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Isolation, identification and antimicrobial resistance of *Cronobacter* spp. isolated from various foods in China

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ABSTRACT

Cronobacter spp. are important foodborne pathogens that can cause severe diseases such as meningitis, sepsis, and necrotizing enterocolitis in neonates. In this study, 195 food samples, including cereals, cereal products, powdered infant formula (PIF), infant food formula, herbs, spices, vegetables, and fruits, were analyzed for the presence of *Cronobacter* spp. by culture-based method. The presumptive isolates were further confirmed by targeting the 16S rDNA gene using PCR. Out of 195 samples, 13 samples (6.7%) were positive for *Cronobacter* species. 12 of 85 cereal and cereal products (14.1%), and 1 of 22 herbs and spices (4.5%) were contaminated. In contrast, no *Cronobacter* was detected in commercial powdered infant formula, infant food formula, vegetables, or fruits. Alignment of 16S rRNA gene sequences showed that 13 isolates was most closely related to the genus *Cronobacter*. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis revealed that *Cronobacter sakazakii* was the only *Cronobacter* species isolated from various food samples. The antimicrobial susceptibility of 13 *Cronobacter* isolates was determined by the standard disk diffusion method. All isolated strains, except one resistant to ampicillin, were sensitive or displayed intermediate susceptibility to the 10 antimicrobial agents investigated. No multiple drug resistance was observed.

1. Introduction

The genus *Cronobacter*, a member of the family Enterobacteriaceae, comprises a group of Gram-negative foodborne pathogens that have been implicated as a cause of necrotizing enterocolitis, sepsis, and meningitis in neonates (Bowen & Braden, 2006; Caubilla-Barron et al., 2007; NazarowecWhite & Farber, 1997). This genus consists of seven species: *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter muytjensii*, *Cronobacter turicensis*, *Cronobacter dubliniensis*, *Cronobacter condimenti* and *Cronobacter universalis* (Iversen et al., 2008; Joseph et al., 2012). Although the incidence of human infections caused by *Cronobacter* spp. is low, these pathogens have a high mortality rate (more than 40%) in infected infants, and the survivors often suffer from severe neurological complications (Gallagher & Ball, 1991; Ries, Harms, & Scharf, 1994; Willis & Robinson, 1988). Outbreaks of *Cronobacter* spp. associated with contaminated PIF have been reported in Europe (Arseni, Malamouladas, Koutsia, Xanthou, & Trikka, 1987; Caubilla-Barron et al., 2007) and USA (Himelright et al., 2002).

While infections caused by *Cronobacter* have been epidemiologically linked to the consumption of contaminated PIF (El-Sharoud, El-Din, Ziada, Ahmed, & Klena, 2008; Lampel & Chen, 2009), *Cronobacter* spp. have been frequently isolated from a wide range of foods, such as cereals, meat, herbs, spices, salads, fruits, and vegetables (Jaradat, Ababneh, Saadoun, Samara, & Rashdan, 2009; Shaker, Osaili, Al-Omary, Jaradat, & Al-Zuby, 2007; Wang, Zhu, Xu, & Zhou, 2012; Ye, Wu, Zhou, Dong, & Zhang, 2008). Investigations on the presence of *Cronobacter* in various foods are therefore necessary to aid epidemiological studies.

Although most *Cronobacter* isolates are susceptible to commonly used antimicrobial agents, resistance has been reported in some isolates from food samples (Chon, Song, Kim, Hyeon, & Seo, 2012; Lee, Park, & Chang, 2012; Molloy et al., 2009). Additionally, inappropriate and irrational use of antimicrobial agents in agriculture has increased the emergence of antimicrobial-resistant strains (Girlich et al., 2001). Antimicrobial resistance, especially multiple drug resistance, is a public health problem, because it may cause failure of conventional treatment, resulting in prolonged illness and greater risk of death.

Although the occurrence of *Cronobacter* in food products and environmental samples has been investigated in several countries (El-Sharoud et al., 2009; Hoque et al., 2010; Lee et al., 2012;

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Terragno et al., 2009), little information is available on the prevalence of these organisms in food products in China. The aim of this study was to investigate the prevalence of *Cronobacter* in various Chinese food products, to identify the isolates by 16S rDNA sequencing and PCR-RFLP analysis, and to check antibiotic resistance patterns of the isolated strains.

2. Materials and methods

2.1. Sample collection

One hundred and ninety-five food samples, including cereals, cereal products, PIF, infant food formula, herbs, spices, vegetables, and fruits, were purchased between May 2010 and March 2013 from local supermarkets in Nanjing, China (Table 1). The samples were produced by different food manufacturers and purchased from different supermarkets. The samples were immediately transported to the laboratory in an ice box with ice packs and examined for the presence of *Cronobacter* spp. on the day of arrival.

2.2. Isolation and identification of Cronobacter spp.

The procedure adopted for the isolation of *Cronobacter* spp. from various food samples was a modification of the ISO method (ISO, 2006). Briefly, 25 g each sample except powders samples were homogenized with 225 mL of buffered peptone water (BPW; OXOID, Hampshire, UK) and then incubated at 37 °C for 18 h. For powders, 25 g portions per sample were mixed with 225 mL of BPW, and incubated at 37 °C for 18 h as a pre-enrichment step. Subsequently, 1 mL of the BPW suspension was transferred to 10 mL modified lauryl sulfate tryptose broth (OXOID, Hampshire, UK), and after further incubation at 42 °C for 24 h, the broth was streaked on chromogenic *Cronobacter* isolation agar (CCI; OXOID, Hampshire, UK). The plates were incubated at 42 °C for 24 h. All the suspected isolates, blue-green colonies on CCI, were subjected to the following biochemical tests for identification of the genus *Cronobacter*: Gram staining, oxidase, catalase, L-Lysine decarboxylase, L-Ornithine decarboxylase, L-Arginine dihydrolase, acid production from malonate, D-sorbitol, L-rhamnose, D-sucrose, D-melibiose, amygdaline, dulcitol and methyl-α-D-glucoside.

Table 1
Cronobacter spp. isolated from various food samples by culture-based method and PCR.

Origin	Sample category	No. of samples	Number of positive samples by culture-based method	Number of positive samples by PCR
Cereals and cereal products	Wheat	38	6	6
	Buckwheat	8	1	1
	Corn	12	4	4
	Rice	10	0	0
	Oatmeal	9	0	0
	Barley	8	1	1
Milk powdered	Powdered infant formula (0–6 months)	16	0	0
	Follow-on formular (6–12 months)	11	0	0
	Growing-up milk (12–36 months)	6	0	0
Infant food formula	Biscuit	15	0	0
Spices	Black pepper	5	1	1
	Red pepper	5	0	0
	Ginger powder	4	0	0
	Illicium verum	4	0	0
	Cymous cuminum fruit	4	0	0
Vegetables and fruits	Vegetables	20	0	0
	Fruits	20	0	0
Total		195	13	13

2.3. DNA extraction

Presumptive *Cronobacter* isolates were grown overnight at 37 °C in Luria–Bertani broth. Genomic DNA was extracted using an EZNA Genomic DNA isolation kit (Omega Bio-Tek, Doraville, USA) according to the manufacturer's instructions. Concentration and purity of DNA samples were estimated by means of a spectrophotometer (GeneQuant 100, GE Healthcare, USA) in order to evaluate the amount and quality of extracted DNA. DNA samples were thereafter stored at −20 °C.

2.4. Confirmation of Cronobacter isolates

PCR confirmation of the presumptive *Cronobacter* isolates was performed as described in Lehner, Tasara, and Stephan (2004) using the primers Esakf (5′-GCTYTGCTGACGAGTGGCGG-3′) and Esakr (5′-ATCTCTGCAGGATTCTCTGG-3′). Because of its high specificity, Esakf/Esakr is the most suitable primer pair for *Cronobacter* detection and identification (Blazkova, Javurkova, Fukal, & Rauch, 2011; Cawthorn, Botha, & Witthuhn, 2008; Lehner et al., 2004). The thermal cycle consisted on initial denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 2 min. A final elongation at 72 °C for 7 min completed the programme. After amplification, PCR products were separated by electrophoresis on a 1% (m/v) agarose gel (MDBio, QingDao, China) in TAE buffer, followed by staining with ethidium bromide. The gel was then visualized and photographed under a UV transilluminator (Nuoding, Hangzhou, China). *Cronobacter* type strains (*C. sakazakii* ATCC 29544 and *C. muytjensii* ATCC 51329) were used as positive controls.

2.5. PCR amplification and 16S rDNA gene sequence analysis

The 16S rDNA gene was PCR-amplified in a T-gradient thermocycler (PTC200, Bio-Rad, USA) using Taq DNA polymerase (Fermentas, Shanghai, China) and the universal primers 5′-AGAGTTTG ATCCTGGCTCAG-3′ and 5′-TACCTTGTTACGACTT-3′ (Weisburg, Barns, Pelletier, & Lane, 1991). PCR conditions were 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and elongation at 72 °C for 2 min. A final extension step of 7 min at 72 °C followed the final cycle. PCR products were purified with an EZ-10 Spin Column PCR product purification kit (Sangon, Shanghai, China). The 16S rDNA products were cloned into a pMD19-T vector (Takara, Dalian, China) and transformed into competent *Escherichia coli* DH5α cells. Partial sequencing of the 16S rDNA gene was then carried out by Genscript (Nanjing, China) using an ABI 3730 Genetic Analyzer. The resulting sequences were subjected to a GenBank BLASTn search (http://www.ncbi.nlm.nih.gov/BLAST/) to determine potentially homologous sequences in GenBank, and also aligned using the ClustalX 2.0 multiple alignment program (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997). Phylogenetic analysis was performed using MEGA 4.0. Branch support was evaluated by a bootstrap analysis of 1000 replicates. Phylogenetic trees of 16S rDNA sequences were inferred using the Neighbor-Joining method (Saitou & Nei, 1987).

2.6. PCR-RFLP

To identify all isolates at the species level, a PCR-RFLP protocol modified from that of Strydom, Cameron, and Witthuhn (2011) was