outbreaks and cases of the infection have been reported. USFDA and ISO method are widely used as standard method for detection of *Cronobacter* species although they are time consuming. Many researchers are dedicating on the development of more fast and sensitive methods for detecting *Cronobacter* species in PIF. Various new methods have been developed for the rapid detection of *Cronobacter* species, and combination of two or more of techniques have become more popular than use of a single method alone, especially combination of the conventional method with new techniques, such as selective media combined with PCR, biosensor or fluorescent. PCR can be also combined with immobilization, electrophoresis-laser induced fluorescence detector, as well as biosensors. Magnetic nanoparticles are a useful research tool of biosensor for detecting *Cronobacter* species, which usually composited with strip, PCR, and immunoassay. These methods enhance sensitivity, reduce detection time, and make great contributions on the PIF safety of *Cronobacter* species.

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- Kosakonia radicincitans* comb. nov., *Kosakonia oryzae* comb. nov. and *Kosakonia arachidis* comb. nov., respectively, and *E. turicensis*, *E. helveticus* and *E. pulveris* into *Cronobacter* as *Cronobacter zurichensis* nom. nov., *Cronobacter helveticus* comb. nov. and *Cronobacter pulveris* comb. nov., respectively, and emended description of the genera *Enterobacter* and *Cronobacter*. Syst Appl Microbiol 36:309-319.
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