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Detection of *Cronobacter* species in powdered infant formula using immunoliposome-based immunomagnetic concentration and separation assay

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A B S T R A C T

Cronobacter species are foodborne pathogens that can affect the human central nervous system. Survivors of *Cronobacter* infections often suffer from severe neurological impairments, including hydrocephalus, quadriplegia, and developmental delays in all ages, especially in infants and the immunocompromised. Moreover, *Cronobacter* species pose a high risk in powdered infant formula (PIF) because PIF is a major source of nutrition for infants worldwide. To develop a rapid and sensitive detection method for *Cronobacter* species in PIF, immunoliposomes and immunomagnetic nanoparticles were synthesized, after which an immunoliposome-based immunomagnetic concentration and separation assay was developed and applied to PIF for the detection of *Cronobacter* species. The detection limits of the developed assay were $5.9 \times 10^3 \pm 0.7$–$4.8 \times 10^4 \pm 0.2$ CFU/mL for *Cronobacter* species in pure culture with no cross-reactivity with 13 other tested non-*Cronobacter* strains. Additionally, the developed assay could provide results in 3 h when the contaminated level was higher than 10^4 CFU/25 g PIF and in 9 h when the contaminated level was 10 CFU/25 g PIF. The developed immunoliposome-based immunomagnetic concentration and separation assay is rapid, sensitive, and simple and thus has great potential for use in the detection of *Cronobacter* species in PIF.

1. Introduction

Cronobacter species belong a newly classified genus, *Cronobacter* genus, that was recently transferred from *Enterobacter*. There are currently 7 species of *Cronobacter*; namely, *Cronobacter sakazakii*, *Cronobacter malonaticus, Cronobacter turicensis, Cronobacter muytjensii, Cronobacter dublinensis* with 3 subspecies, *dublinensis, lausannensis*, and *lactaridi*, *Cronobacter condimenti*, and *Cronobacter universalis* (Iversen and Forsythe, 2003; Iversen et al., 2008; Joseph et al., 2012; Jackson et al., 2014). *Cronobacter* species have been linked to life-threatening infection such as meningitis, enterocolitis, and septicemia in neonates and infants (Iversen et al., 2008). *Cronobacter* species have been detected frequently in various foods, including dry powdered food, agricultural products, and fresh-cut food (Lee et al., 2010, 2012; Holy and Forsythe, 2014). Especially, *Cronobacter sakazakii*, *Cronobacter malonaticus*, and *Cronobacter turicensis* have been isolated from infected neonates; however, all species of *Cronobacter* should be considered pathogenic because they have all been linked retrospectively to clinical cases of infection in infants or adults (Food and Agriculture Organization/World Health Organization, 2004, 2006, 2007; Xu et al., 2014). It is reported that powdered infant formula (PIF) is the major source of contaminants of *Cronobacter* species (Cahill et al., 2008), therefore, methods for new, rapid, and sensitive detection of *Cronobacter* species are urgently required to prevent its contamination in the food industry, especially in PIF.

A number of methods for the detection of *Cronobacter* species have been reported. According to the Korean Food Code of the Ministry of Food and Drug Safety (Ministry of Food and Drug Safety, 2005), isolating and confirming *Cronobacter sakazakii* from PIF involves a pre-enrichment step in buffered peptone water (BPW), selective enrichment by *Enterobacteriaceae* enrichment broth, selective differential plating on violet red bile glucose agar, and biochemical confirmation of the isolated colony, which requires 5–7 days. A revised method by the United States Food and Drug Administration (2012) includes real-time polymerase chain

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reaction (PCR) with an internal amplification control, which needs 2–3 days to detect the *Cronobacter* species in PIF. Lampel and Chen (2009) described a method for isolation and detection of *Cronobacter* species from PIF by using chromogenic agars and a real-time PCR-based assay. In their study, the suspended cells were isolated from selective media after enrichment in BPW and confirmed by real-time PCR assay (Lampel and Chen, 2009). Mullane et al. (2006) developed a method using a cationic-magnetic-bead to capture the *Cronobacter* cells, while subsequent identification was conducted after plating the captured cells onto Druggan-Forsythe-Iversen formulation agar, which can detect 1–5 colony forming unit (CFU)/500 g PIF within 24 h. Blazkova et al. (2011) reported a method in which DNA isolated from culture was used for genus *Cronobacter*-specific PCR with labeled primers, after which the obtained amplicons were applied to an immunochromatographic strip with a carbon-neutravidin conjugation. Zimmermann et al. (2014) reported a PCR-based detection system for *Cronobacter* species, including enrichment, DNA-isolation, and detection by real-time PCR, using the outer membrane protein gene *ompA* as a target.

Immunological methods have been widely applied for the determination of foodborne pathogens because they require less assay time than traditional culture techniques and have high specificity toward the target pathogen (Velusamy et al., 2010). Liposome, an artificial particle, composed of phospholipids encapsulating a volume of aqueous compounds, has been successfully applied in immunoassays and biosensors (Diaz-Gonzalez et al., 2005; Jesorka and Orwar, 2008). We previously reported a fluorescence-based liposome immunoassay using liposomes tagged with developed antibodies for easy and rapid detection of *Cronobacter muytjensii* (Song et al., 2015) and *Cronobacter sakazakii* (Shukla et al., 2016a). Magnetic nanoparticles conjugated with antibody were also successfully used as a tool for the detection of foodborne pathogens such as *Escherichia coli* O157:H7 (DeCory et al., 2005), *Salmonella* (Liu et al., 2001), and *Cronobacter* (Mullane et al., 2006; Jans et al., 2009; Shukla et al., 2016a, 2016b). The present study was conducted to develop an easy, simple, and rapid method for the detection of 7 *Cronobacter* species. To accomplish this object, an antibody against 7 *Cronobacter* species was developed and applied into an immunomagnetic nanoparticle and immunoliposome.

2. Materials and methods

2.1. Bacterial strains and reagents

The bacterial strains used in this study were purchased from the American Type Culture Collection (ATCC), Belgian Coordinated Collections of Microorganisms (strains indicated by LMG), Korean Collection for Type Cultures (KCTC), and Korean Culture Center of Microorganisms (KCCM), except for *Cronobacter muytjensii* (CDC 3523-75) donated by Dr. Carol Iversen from University College Dublin, Ireland. *Bacillus cereus* (KCCM 40935), *Buttiauxella noackiae* (ATCC 51713), *Citrobacter freundi* (ATCC 8090), *Cronobacter condimenti* (LMG 26250), *Cronobacter dublinensis* (LMG 23823), *Cronobacter malonaticus* (LMG 23826), *Cronobcter muytjensii* (ATCC 51329), *Cronobacter muytjensii* (CDC 3523-75), *Cronobacter sakazakii* (ATCC 29004), *Cronobacter sakazakii* (ATCC 29544), *Cronobacter turicensis* (LMG 23827), *Cronobacter universalis* (LMG 26249), *Enterobacter aerogenes* (ATCC 15038), *Escherichia coli* (ATCC 39418), *Escherichia coli* O157:H7 (ATCC 43888), *Franconibacter helveticus* (LMG 23732), *Franconibacter pulveris* (LMG 24057), *Lactobacillus brevis* (KCTC 3498), *Lactobacillus paracasei* (KCTC 3260), *Listeria monocytogenes* (ATCC 19115), *Salmonella* Typhimurium (ATCC 13311), and *Siccibacter turicensis* (LMG 23730) were used in this study. All strains were cultured in nutrient broth (NB) for 18 h at 37 °C on a shaking incubator (150 rpm).

NB, bactopeptone, and skim milk were purchased from Difco (Franklin Lakes, NJ, USA). Phosphatase-labeled goat anti-rabbit immunoglobulin (IgG) was purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD, USA). PIF used for the food test was purchased from a local market in Gyeongsan-si, Republic of Korea.

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol, tris(hydroxymethyl)aminomethane (Tris), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), *n*-octyl-*β*-D-glucopyranoside (OG), potassium phosphate dibasic, potassium phosphate monobasic, sucrose, Tween 20, and sodium chloride were purchased from Sigma (St. Louis, MO, USA). *N*-Succinimidyl-*s*-acetylthioacetate (SATA) and sulforhodamine B (SRB) were purchased from Pierce (Rockford, MD, USA). Carboxyl magnetic iron oxide nanoparticles conjugation kits, containing carboxyl magnetic iron oxide nanoparticles, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), and *N*-hydroxysuccinimide (NHS), were purchased from Ocean Nano Tech (Springdale, AR, USA). Ninetysix-well microtiter plate was purchased from SPL Life Sciences (Pocheon, Gyeonggi-do, Korea). Magnetic particle concentrator was purchased from Dynal Inc. (Lake Success, NY, USA).

2.2. Preparation of immunomagnetic nanoparticles with anti-*Cronobacter* IgG

The polyclonal rabbit anti-*Cronobacter* IgG used in this study was prepared in the Laboratory of Food Safety and Microbiology, Yeungnam University, Republic of Korea, under Animal Ethics License No. 2013-012 and 2012-010 (Song et al., 2016). The purified rabbit anti-*Cronobacter* IgG was conjugated to the carboxyl magnetic iron oxide nanoparticles according to the manufacturer's instruction and the method described in our previous study (Shukla et al., 2016a). Briefly, 0.2 mL of the magnetic iron oxide nanoparticles (30 nm diameter) was added into a 1.5 mL eppendorf tube, after which 0.2 mL activation buffer was added to the magnetic iron oxide nanoparticles. Next, 100 μL of the EDAC/NHS (containing 0.5 mg/mL EDAC and 0.25 mg/mL NHS) solution was added into the magnetic iron oxide nanoparticles, mixed well, and allowed to react at room temperature for 5–10 min with continuous mixing. Next, 0.5 mL of coupling buffer was added to the activated magnetic iron oxide nanoparticles, mixed well, and amended with 0.5 mL of a various of concentrations of anti-*Cronobacter* IgG at 3.0 mg/mL, 3.4 mg/mL, and 4.0 mg/mL. The reactant of anti-*Cronobacter* IgG and magnetic iron oxide nanoparticles was then allowed to react at room temperature for 2 h with continuous mixing. The reactant was subsequently incubated for 10 min at room temperature after added with 10 μL quenching solution. Next, the reaction mixture was transferred into a disposable glass tube, after which 3 mL wash/storage solution was added, and the reactant was mixed gently. The disposable glass tube was then inserted into the magnetic particle concentrator, and the conjugated magnetic iron oxide nanoparticles were allowed to separate at 4 °C for 4 h, after which the buffer was carefully transferred to a new disposable glass tube to check unconjugated anti-*Cronobacter* IgG. Finally, anti-*Cronobacter* IgG conjugated magnetic iron oxide nanoparticles were resuspended with 1 mL wash/storage buffer to store up to 3 months at 4 °C. The conjugation of anti-*Cronobacter* IgG and magnetic nanoparticles was confirmed by measuring the average particle size change before and after conjugation (Jans et al., 2009; Chen et al., 2016).