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An immunoliposome-based immunochromatographic strip assay for the rapid detection of *Cronobacter* species

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ABSTRACT

Cronobacter species are foodborne pathogens that pose a high risk in infant formula and can cause fatality rates of 40–80% in infected infants. To develop a rapid and easy detection method for *Cronobacter* species, especially in powdered infant formula (PIF), an immunoliposome-based immunochromatographic strip assay was developed using an anti-*Cronobacter* immunoglobulin G (IgG)-conjugated liposome and an anti-*Cronobacter* IgG-coated nitrocellulose membrane. The developed assay could detect *Cronobacter* species in both pure culture and artificially contaminated PIF. The detection limits of the developed assay were 10^6–10^7 colony forming units (CFU)/mL in pure culture and 10^7–10^8 CFU/g in PIF by visual judgment, respectively. When the immunoliposome-based immunochromatographic strip assay results were analyzed using QuantiScan, the detection limit decreased to 10^5–10^7 CFU/mL in pure culture and 10^6–10^8 CFU/g in PIF, except for *Cronobacter malonaticus*. Furthermore, visual judgment showed that the developed immunochromatographic strip could not detect *Cronobacter malonaticus* in pure culture or PIF. However, *Cronobacter malonaticus* could be detected after QuantiScan analysis, and the detection limits were 10^8 CFU/mL and 10^8 CFU/g in both pure culture and PIF. This developed immunoliposome-based immunochromatographic strip assay is simple, easy, and effective method to detect *Cronobacter* species and thus could be widely applied in the food industry, research institutes, and even for onsite detection.

1. Introduction

Cronobacter species, *Cronobacter condimenti*, *Cronobacter dublinensis*, *Cronobacter malonaticus*, *Cronobacter muytjensii*, *Cronobacter sakazakii*, *Cronobacter turicensis*, and *Cronobacter universalis*, are members of the family *Enterobacteriaceae* (Iversen et al., 2008; Joseph et al., 2012; Jackson et al., 2014). Species of this genus are foodborne pathogens that pose a high risk of infection to neonates and immune-compromised individuals. *Cronobacter* species can cause serious diseases, such as severe necrotizing enterocolitis, bacteremia, and meningitis, in infants, with fatality rates of 40 to 80% (Van Acker et al., 2001; Centers for Disease Control and Prevention, 2002; Bowen and Braden, 2006). *Cronobacter* species have been isolated from a range of environments, in clinical settings, and in food and beverage sources, such as water, vegetables, cheese, and meat, even in ready-to-eat foods (Kim and Beuchat, 2005; Beuchat et al., 2009; Lee et al., 2012; Pei et al., 2019). Powdered infant formula (PIF) is the only food source that has been linked to epidemic disease outbreaks caused by *Cronobacter* species (Food and Agriculture Organization/World Health Organization, 2004). Recent outbreaks and the increased prevalence of *Cronobacter* species have led *Cronobacter* species to become the most risky foodborne pathogens in PIF. In 2004, an outbreak of this disease occurred in New Zealand, which was caused by PIF used in a nursery, resulted in the deaths of premature infants that contracted meningitis (Food and Agriculture Organization/World Health Organization, 2007). Another outbreak occurred in France resulted in two deaths out of nine infected individuals (Food and Agriculture Organization/World Health Organization, 2006). Additionally, there have been a number of recalls of PIF worldwide since 2004 due to contamination with *Cronobacter* species (Food and Agriculture Organization/World Health Organization, 2007). Jung and Park (2006) analyzed PIF samples in the Republic of Korea and reported a 20% contamination rate with *Cronobacter* species. Lee et al. (2012) also reported the isolation of *Cronobacter* species from 18.6% of detected food samples. Yao et al. (2016) isolated *Cronobacter* species from 16 of 133 indigenous infant formulas sold in public heath care centers in Abidjan, Côte d'Ivoire.

Therefore, a rapid and convenient method to monitor *Cronobacter* species in PIF is required. All *Cronobacter* species are considered

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pathogenic, even if only several species have been isolated from infected neonates, such as *Cronobacter sakazakii*, *Cronobacter malonaticus*, and *Cronobacter turicensis*. The classical method to detect and identify *Cronobacter* species requires 5 to 7 days to get a final result (United States Food and Drug Administration, 2012), which is laborious and time consuming. Several methods used in combination with other technologies, such as polymerase chain reaction (PCR) and immunology have been reported for the detection of *Cronobacter* in food samples (Wang et al., 2012; Zhao et al., 2013; Xu et al., 2014; Zimmermann et al., 2014; Song et al., 2016a). Zhou et al. (2008) developed an immobilization and detection method for *Cronobacter* species utilizing species-specific duplex PCR, based on the 16s rDNA internal transcribed spacer and the *omp*A gene. The method had a detection limit of 3×10^5 colony forming units (CFU)/mL in pure culture. Similarly, Ruan et al. (2013) reported a duplex PCR method for the detection of *Cronobacter* species. Chen et al. (2014) reported an immunochromatographic strip used in combination with silica-coated magnetic nanoparticles and a 16S rRNA probe to detect *Cronobacter* species. The detection limit was 10^5 CFU/mL in pure culture. These PCR-based methods are sensitive but are limited by the need for PCR instruments and sophisticated techniques.

Biosensors are analytical devices that have been developed for a wide spectrum of analytes, including microbes, toxins, proteins, drugs, and ions (Mazur et al., 2017). Liposomes are lipid-based vesicular carriers comprised of a phospholipid outer shell bilayer and an internal chamber that encapsulates signal markers (Liu et al., 2019). Liposome-based assays have many advantages, such as sensitivity, rapid processing times, wide dynamic range, and simple operation without any expensive equipment (Petaccia et al., 2017).

The present study explored the use of a novel immunoliposome conjugated with a genus-specific polyclonal antibody against *Cronobacter* species to detect *Cronobacter* species in food samples, especially in PIF. This assay is simple, easy, and effective for onsite detection of *Cronobacter* species.

2. Materials and methods

2.1. Strains and reagents

Cronobacter muytjensii (CDC 3523-75) was donated by Dr. Carol Iversen, University College Dublin, Ireland. All other strains were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA), the Belgian Coordinated Collections of Microorganisms (BCCM; Brussels, Belgium; strains are indicated as LMG), and the Korean Culture Center of Microorganisms (KCCM; Seoul, Republic of Korea). The strains used were: *Bacillus cereus* (KCCM 40935), *Buttiauxella noackiae* (ATCC 51713), *Citrobacter freundi* (ATCC 8090), *Cronobacter condimenti* (LMG 26250), *Cronobacter dublinensis* (LMG 23823), *Cronobacter malonaticus* (LMG 23826), *Cronobacter muytjensii* (CDC 3523-75), *Cronobacter sakazakii* (ATCC 29544), *Cronobacter sakazakii* (ATCC 29004), *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), *Salmonella* Typhimurium (ATCC 13311), and *Yersinia enterocolitica* (KCCM 41657). All strains were cultured in nutrient broth (NB) for 18 h at 37 °C on a shaking incubator (150 rpm). NB and bactopeptone were purchased from Difco (Franklin Lakes, NJ, USA). Nitrocellulose membranes were purchased from Millipore (Tullagreen, Carrigtwohill, Ireland). Goat anti-rabbit immunoglobulin G (IgG) was purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD, USA). PIF used for food testing was purchased from a local market in Gyeongsan-si, Republic of Korea.

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-bis(diphenylphosphino)ethane (DPPE), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphor-(1'-rac-glycerol) (DPPG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Casein, cholesterol, chlorofrom, *N*-ethylmaleimide, dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), membrane filters (0.4 μm, 0.8 μm), methanol, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), Sepharose CL-4B, *N*-succinimidyl-*s*-acetylthioacetate (SATA), sodium azide, sodium chloride, sulfo-KMUS, polyvinylpyrrolidone, potassium phosphate monobasic, potassium phosphate dibasic, and Tris were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dialysis membrane (MWCO: 12-14 kD) was purchased from Spectrum laboratories, Inc. (San Diego, CA, USA). Sulforhodamine B (SRB) dye was purchased from Pierce (Rockford, IL, USA). Disposable glass tubes (12 mm × 78 mm) were purchased from Kimble Chase (Rockwood, TN, USA).

2.2. Preparation and conjugation of SRB-encapsulated liposomes with rabbit anti-Cronobacter IgG

DPPE-ATA, DPPC, DPPG, and cholesterol were used to prepare the liposomes as previously described (Song et al., 2016b). Briefly, the DPPE-ATA was prepared by dissolving DPPE (7.2 μM) and SATA (14.3 μM) in 1 mL of 0.7% triethylamine in chloroform (v/v). The lipid mixture of DPPC (40.3 μM), DPPG (4.2 μM), and cholesterol (40.9 μM) was dissolved in a mixture of 3 mL chloroform and 0.5 mL methanol. Two milliliters of encapsulant (100 mM SRB in 0.02 M HEPES buffer; pH 7.5) was immediately added to the lipid mixture after 1 min of sonication at 45 °C. The organic solvent was removed by evaporation at 45 °C, leaving a dark purple, gel-like suspension. An additional 2 mL of the encapsulant was added to the suspension, followed by 1 min of sonication. Vortex, evaporation, and sonication were alternately repeated until a uniform suspension was obtained. The suspension was extruded through 0.8 μm and 0.4 μm membrane filters to produce a more uniform size. The SRB-encapsulated liposomes were collected and dialyzed overnight in 0.01 M HEPES buffer containing 2 M NaCl and 0.01% NaN_3 (pH 7.5). The liposome solution was kept at 4 °C in the dark until use. Liposome particle size was measured using a Zetasizer (Malvern; Worcestershire, UK).

Anti-*Cronobacter* IgG-tagged liposomes (immunoliposomes) were prepared as previously described (Song et al., 2016b). The rabbit anti-*Cronobacter* IgG used in this study was prepared at Yeungnam University Laboratory Animal Center under Animal Ethics License No. 2013-012 and 2012-010 (Song et al., 2016c). Firstly, purified rabbit anti-*Cronobacter* IgG (1 mg) was dissolved in 1 mL of 0.05 M phosphate buffered saline (PBS) containing 1 mM EDTA and 0.01% NaN_3 (pH 7.8). Sulfo-KMUS solution prepared by dissolving 2 mg of sulfo-KMUS in 0.1 mL of a solvent mixture of DMSO and methanol (2:1, v/v) was then added and allowed to react with orbital shaking (70 rpm) at room temperature for 3 h. IgG derivatized with maleimide groups was dialyzed overnight in 0.02 M HEPES containing 0.15 M NaCl and 0.01% NaN_3 (pH 7.0) at 4 °C in the dark. The SH-containing liposome solution was adjusted to pH 7.0 by adding 0.5 M HEPES and then mixed with the maleimide-derivatized IgG solution. The mixture was flushed with nitrogen gas for 1 min and incubated at room temperature for 4 h, followed by incubation in the dark at 4 °C overnight. Ethylmaleimide (100 mM) dissolved in 0.02 M Tris buffer containing 0.15 M NaCl and 0.01% NaN_3 (TBS, pH 7.0) was added to the conjugated liposome solution and reacted at 70 rpm for 30 min to quench unreacted sulfhydryl groups. The immunoliposome solution was separated by filtering through a Sepharose CL-4B column equilibrated with 0.02 M TBS. The collected immunoliposomes were dialyzed with the same buffer of 0.02 M TBS and then stored at 4 °C in the dark until use. The conjugation of anti-*Cronobacter* IgG and the liposomes was confirmed by measuring the average change in particle size of the liposomes before and after conjugation (Jans et al., 2009; Chen et al., 2016).

2.3. Preparation of immunochromatographic strip

The immunochromatographic strip was prepared as previously

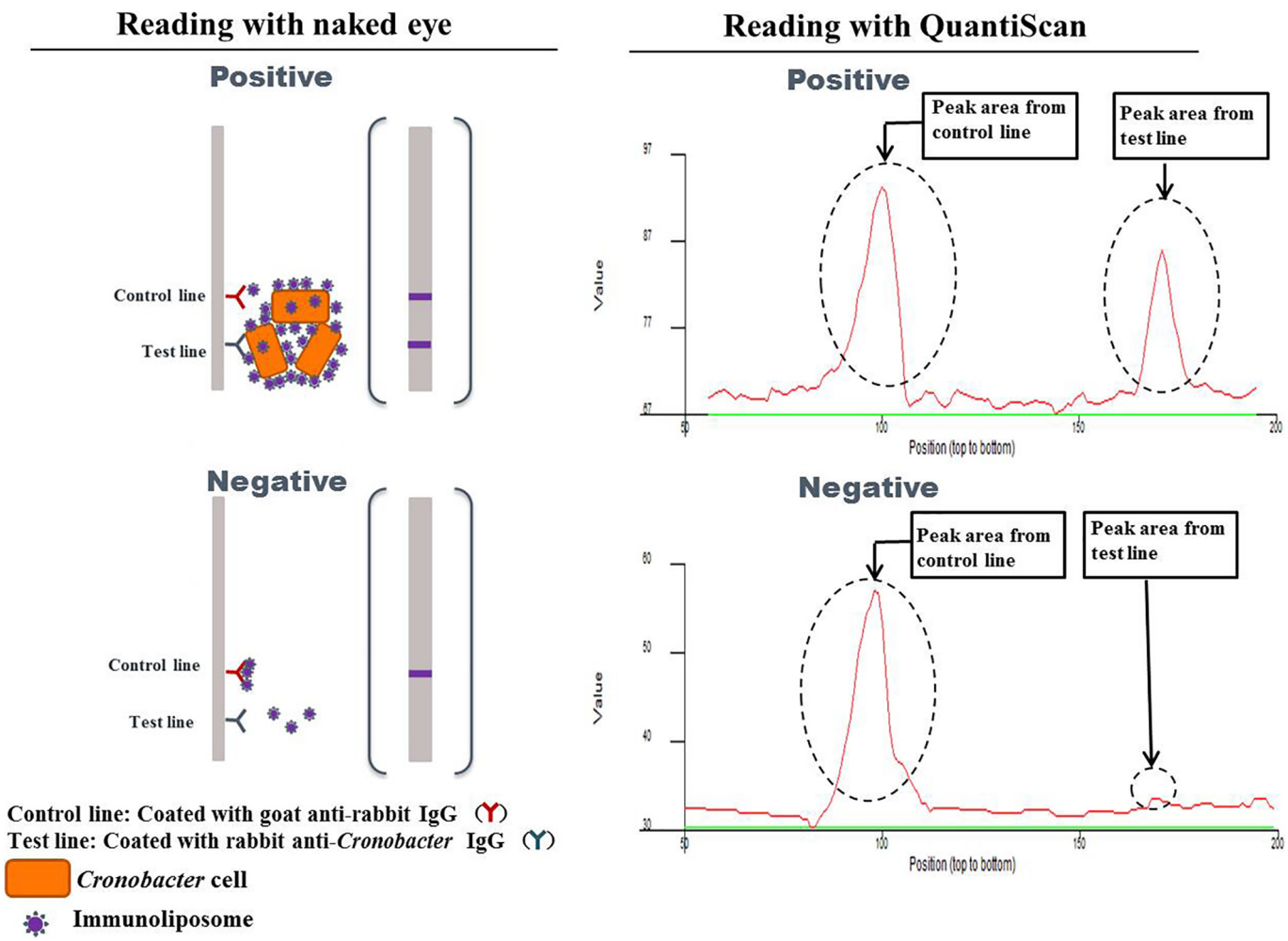

Fig. 1. Format of data analysis.

described (Song et al., 2016b). A plastic-backed nitrocellulose membrane was cut into pieces (8.5 cm × 10 cm) and wetted with 10% methanol in PBS (pH 7.0) at room temperature for 15 min with shaking at 70 rpm to maintain the hydrophobicity of the membrane. The membrane pieces were dried for 2 h under vacuum (15 psi) at 30 °C. The coating process was performed using a Linomat IV apparatus (Camag; Muttenz, Switzerland). The developed genus-specific antibody (rabbit anti-*Cronobacter* IgG) was coated on the test line, and goat anti-rabbit IgG was coated on the control line. After coating, the membrane was blocked in 0.02 M TBS (pH 7.0) containing 0.5% polyvinylpyrrolidone and 0.02% casein. The membrane was dried overnight under vacuum (15 psi) at 30 °C. The prepared membrane was cut into test strips, with a width of 0.5 cm and a length of 8.5 cm, and stored at 4 °C until use.

2.4. Detection of Cronobacter species by immunoliposome-based immunochromatographic strip assay in pure culture

Seven *Cronobacter* species were cultured in NB at 37 °C and 150 rpm for 18 h, respectively, and then serially diluted (10^3–10^8 CFU/mL) with buffered peptone water (BPW) for further use. A stock solution of prepared immunoliposomes was diluted to the appropriate ratio (1:5) with 0.02 M TBS. The immunochromatographic strip was dipped in a mixture of 50 μL diluted culture and 50 μL immunoliposome solution in a disposable glass tube and left for 10 min to let the liquid flow by capillary action. The intensity of the purple color for both the test and control lines of the developed test strip was examined visually and then measured using QuantiScan software. The presence of a purple color in the test and control lines indicated a positive test result. No color in the test line and a purple color in the control line of the developed strip was interpreted as a negative test result.

2.5. Cross-reactivity of immunoliposome-based immunochromatographic strip assay

The specificity of the developed immunoliposome-based immunochromatographic strip assay was tested using foodborne pathogens. These included *Bacillus cereus, Citrobacter freundi, Enterobacter aerogenes, Escherichia coli, Escherichia coli O157:H7, Franconibacter helveticus, Franconibacter pulveris, Salmonella Typhimurium, and Yersinia enterocolitica*. Fresh cultures of the pathogens were adjusted to 10^8 CFU/mL with BPW and 50 μL of each suspension was mixed with 50 μL of immunoliposomes in a disposable glass tube. The prepared immunochromatographic strip was dipped into the mixture and incubated for 10 min to develop the result.

2.6. Detection of Cronobacter species in PIF

An important aim of this study was to develop an immunochromatographic strip that could be used in PIF samples. Therefore, *Cronobacter* species-spiked PIF samples were tested using the developed immunochromatographic strip. First, fresh cultures of seven *Cronobacter* species were separately prepared with NB media (18 h incubation at 37 °C, 150 rpm). Each culture was serially diluted (10^3–10^8 CFU/mL) in BPW. PIF samples (25 g) were homogenized in

225 mL BPW and each was spiked with various dilutions of each *Cronobacter* culture (10^3–10^8 CFU/g) for direct analysis. Simultaneously, PIF samples (25 g) spiked with a low concentration (< 10 *Cronobacter* bacterial cells) of *Cronobacter* were enriched in 225 mL BPW, and then incubation times of 10 h and 14 h were investigated. Fifty microliter aliquots of each enrichment culture were taken and mixed with 50 μL of appropriately diluted immunoliposomes. The immunochromatographic strip was dipped in this mixture and left for 10 min to obtain the result.

2.7. Result judgment and statistical analysis

The assay results were analyzed by both the visual observation and QuantiScan software (Biosoft;Cambridge, UK) (Fig. 1). For QuantiScan analysis, the average of the peak area of the test line on the tested immunochromatographic strip of an un-spiked sample was used as the negative control. The result was presumptively positive when the peak area of test line on the tested strip of spiked sample was more than three times greater than that of the negative control. All experiments and results were analyzed in at least three trials to provide reliable and reproducible data.

3. Results and discussion

3.1. Detection of Cronobacter species by immunoliposome-based immunochromatographic strip assay

When immunoliposomes and *Cronobacter* bacterial cells are mixed, the immunoliposomes can bind to the *Cronobacter* surface. The immunoliposome-*Cronobacter* complexes flow up the immunochromatographic strip via capillary migration and are captured by the rabbit anti-*Cronobacter* IgG of the test line to produce a purple signal. The remaining free immunoliposomes flow further up on the strip and bind with the goat anti-rabbit IgG of the control line. This binding is also apparent by a purple color. Both the test line and control line appear purple for positive samples. In the negative samples which lack *Cronobacter* species, the immunoliposomes cannot complex with *Cronobacter* species and bind only with the goat anti-rabbit IgG on the control line. The result is one purple line on the strip. The results of the visual and QuantiScan analysis of seven *Cronobacter* species in pure culture are shown in Fig. 2 and Table 1, respectively.

The visually determined detection limits of the developed immunochromatographic strip for *Cronobacter condimenti*, *Cronobacter dublinensis*, *Cronobacter muytjensii*, *Cronobacter sakazakii*, *Cronobacter turicensis*, and *Cronobacter universalis* were as 10^7 CFU/mL for all except *Cronobacter muytjensii* (10^6 CFU/mL) (Fig. 2). The detection limit for the respective species using the QuantiScan analysis was 10^6, 10^6, 10^8, 10^5, 10^6, 10^6, and 10^6 CFU/mL (Table 1). The QuantiScan analysis evidently enhanced the detection sensitivity compared with the visual judgment. In particular, *Cronobacter malonaticus* could be detected using QuantiScan analysis while it was difficult to judge visually. Shukla et al. (2014) reported on a liposome-based immunochromatographic test strip, which showed a limit of detection 10^6–10^7 CFU/mL for *Salmonella Typhimurium*. Song et al. (2016b) reported that an immunochromatographic strip coated with IgG to *Cronobacter sakazakii* had a detection limit of 10^7 CFU/mL.

3.2. Cross-reactivity of immunoliposome-based immunochromatographic strip assay in pure culture

The cross-reactivity results of the developed immunoliposome-based immunochromatographic strip were assessed visually and using the QuantiScan analysis (Fig. 3 and Table 2, respectively). The developed assay showed no cross-reactivity with the nine tested species (*Bacillus cereus*, *Citrobacter freundi*, *Enterobacter aerogenes*, *Escherichia coli*, *Escherichia coli* O157:H7, *Franconibacter helveticus*, *Franconibacter pulveris*, *Salmonella* Thphimurium, and *Yersinia enterocolitica*). These findings indicated that the novel strip assay was specific for the detection of *Cronobacter* species. These results agreed well with the result of a competitive enzyme-linked immunosorbent assay developed using anti-*Cronobacter* IgG (Song et al., 2016c). Several methods have been reported for the species-specific detection of *Cronobacter sakazakii*, including an immunoliposome-based immunomagnetic concentration and separation assay (Shukla et al., 2016) and an immunochromatographic strip assay (Song et al., 2016b). Hu et al. (2015) developed a real-time PCR assay based on the *cgcA* gene of *Cronobaccter sakazakii* and a TaqMan probe with internal amplification control. The method displayed very good species specificity. Zimmermann et al. (2014)

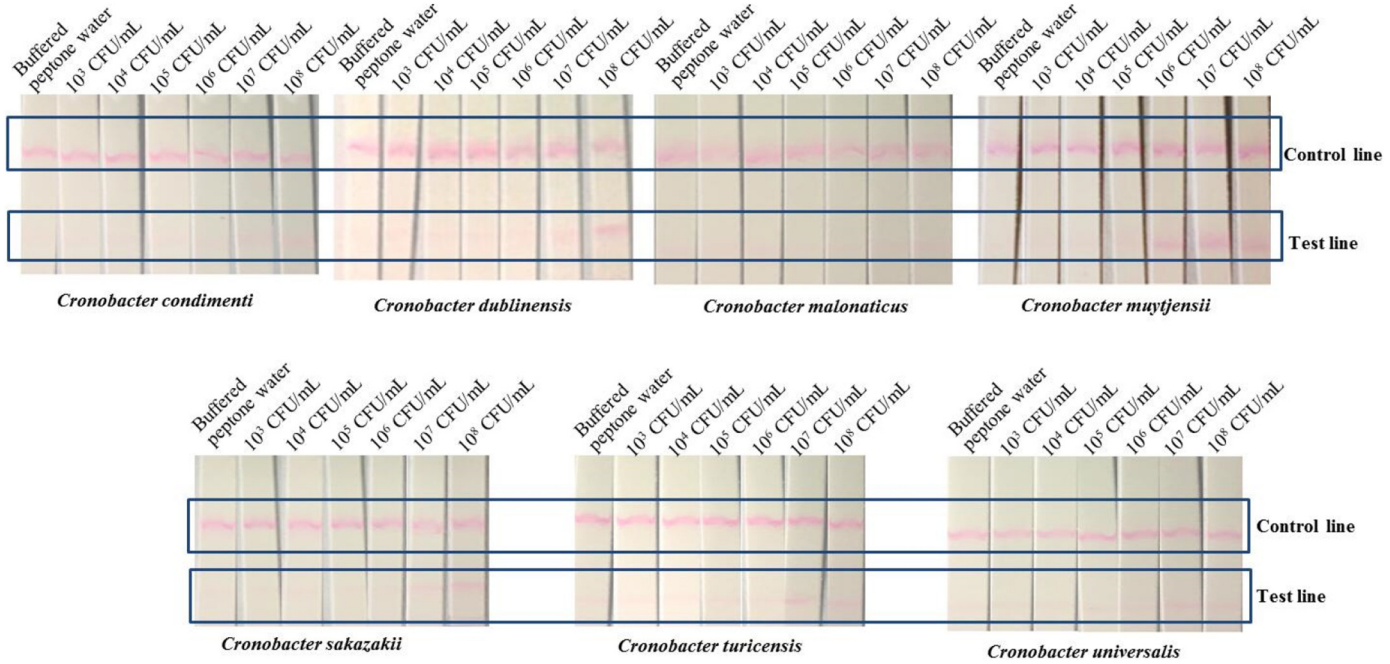

Fig. 2. Detection of *Cronobacter* species by immunoliposome-based immunochromatographic strip assay in pure culture.

Table 1
The QuantiScan result of detecting *Cronobacter* species in pure culture using immunoliposome-based immunochromatographic strip assay.

Cronobacter species	Concentration of *Cronobacter* species (CFU/mL)									
	0		10^3		10^4		10^5	10^6	10^7	10^8
	Peak area/Interpretation		Peak area/Interpretation		Peak area/Interpretation		Peak area/Interpretation	Peak area/Interpretation	Peak area/Interpretation	Peak area/Interpretation
Cronobacter condimenti	0/−		0/−		0/−		0/−	17.94 ± 2.11/+	45.39 ± 1.26/+	73.40 ± 1.54/+
Cronobacter dublinensis	0/−		0/−		0/−		0/−	43.67 ± 1.63/−	50.60 ± 1.27/+	107.82 ± 1.71/+
Cronobacter malonaticus	0/−		0/−		0/−		0/−	0/−	0/−	33.80 ± 3.05/+
Cronobacter muytjensii	0/−		0/−		0/−		34.31 ± 1.85/+	85.09 ± 3.03/+	146.40 ± 2.88/+	91.38 ± 2.39/+
Cronobacter sakazakii	0/−		0/−		0/−		0/−	30.31 ± 2.15/+	58.18 ± 1.71/+	89.67 ± 3.65/+
Cronobacter turicensis	0/−		0/−		0/−		0/−	24.40 ± 0.87/+	62.69 ± 2.88/+	85.92 ± 3.27/+
Cronobacter universalis	0/−		0/−		0/−		0/−	29.69 ± 2.60/+	42.90 ± 1.59/+	74.08 ± 0.94/+

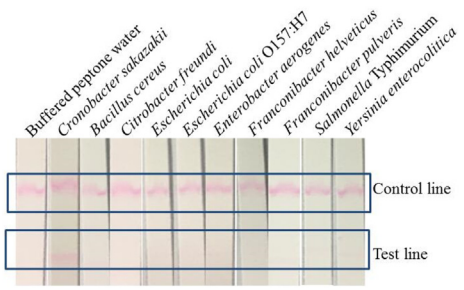

Fig. 3. Cross-reactivity of immunoliposome-based immunochromatographic strip assay in pure culture.

Table 2
The QuantiScan result of cross-reactivity of developed immunoliposome-based immunochromatographic strip assay in pure culture.

Strains	Collection number	Peak area	Interpretation
Buffered peptone water		0	−
Cronobacter sakazakii	ATCC 29544	125.84 ± 5.10	+
Bacillus cereus	KCCM 40935	0	−
Citrobacter freundii	ATCC 8090	0	−
Enterobacter aerogenes	ATCC 15038	0	−
Escherichia coli	ATCC 39418	0	−
Escherichia coli O157:H7	ATCC 43888	0	−
Franconibacter helveticus	LMG 23732	0	−
Franconibacter pulveris	LMG 24057	0	−
Salmonella typhimurium	ATCC 13311	0	−
Yersinia enterocolitica	KCCM 41657	0	−

reported a rapid system for the detection of *Cronobacter* species that included enrichment, DNA-isolation, and detection by real-time PCR using the outer membrane protein gene *ompA* as the target. Wang et al. (2012) reported a TaqMan real-time PCR assay with a detection limit of 10^3 CFU/mL in pure culture. For all these methods, PCR was indispensable for the genus-specific detection of *Cronobacter* species. While accurate, the requirement for PCR makes these detections relatively complex. The present immunoliposome-based immunochromatographic strip assay for *Cronobacter* species is very convenient. The test strip needs to be dipped in the test sample-immunoliposome mixture, and the result is available in 10 min. Thus, it might be applicable for the rapid detection of *Cronobacter* species in the food industry, in research, and even in the field for onsite detection.

3.3. Detection of Cronobacter species in PIF

The results of the visual and QuantiScan detection of the seven *Cronobacter* species in spiked PIF are shown in Fig. 4 and Table 3, respectively. When the PIFs were spiked with high levels of *Cronobacter* species (10^3–10^8 CFU/g), enrichment was not necessary, and the detection limits were 10^8 CFU/g for *Cronobacter condimenti*, *Cronobacter muytjensii*, *Cronobacter turicensis*, and *Cronobacter universalis* and 10^7 CFU/g for *Cronobacter dublinensis* and *Cronobacter sakazakii* (Fig. 4). *Cronobacter malonaticus* could not be detected visually. QuantiScan analysis revealed detection limits of 10^7, 10^6, 10^8, 10^6, 10^7, 10^6, and 10^7 CFU/g for *Cronobacter condimenti*, *Cronobacter dublinensis*, *Cronobacter malonaticus*, *Cronobacter muytjensii*, *Cronobacter sakazakii*, *Cronobacter turicensis*, and *Cronobacter universalis*, respectively (Table 3). When the PIF samples (25 g) were spiked with < 10 *Cronobacter* bacterial cells, enrichment of the PIF samples for 14 h produced positive results, while enrichment of the PIF samples for 0 h and 10 h did not show color development on the test line (Fig. 5). The detection limit using the 14-h enrichment was < 10 cells in 25 g of PIF. The only exception was *Cronobacter malonaticus*, which could not be detected in both the 10 h and 14 h enrichment samples. The QuantiScan analysis results agreed with the visual judgment (Table 4). Recently, Song et al.

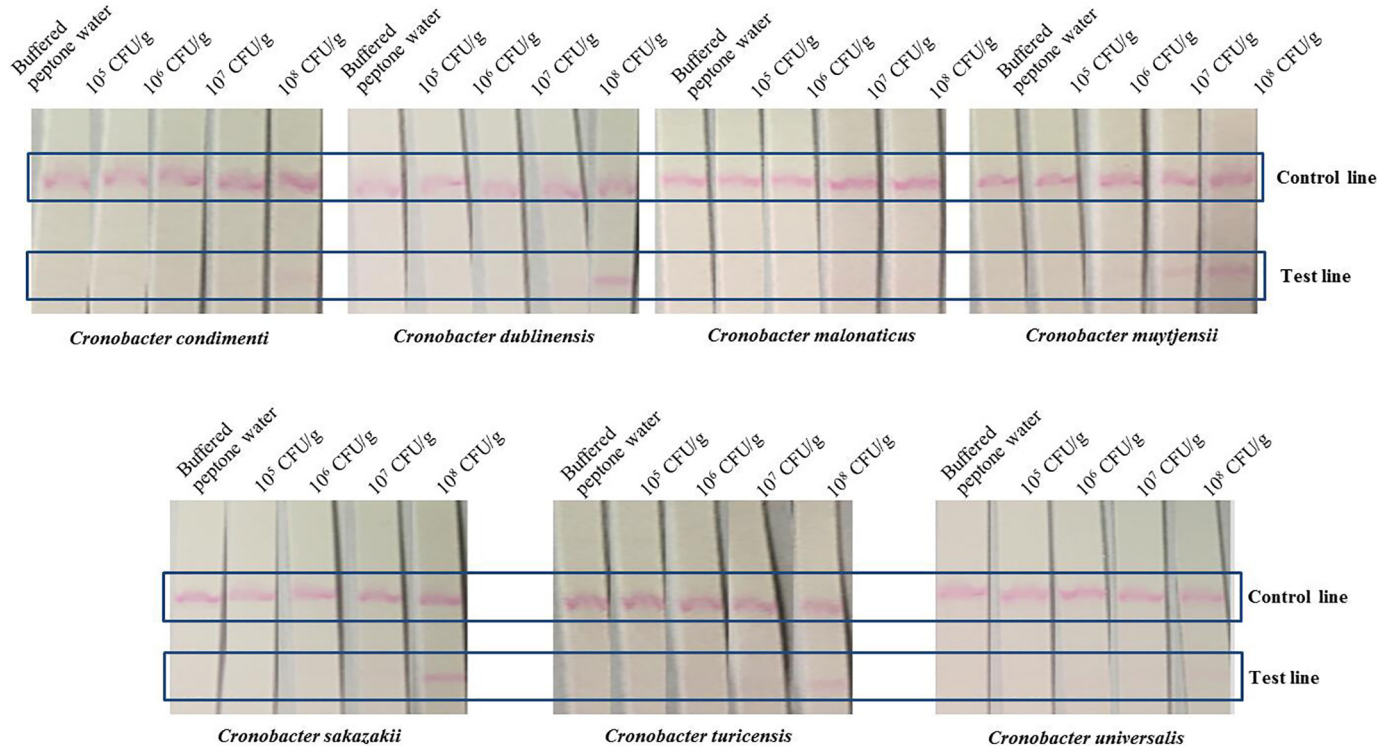

Fig. 4. Detection of *Cronobacter* species by immunoliposome-based immunochromatographic strip assay in powdered infant formula (without enrichment).

Table 3
The QuantiScan result of detecting *Cronobacter* species in powdered infant formula using immunoliposome-based immunochromatographic strip assay.

Spiked *Cronobacter* species	Concentration of spiked *Cronobacter* species (CFU/g)				
	0	10^5	10^6	10^7	10^8
	Peak area/Interpretation	Peak area/Interpretation	Peak area/Interpretation	Peak area/Interpretation	Peak area/Interpretation
Cronobacter condimenti	0/−	0/−	0/−	27.67 ± 1.97/+	50.27 ± 2.15/+
Cronobacter dublinensis	0/−	0/−	45.40 ± 1.61/+	72.14 ± 1.22/+	68.26 ± 0.68/+
Cronobacter malonaticus	0/−	0/−	0/−	0/−	33.60 ± 1.58/+
Cronobacter muytjensii	0/−	0/−	51.08 ± 2.28/+	85.47 ± 1.38/+	122.47 ± 1.64/+
Cronobacter sakazakii	0/−	0/−	0/−	30.87 ± 2.47/+	36.24 ± 1.06/+
Cronobacter turicensis	0/−	0/−	34.24 ± 1.37/+	38.67 ± 3.04/+	110.24 ± 3.31/+
Cronobacter universalis	0/−	0/−	0/−	32.81 ± 2.14/+	64.10 ± 3.00/+

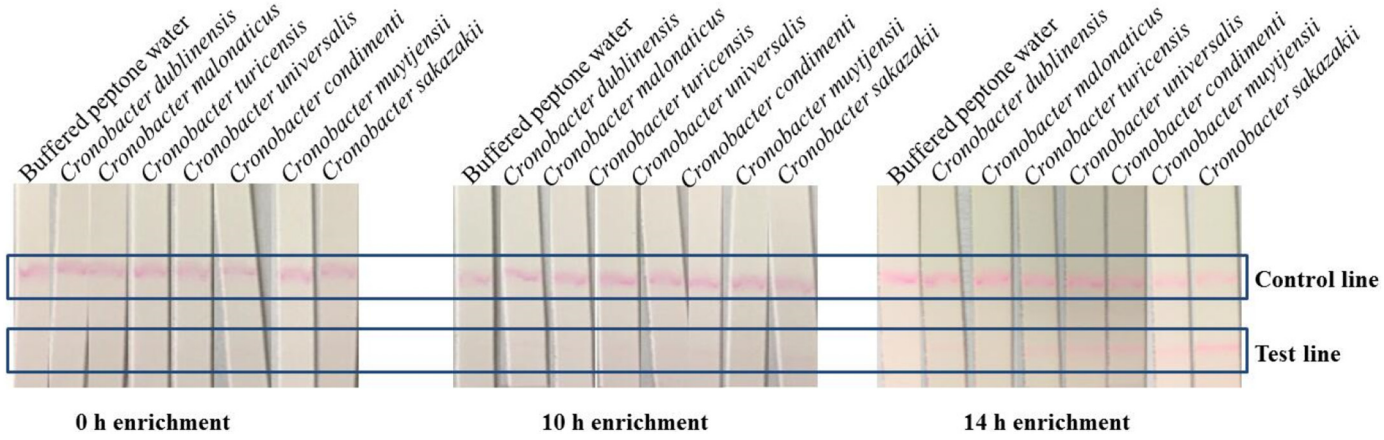

Fig. 5. Detection of *Cronobacter* species by immunoliposome-based immunochromatographic strip assay in spiked powdered infant formula.

Table 4
The QuantiScan result of detecting *Cronobacter* species in powdered infant formula after enrichment using immunoliposome-based immunochromatographic strip assay.

Spiked *Cronobacter* species	Enrichment time		
	0 h	10 h	14 h
	Peak area/Interpretation	Peak area/Interpretation	Peak area/Interpretation
Buffered peptone water	0/−	0/−	0/−
Cronobacter condimenti	0/−	0/−	111.52 ± 2.55/+
Cronobacter dublinensis	0/−	0/−	83.86 ± 1.73 /+
Cronobacter malonaticus	0/−	0/−	0/−
Cronobacter muytjensii	0/−	0/−	52.90 ± 2.43/+
Cronobacter sakazakii	0/−	0/−	73.38 ± 2.74/+
Cronobacter turicensis	0/−	0/−	54.34 ± 1.17/+
Cronobacter universalis	0/−	0/−	31.05 ± 2.33/+

(2016a) reported the use of an immunofluorescence strip to detect *E. coli* O157:H7, based on the reaction of fluorescent bacteria and an unlabeled monoclonal antibody immobilized on the test line. The visual detection limit of the strip was 10^6 CFU/mL, and the detection limit for semi-quantitative detection using a fluorescence scan reader was 10^5 CFU/mL. In the rapid detection method developed by Zimmermann et al. (2014), as few as 10 *Cronobacter* cells in PIF could be detected. However, for this detection, enrichment, DNA-isolation, and real-time PCR were required, which limited the application of the developed method. Blazkova et al. (2011) reported an immunochromatographic strip test that could detect 10 cells of *Cronobacter* species in PIF in 16 h, in which the DNA of *Cronobacter* was isolated, and the 16s rRNA gene was amplified by PCR. Wang et al. (2012) reported a TaqMan real-time PCR assay with a detection limit of 10^0 CFU/mL in PIF in which 24 h of enrichment was needed. All these reported detection methods are based on PCR and are more rapid than the conventional methods. However, the limitation of the PCR-based methods is obvious; they require specific instruments and user training. In contrast, the presently-developed assay is rapid (10 min) and simple. This assay could be used widely in food, in research, and in domestic settings.

PIF is an important source of nutrition for infants, and its safety is closely associated with infant heath. Concerns regarding the contamination of PIF with *Cronobacter* species have increased in recent years because of the high risk of fatality due to contaminated infant formula. Controlling and monitoring the contamination of *Cronobacter* species in PIF is very important in the food industry. To develop a rapid and simple method to detect *Cronobacter* species in PIF and pure culture, immunoliposome-based immunochromatographic strip assay were developed. This strip assay could detect *Cronobacter* species both in pure culture and PIF, and the results were visualized in 10 min. The detection limit ranges of the strip assay for *Cronobacter* species, except for *Cronobacter malonaticus*, was 10^6 to 10^7 CFU/mL in pure culture and 10^7 to 10^8 CFU/g in PIF. The detection limit range of our developed method was improved to 10^5 to 10^7 CFU/mL in pure culture and 10^6 to 10^8 CFU/g in PIF using QuantiScan. The developed immunochromatographic strip could detect < 10 *Cronobacter* cells in 25 g of spiked PIF when 14 h enrichment was applied. Visual assessment could not detect *Cronobacter malonaticus* in both pure culture and PIF, but detection was achieved using QuantiScan analysis, and the detection limits were 10^8 CFU/mL and 10^8 CFU/g in both pure culture and PIF.

In conclusion, the developed immunoliposome-based immunochromatographic strip assay could detect *Cronobacter* species in 10 min when the contaminated bacteria were present at numbers exceeding 10^7 CFU/mL. The assay could detect < 10 cells in 25 g when 14 h of enrichment was included. The developed assay is simple, easy, and effective for onsite the detection of *Cronobacter* species and could be acceptable as a commercial platform.

Conflicts of interest

No potential conflict of interest was reported by the authors.

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