Development and application of a sensitive, rapid, and reliable immunomagnetic separation-PCR detection method for *Cronobacter* spp.

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ABSTRACT

Cronobacter spp. have been linked to clinical cases of infection in both adults and infants. Enrichment of *Cronobacter* spp. before detection has been necessary but is quite time consuming. Hence, we sought to develop an immunomagnetic separation (IMS) PCR method that could shorten the time of enrichment before the detection of *Cronobacter* spp. The polyclonal antibody used in this immunomagnetic separation was prepared based on the outer membrane protein A of *Cronobacter sakazakii* China Center of Industrial Culture Collection 21560 and had high specificity to the target. The primers used in the IMS-PCR method also showed high specificity. The detection limit of IMS-PCR for pure *C. sakazakii* culture was 5.2×10^2 cfu/mL. *Cronobacter sakazakii* in artificially contaminated powdered infant formula (PIF) was also detected at a detection limit of 5.2×10^2 cfu/mL. After 8 h of enrichment, the detection limit in PIF was lower than 5.2×10^1 cfu/mL. An interference test using *Escherichia coli* in artificially contaminated PIF showed that the IMS-PCR method developed in this study had a good ability to resist interference. Finally, the IMS-PCR method was applied to the detection of *Cronobacter* in food samples and was shown to be reliable. Thus, this newly developed IMS-PCR detection method was quite sensitive, rapid, and reliable and could be applied to the detection of *Cronobacter* in foods.

Key words: immunomagnetic separation, *Cronobacter* spp., PCR, polyclonal antibody

INTRODUCTION

Cronobacter was identified as a new genus in the family *Enterobacteriaceae* in 2008 (Joshi et al., 2014; Chen et al., 2016). This genus of gram-negative, rod-shaped bacteria consists of 7 species: *Cronobacter sakazakii*, *Cronobacter dublinensis*, *Cronobacter malonaticus*,

Cronobacter muytjensii, *Cronobacter turicensis*, *Cronobacter universalis*, and *Cronobacter condimentii* (Iversen et al., 2008; Joseph et al., 2012). Infection with *Cronobacter* spp. can cause meningitis, sepsis, and necrotizing enterocolitis, with a fatality rate of 80% (Townsend et al., 2008; Healy et al., 2010). Immunocompromised infants and pre-term or low-birth-weight infants under 2 mo of age are especially high-risk groups (Chiang et al., 2012; Li et al., 2013; Álvarez-Ordóñez et al., 2014). Moreover, many infant infection cases have been associated with contaminated powdered infant formula (PIF; Arseni et al., 1987; Friedemann, 2009; Joseph and Forsythe, 2012).

Conventional culture-based methods play an important role in the detection of *Cronobacter* spp. (Druggan and Iversen, 2009) and have been widely adopted in many standardized detection methods (Wang et al., 2012). These methods usually consist of a series of steps, including nonselective enrichment, selective enrichment, differential plating, and final biochemical confirmation. However, these methods are time consuming (the enrichment steps in particular), have low sensitivity, and are not easy to use (Cawthorn et al., 2008). Polymerase chain reaction and its related technologies have also been widely used in detecting foodborne pathogens. Compared with culture-based methods, PCR methods are more sensitive and have fewer steps. In the case of *Cronobacter* spp., PCR detection methods based on specific targets such as 16S rDNA (Iversen et al., 2004), 16S-23S rDNA internal transcribed spacer (ITS) (Liu et al., 2006), 23S rDNA (Derzelle et al., 2007), tRNAGlu (Hassan et al., 2007), *dnaG* (Seo and Brackett, 2005), *OmpA* (Mohan Nair and Venkitanarayanan, 2006), and *gluA* (Iversen et al., 2007) have been previously reported. In addition, polyclonal and monoclonal antibodies specific to *Cronobacter* have also been used in detection (Park et al., 2012; Chen et al., 2014; Xu et al., 2014b).

Both PCR and immunological methods can provide rapid, sensitive, specific, and efficient ways to detect *Cronobacter*. However, the enrichment time necessary before detection is still time-consuming. Immunomagnetic separation (IMS) is a method that isolates cells

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using paramagnetic particles coated with specific antibodies and offers an attractive alternative by shortening the time of enrichment and removing impurities. After separation by IMS, the enriched bacterial cells are detected by PCR. This new combinative method improves the speed, sensitivity, and efficiency of detection (Leon-Velarde et al., 2009). Immunomagnetic separation PCR has been used to detect foodborne pathogens, such as *Campylobacter jejuni* in chicken samples (Tram et al., 2012), *Escherichia coli* O157:H7 in raw ground beef (Fedio et al., 2011), *Salmonella* in raw duck wings (Zheng et al., 2014) and egg products (Jenikova et al., 2000), and *Listeria monocytogenes* in ready-to-eat foods (Wadud et al., 2010). To our knowledge, no reports are available regarding the detection of *Cronobacter* spp. by the IMS-PCR method. Only a probe-magnetic separation (PMS) PCR method was reported in a previous study, which used DNA probes conjugated to magnetic beads to isolate genomes of bacterial cells from samples and detect target DNA by PCR (Xu et al., 2014a). However, this method did not include the main species of the *Cronobacter* genus and lacked application to actual food samples.

In this study, a polyclonal antibody specific to protein outer membrane protein A (ompA) of *Cronobacter* spp. was prepared and conjugated to magnetic beads. The immunomagnetic beads were used to separate *Cronobacter* cells to shorten the time of enrichment.

The PCR combined with IMS was used to improve the specificity and sensitivity. Thus, we developed a rapid and reliable IMS-PCR detection method for *Cronobacter* spp.

MATERIALS AND METHODS

Bacteria Strains and Growth

In total, 6 *Cronobacter* strains and 12 non-*Cronobacter* strains (Table 1) were tested in this study. These strains were purchased from the American Type Culture Collection (ATCC, Manassas, VA), the China Center of Industrial Culture Collection (CICC, Beijing, China), the China General Microbiological Culture Collection Center (CGMCC, Beijing, China), the National Center for Medical Culture Collections (CMCC, Beijing, China), the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), and the National Collection of Type Cultures (NCTC, Salisbury, UK). All strains were retrieved from frozen stock cultures and grown in an appropriate broth at 37°C at 180 rpm.

Preparation of OmpA Protein

The ompA protein was prepared according to our previous study (Chen et al., 2016). *Escherichia coli*

Table 1. Specificity of the polyclonal antibody by ELISA and immunomagnetic separation (IMS) PCR

Strain	Strain identification ¹	OD ₄₅₀ ²	P/N ³	IMS-PCR result ⁴
<i>Cronobacter muytjensii</i>	CICC 21563	1.346	5.40	+
<i>Cronobacter malonicus</i>	DSM 18702	1.521	6.21	+
<i>Cronobacter turicensis</i>	DSM 18703	1.348	5.41	+
<i>Cronobacter dublinensis</i>	DSM 18705	1.163	4.67	+
<i>Cronobacter universalis</i>	NCTC 9529	1.454	5.83	+
<i>Cronobacter sakazakii</i> (positive control)	CICC 21560	1.586		+
Negative control		0.249		—
<i>Bacillus cereus</i>	AS 1.1846	0.264	1.06	—
<i>Enterobacter cloacae</i>	ATCC 13047	0.432	1.73	—
<i>Escherichia coli</i> BL ₂₁ (DE3)		0.553	2.20	—
<i>E. coli</i> DH5α		0.457	1.84	—
<i>Serratia marcescens</i>	CICC 10187	0.321	1.28	—
<i>Salmonella enteritidis</i>	CICC 21482	0.522	2.09	—
<i>Listeria monocytogenes</i>	ATCC 15313	0.281	1.12	—
<i>Escherichia coli</i>	ATCC 35150	0.453	1.81	—
<i>Bacillus coagulans</i>	CICC 10144	0.254	1.02	—
<i>Bacillus licheniformis</i>	CICC 23584	0.268	1.07	—
<i>Staphylococcus aureus</i>	AS 1.2465	0.325	1.3	—
<i>Pseudomonas fluorescens</i>	AS 1.1802	0.259	1.04	—

¹CICC = China Center of Industrial Culture Collection; DSMZ = Deutsche Sammlung von Mikroorganismen; NCTC = National Collection of Type Cultures; AS = China General Microbiological Culture Collection Center; ATCC = American Type Culture Collection.

²OD₄₅₀ = optical density at 450 nm.

³P/N, positive value/negative value using a ratio of the optical density of the test sample to the negative sample; P/N value greater than or equal to 2.1: positive result, P/N value less than 2.1: negative result.

⁴+, positive result; —, negative result.

BL21 (DE3) with pET-32a-ompA grown in Luria-Bertani (LB) broth with ampicillin (100 µg/mL) at 37°C and 180 rpm. Isopropyl-β-D-thiogalactopyranoside was added at a final concentration of 1 mM when the optical density at 600 nm value of the culture reached 0.6. After induced expression by isopropyl-β-D-thiogalactopyranoside at 16°C and 180 rpm for 20 h, the bacterial cells were collected by centrifugation (10,000 × g, 4°C, 10 min), washed with normal saline, and broken by sonication. The supernatant and the precipitation were then separated by centrifugation, and ompA was purified from supernatant by Ni-chelating affinity chromatography.

The process of immunization of male New Zealand rabbits was carried out partly according to the study of Chen and Zhuang (2012). One immunization dose for rabbits was 1 mL of ompA solution (0.91 mg/mL) mixed with isovolumetric adjuvant (Freund's complete adjuvant for first immunization and Freund's incomplete adjuvant for boosted immunization). The antigen was injected into rabbits at 2 sites in the back, 2 sites in the groin, and 2 sites in the armpit. Boosts were given every 2 wk after first immunization. Blood samples were drawn regularly and the titer was checked.

Preparation of a Polyclonal Antibody Specific to *Cronobacter* spp.

The antibody titer of the immunized rabbit serum was determined by an indirect ELISA and the blood of the rabbit was isolated when the titer reached 1:100,000. The optimized indirect ELISA process was according to our previous study (Chen et al., 2016). One hundred microliters per well of *C. sakazakii* culture with concentration of 10⁷ cfu/mL was coated on a 96-well plate by coating buffer (carbonate sodium buffer, pH 9.6) at 4°C overnight. After washing in washing buffer (0.1 M PBS buffer and 0.05% Tween 20, 200 µL/well) 3 times, the plate was blocked with BSA solution (1% BSA, wt/vol) at 37°C for 60 min. Immunized rabbit serum was serially diluted and then added to wells (100 µL/well) as primary antibody after washing step and horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000, vol/vol) was used as secondary antibody (100 µL/well). Finally, 100 µL of tetramethylbenzidine was added per well and 50 µL of 2 M H₂SO₄ was added to terminate the reaction after incubation at 25°C for 10 min. The absorbance values at 450 nm wavelength were measured.

The rabbit anti-serum was prepared by centrifugation at 10,000 × g for 10 min at 4°C after 24 h to allow for clotting. The polyclonal antibody was obtained from anti-serum by ammonium sulfate fractionation and fur-

ther purified by HiTrap Protein G HP (GE Healthcare, Chicago, IL). After desalination by HiTrap desalting (GE Healthcare), NaN₃ was added to the purified antibody at a final concentration of 0.02%. The antibody was stored at 4°C and later analyzed by SDS-PAGE and an indirect ELISA.

Conjugation of the Polyclonal Antibody to IMB

The purified polyclonal antibody specific to *Cronobacter* was conjugated to magnetic beads according to the protocol of Dynabeads M-280 Tosylactivated (Invitrogen, Thermo Fisher Scientific, Waltham, MA). The magnetic beads were resuspended by vortexing and placed on a magnet for 2 min to allow the beads to gather at bottom of the tube. The supernatant was removed, and the magnetic beads in the tube were washed with 130 µL each of buffer A (0.1 M borate buffer, pH 9.5) and buffer B (0.1 M Na-phosphate buffer, pH 7.4). Twenty microliters of the purified polyclonal antibody (20 µg/µL) and 100 µL of buffer C (3 M ammonium sulfate in buffer A or B) were added to the tube and mixed lightly with the magnetic beads. After incubation for 12 to 18 h, the tube was placed on a magnet for 2 min and the supernatant was removed. The sites on the beads that had not been bound with the antibody were blocked with 1 mL of buffer D (PBS with 0.5% [wt/vol] BSA, pH 7.4). The magnetic beads bound with antibody were washed 3 times with buffer E (PBS with 0.1% [wt/vol] BSA, pH 7.4) and resuspended with 240 µL of buffer E.

Optimized IMS-PCR Conditions

Twenty microliters of the prepared immunomagnetic bead suspension was added to a 1.5-mL tube and mixed with 1 mL of bacterial cell culture. The tube was incubated for 30 min at 37°C at 180 rpm and placed on a magnet for 2 min. The supernatant was removed and beads were washed 3 times with 1 mL of buffer D. The immunomagnetic beads were then resuspended in 100 µL of normal saline.

The primers (Esakf: 5'-GCTYTGTCTGAC-GAGTGGCGG-3'; Esakr: 5'-ATCTCTGCAG-GATTCTCTGG-3') used in the IMS-PCR method were from a previous study (Lehner et al., 2004). The PCR amplification was carried out in a 25-µL reaction mixture containing 12.5 µL of 2 × Taq Master (Vazyme Biotech Co., Jiangsu, China), 9.5 µL of sterile distilled water, 1 µL of each primer (0.25 µM), and 2 µL of the immunomagnetic beads. The PCR conditions included an initial incubation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 58°C for 40 s, 72°C for 1 min,

and the final step of 72°C for 7 min. The PCR products were analyzed by agarose gel electrophoresis and visualized using a UV transilluminator.

Specificity Test and Sensitivity in Pure Culture of the IMS-PCR Method

A total of 18 bacterial strains were cultured in appropriate medium overnight at 37°C and 180 rpm. One milliliter of each culture was sampled and detected by IMS-PCR.

The *C. sakazakii* CICC 21560 culture was serially diluted 10-fold with 0.9% normal saline, and the cell concentration was determined by plating on LB agar and counting the colonies. The cells from 1 mL of *C. sakazakii* CICC 21560 culture at different dilutions were isolated by IMS and detected by the plate cultivation method and PCR.

Testing for IMS-PCR Interference Using *E. coli* in Artificially Contaminated PIF

Cronobacter sakazakii CICC21560 and *E. coli* CMCC (National Center for Medical Culture Collections, Beijing, China) 23657 were grown in LB broth at 37°C overnight. Their cell concentrations were determined by serially diluting the cultures 10-fold, plating on LB agar, and counting the colonies.

Twenty-five grams of PIF was mixed with 225 mL of nutrient broth. Then, 8 mL of nutrient broth-PIF mixture, 1 mL of *E. coli* CMCC 23657 culture (10^8 cfu/mL), and 1 mL of *C. sakazakii* CICC 21560 culture (10^2 – 10^8 cfu/mL) were mixed. Sterile water was used as a negative control. *Cronobacter muytjensii* CICC 21563 and *C. sakazakii* CICC 21560 were used as positive controls. The cell culture mixture was detected by the IMS-PCR method developed in this study.

Use of the IMS-PCR Method for Detection of *C. Sakazakii* in Artificially Contaminated PIF

An overnight culture of *C. sakazakii* CICC 21560 was serially diluted 10-fold, and the cell concentration was determined by the plate counting method. Afterward, 25 g of PIF was mixed with 225 mL of nutrient broth; 1 mL of *C. sakazakii* CICC 21560 culture (5.2×10^6 to 5.2×10^1 cfu/mL) was added to 9 mL of the nutrient broth-PIF mixture. One milliliter of artificially contaminated PIF was separately sampled and detected with IMS-PCR directly.

Similarly, 1 mL of *C. sakazakii* CICC21560 culture (5.2×10^6 to 5.2×10^2 cfu/mL) was added to 9 mL of the nutrient broth-PIF mixture. After culturing for 8 h

at 37°C, 1 mL of cell culture was removed and detected by IMS-PCR.

Application of the IMS-PCR Method in Various Food Samples

Seventy food samples, including 17 PIF samples, infant formula foods (9 rice flour samples, 2 noodle samples, and 6 cookie samples), 6 formulated milk powder samples for pregnant and breastfeeding mothers, and 30 cereals and their product samples were purchased from local grocery stores and supermarkets in Nanjing. Twenty-five grams of each sample was mixed with 225 mL of nutrient broth and incubated at 37°C and 180 rpm for 8 h. One milliliter of each culture was sampled twice and detected by IMS-PCR and culture-based method from ISO standard (ISO, 2006), respectively. The rest of the immunomagnetic beads of positive samples were spread on solid medium and isolates were recovered (the isolates were detected by primers of IMS-PCR again). The target strains were further identified by 16S rDNA identification.

RESULTS AND DISCUSSION

Serum Purification of the Polyclonal Antibody Specific to *Cronobacter*

The sequence of ompA was relatively conserved throughout the *Cronobacter* genus but was not conserved compared with other genera of common food-borne pathogens (Chen et al., 2016). Thus, ompA from *C. sakazakii* was used to prepare the antibody. The crude polyclonal antibody obtained by ammonium sulfate precipitation was further purified by HiTrap Protein G HP (Figure 1A) and analyzed by SDS-PAGE. The result of SDS-PAGE showed that the purified antibody consisted of 2 bands at approximately 25 kDa (light chain) and 55 kDa (heavy chain; Figure 1B). Also, 2 impurity bands (red box in Figure 1B) had been removed after being purified by HiTrap Protein G HP. Therefore, the purified polyclonal antibody had higher purity than the product of saturated ammonium sulfate precipitation. The titer of antibody was tested by an indirect ELISA and was over 1:80,000 (Figure 1C).

The specificity of the polyclonal antibody was tested and showed very strong reactivity with *Cronobacter*, weak cross-reactivity with *S. enteritidis* and *E. coli* BL21 (DE3), and no reactivity with other bacteria tested (Table 1). Considering that PCR is also used in the IMS-PCR detection method, the weak cross-reactivity could reduce false-negative results, while not increasing false positives in the final detection. Finally, the antibody was conjugated to magnetic beads.

IMS-PCR Procedure

The polyclonal antibody was added to the surface of magnetic beads. The IMS and PCR together create the IMS-PCR method to detect *Cronobacter* spp. The primers used in IMS-PCR are widely used (Lehner et

al., 2004; Caubilla-Barron et al., 2007; Cawthorn and Witthuhn, 2008) and better identify the *Cronobacter* genus as compared with other specific primers (Cawthorn et al., 2008). The PMS-PCR must first lyse the bacterial cell wall to release the genomic DNA, which is then isolated by a DNA probe targeting 16S rRNA

Figure 1. Sodium dodecyl sulfate-PAGE analysis of the purified rabbit anti-ompA (outer membrane protein A) polyclonal antibody and the antibody titers. (A) Elution profile of polyclonal antibody purified from a HiTrap Protein G HP column (GE Healthcare, Chicago, IL); (B) SDS-PAGE analysis of the rabbit anti-ompA polyclonal antibody purified by ammonium sulfate and HiTrap Protein G HP. Lane M: Protein molecular weight marker (kDa); lane 1: polyclonal antibody purified by saturated ammonium sulfate; the 2 bands in the white (red) box were impurities that had not been removed by ammonium sulfate precipitation; lanes 2 to 6: purified fractions 1 to 5 in panel A. The heavy chain and light chain were approximately 55 and 25 kDa, respectively; (C) titer of rabbit anti-ompA polyclonal antibody. OD450 = optical density at 450 nm. Color version available online.

Figure 2. Detection of *Cronobacter sakazakii* using the immunomagnetic separation (IMS) PCR method in the presence of *Escherichia coli* American Type Culture Collection (ATCC) 35150. Lane M: 100-bp DNA ladder; lane 1: positive control [*C. sakazakii* China Center of Industrial Culture Collection (CICC) 21560]; lane 2: positive control (*Cronobacter muytjensii* CICC 21563); lane 3: negative control (samples of powdered infant formula without inoculation); lane 4 to lane 10: artificially contaminated powdered infant formula with different levels of *C. sakazakii* (10^7 – 10^1 cfu/mL) and a fixed level of *E. coli* CMCC (National Center for Medical Culture Collections, Beijing, China) 23657 (10^7 cfu/mL).

gene sequence conjugated to magnetic beads (Xu et al., 2014a). Compared with this method, IMS-PCR does not need to lyse bacterial cells during IMS; therefore, the entire bacterial cell can be used for further study.

Sensitivity Evaluation and Specificity Test of IMS-PCR

A total of 18 bacterial strains were used to test the specificity of the IMS-PCR method. Only target bands appeared on a gel run of 6 *Cronobacter* spp., and no target bands appeared on gel of other genus bacterial strains (Table 1). Therefore, the IMS-PCR method developed in this study has good specificity. Compared with the PMS-PCR method (Xu et al., 2014a), our specificity test covers common foodborne pathogens and the main species of *Cronobacter* spp., except *C. condimentii*, which has only been isolated once before at this time.

The capture rate of the immunomagnetic beads trended down as the cell concentration of *C. sakazakii* went down. When the cell concentration reached 5.2×10^2 cfu/mL, the cell concentration could not be measured by the plate cultivation method. However, the PCR still showed a positive result. Therefore, the lower detection limit for pure cell culture of the IMS-PCR method was 5.2×10^2 cfu/mL (Table 2). The PMS-PCR method in a previous report (Xu et al., 2014a) showed a lower detection limit (10^1 cfu/mL).

Testing for IMS-PCR Interference with a High Cell Concentration of *E. Coli* in Artificially Contaminated PIF

Genomic DNA from non-*Cronobacter* bacteria may affect IMS-PCR detection of *Cronobacter* and lead to a false-positive result. Hence, *C. sakazakii* CICC21560 with a cell concentration of 5.2×10^5 to 5.2×10^0 cfu/mL was used as a template, and *E. coli* CMCC 23657 with a cell concentration of 10^7 cfu/mL was used as background to evaluate possible interference. As shown in Figure 2, all target bands appeared, indicating that the IMS-PCR method developed in this study was not affected by genomic DNA of other bacteria (*E. coli* in this case).

Application and Evaluation of the IMS-PCR Method in Artificially Contaminated PIF

Food samples may contain inhibitors for the IMS and PCR reactions. Hence, the detection limit of IMS-PCR in artificially contaminated PIF was determined. As shown in Figure 3A, the target band still appeared when the cell concentration of *C. sakazakii* was down to 5.2×10^2 cfu/mL in PIF, which was the same as the detection limit in pure cell culture. Correspondingly, the PMS-PCR method used in previous studies showed a higher detection limit of 10^3 cfu/mL in artificially contaminated PIF (Xu et al., 2014a). Although the IMS-PCR detection limit for pure cell culture was higher than that of PMS-PCR, the detection limit of IMS-PCR in artificially contaminated PIF was lower

Table 2. Sensitivity of immunomagnetic separation PCR for *Cronobacter sakazakii* detection in pure culture

Initial number of <i>C. sakazakii</i> (cfu)	Capture number of <i>C. sakazakii</i> (cfu)	Capture rate (%)	PCR result ¹
5.2×10^6	3.5×10^5	67.3	+
5.2×10^5	3.9×10^4	75	+
5.2×10^4	1.4×10^3	26.9	+
5.2×10^3	89	17.1	+
5.2×10^2	ND	ND	+
5.2×10^1	ND	ND	ND

¹+, positive result; –, negative result; ND, not detected.

Figure 3. Sensitivity of immunomagnetic separation (IMS) PCR for *Cronobacter sakazakii* detection in artificially contaminated powdered infant formula (PIF) and detection of *C. sakazakii* after an 8-h enrichment step in artificially contaminated PIF. (A) Sensitivity of IMS-PCR for *C. sakazakii* detection in artificially contaminated PIF. Lane M: 100-bp DNA ladder; lane N: negative control (samples of PIF without inoculation); lane 1 to lane 6: artificially contaminated PIF with different levels of *C. sakazakii* (5.2×10^5 to 5.2×10^0 cfu/mL). (B) Detection of *C. sakazakii* from artificially contaminated PIF samples using the IMS-PCR method after an 8-h enrichment step. Lane M: 100-bp DNA ladder; lane N: negative control (samples of PIF without inoculation); lane 1 to lane 5: artificially contaminated PIF with different levels of *C. sakazakii* (5.2×10^5 to 5.2×10^1 cfu/mL).

than that of the PMS-PCR method. This indicated that the IMS-PCR method had a better ability to resist interference from inhibitors in the reaction. After 8 h of enrichment, all target bands on the gel were clear and bright, even when the initial cell density was 5.2×10^1 cfu/mL (Figure 3B), which indicated that the detection limit IMS-PCR was lower than 5.2×10^1 cfu/mL. This detection limit is lower than the infection titer of *Cronobacter* (10^3 or 10^4 cfu; Wang et al., 2012); thus, this method could be applied to the detection of *Cronobacter* in PIF.

Application of the IMS-PCR Method in Various Food Samples

Most infectious cases of *Cronobacter* were associated with PIF, thus PIF was chosen as the main food

sample for artificial contamination to test the detection methods outlined in previous reports (Park et al., 2012; Chen et al., 2014, 2015). However, several other foods may potentially be sources of *Cronobacter* during food preparation for infants. Hence, the IMS-PCR method was applied to the detection of actual food samples and culture-based methods served as a control. Nine food samples showed positive results for *Cronobacter* spp., including 1 infant food formula, 5 formulated milk powder for pregnant and breastfeeding mothers, and 3 dry cereal products (Table 3). The culture-based method showed slightly different results. The positive results for the culture-based method were from 1 infant food formula, 3 milk products for pregnant and breastfeeding mothers, and 5 dry cereals. Both the IMS-PCR method and the culture-based method had not detected *Cronobacter* spp. from the PIF. The 11 target bacterial

Table 3. Detection of *Cronobacter* spp. in various food samples by immunomagnetic separation (IMS) PCR and culture-based method

Sample category	No. of samples	IMS-PCR method		Culture-based method	
		Positive	Negative	Positive	Negative
Cereal foods					
Corn meal (grain)	6	0	6	1	5
Buckwheat meal (grain)	2	1	1	1	1
Wheat meal (grain)	3	0	3	0	3
Soybean meal	2	0	2	0	2
Rice	11	1	10	1	10
Black rice	2	0	2	0	2
Pearl barley	2	1	1	2	0
Glutinous millet	2	0	2	0	2
Powered infant formula	17	0	17	0	17
Formulated milk powder for pregnant and breastfeeding mothers	6	5	1	3	3
Infant formula foods	17	1	16	1	16
Total	70	9	61	9	61

Table 4. Origin and strain number of presumptive *Cronobacter* isolates screened from foods

Strain number	Sample	Origin	IMS-PCR method ¹	Culture-based method ¹	16S rDNA sequencing
C1	S5	Buckwheat meal	+	+	<i>Cronobacter</i> spp.
C2	S19	Pearl barley	+	+	<i>Cronobacter</i> spp.
C3	S23	Corn	–	+	<i>Enterobacter hormaechei</i>
C4	S24	Pearl barley	–	+	<i>Klebsiella pneumoniae</i>
C5	S29	Rice	+	+	<i>Cronobacter</i> spp.
C6	S39	Milk powder ²	+	+	<i>Cronobacter</i> spp.
C7	S46	Milk powder ²	+	+	<i>Cronobacter</i> spp.
C8	S48	Milk powder ²	+	+	<i>Cronobacter</i> spp.
C9	S49	Milk powder ²	+	–	<i>Cronobacter</i> spp.
C10	S50	Milk powder ²	+	–	<i>Cronobacter</i> spp.
C11	S66	Infant formula ³	+	+	<i>Cronobacter</i> spp.

¹+, positive result; –, negative result. IMS = immunomagnetic separation.

²Formulated milk powder for pregnant and breastfeeding mothers.

³Formulated noodle for infants.

strains were further identified by 16S rDNA sequencing technology. The results (Table 4) showed that isolated strains C1, C2, and C5–C11 showed a similarity of 99% to *C. sakazakii* and isolated strains C3 and C4 showed 99% similarity with *Enterobacter hormaechei* and *Klebsiella pneumoniae*, respectively. The IMS-PCR detection method showed no false-positive or false-negative results. Thus, IMS-PCR is a reliable method that could be applied to *Cronobacter* in foods.

CONCLUSIONS

In this study, a polyclonal antibody with high specificity to *Cronobacter* spp. was created and attached to the surface of magnetic beads to obtain immunomagnetic beads, which were then used to isolate *Cronobacter* spp. A specific pair of primers further identified the isolated cells. Thus, we developed an IMS-PCR method for *Cronobacter* spp. detection. To our knowledge, this is the first report of an IMS-PCR method for detecting *Cronobacter* spp. We evaluated the specificity of the IMS-PCR method, as well as its ability to avoid interference from off-target bacteria. This method's sensitivity and resistance to interference were determined in artificially contaminated PIF, as well as in actual food samples. These tests proved that the IMS-PCR detection method developed in this study is sensitive, rapid, and reliable and could be applied to the detection of *Cronobacter* in foods.

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