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Immunochromatographic Strip Assay for Detection of *Cronobacter sakazakii* in Pure Culture

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Cronobacter sakazakii (*C. sakazakii*) is a foodborne pathogen, posing a high risk of disease to infants and immunocompromised individuals. In order to develop a quick, easy, and sensitive assay for detecting *C. sakazakii*, a rabbit anti-*C. sakazakii* immunoglobulin G (IgG) was developed using sonicated cell protein from *C. sakazakii*. The developed anti-*C. sakazakii* (IgG) was of good quality and purity, as well as species-specific. The developed rabbit anti-*C. sakazakii* IgG was attached to the surface of a sulforhodamine B-encapsulated liposome to form an immunoliposome. A test strip was then prepared by coating goat anti-rabbit IgG onto the control line and rabbit anti-*C. sakazakii* IgG onto the test line, respectively, of a plastic-backed nitrocellulose membrane. A purple color signal both on the test line and the control line indicated the presence of *C. sakazakii* in the sample, whereas purple color only on the control line indicated the absence of *C. sakazakii* in the sample. This immunochromatographic strip assay could produce results in 15 min with a limit of detection of 10^7 CFU/ml in *C. sakazakii* culture. The immunochromatographic strip assay also showed very good specificity without cross-reactivity with other tested *Cronobacter* species. Based on these results, the developed immunochromatographic strip assay is efficient for the detection of *C. sakazakii* and has high potential for on-site detection.

Keywords: Antibody, *Cronobacter sakazakii*, immunochromatographic strip assay, liposome, rapid detection

Introduction

Cronobacter species belong to the family Enterobacteriaceae. There are a total of seven species of *Cronobacter*; *C. sakazakii*, *C. malonaticus*, *C. condimenti*, *C. dublinensis*, *C. muytjensii*, *C. turicensis*, and *C. universalis* [16–18, 20, 30]. *Cronobacter* species are foodborne pathogens that pose a high risk of infection to neonates and immune-compromised individuals. These organisms can cause serious diseases, including severe necrotizing enterocolitis, meningitis, and bacteremia in infants, with fatality rates between 40% and 80% [4, 34]. These pathogens have been isolated from the clinical environment and food and beverage sources such as water, vegetables, cheese, meat, and ready-to-eat foods [1, 14, 15, 22, 23]. Powdered infant milk formula (PIF) is a main food source that has been linked with epidemic diseases caused by *Cronobacter* species [8, 36, 37]. In 2004, a disease outbreak occurred in New Zealand because of the use of PIF at nurseries, causing the death of premature infants after contracting meningitis [9]. A similar incident occurred in France, resulting in two deaths out of nine infected cases [8]. Globally, there have been cases of PIF contaminated with *Cronobacter* species since 2004 [9]. According to a report by Jung and Park [21], 20% of PIF in Korea has been contaminated with *Cronobacter* species. Lee *et al*. [23] also reported that *Cronobacter* species isolated from 18.6% of detected food samples can result in disease outbreaks. Among the three *Cronobacter* species considered to be the most common opportunistic human pathogens (*C. sakazakii*, *C. malonaticus*, and *C. turicensis*), *C. sakazakii* is the most prevalent [38].

The classical detection method for *Cronobacter* species is laborious and requires 5 to 7 days [33]. Recently, concerns over *Cronobacter* species in food have increased, and thus

rapid detection methods have been developed [35, 40]. As an important species of *Cronobacter*, *C. sakazakii* poses a great health risk and has spurred the development of several detection methods [5, 13, 25, 31, 39, 41].

Liposomes are tiny vesicles, which include several concentric lipid bilayers surrounding aqueous components, and are widely used in various areas of chemistry, medicine, and biotechnology [32]. Our research group has developed an immunoliposome-based assay and liposome-based strip assay for quick detection of pathogens [24, 26, 27]. In addition, an immunoliposome-based fluorescence assay was developed for *C. muytjensii* [29]. Specifically, development of a fast, convenient, and easy strip assay would be very helpful for the monitoring of *C. sakazakii* both in the laboratory and in the field. Therefore, in this study, a species-specific antibody against *C. sakazakii* was produced and then an immunochromatographic strip assay based on immunoliposomes was developed for a specific detection for *C. sakazakii*.

Materials and Methods

Strains and Reagents

Strains indicated with LMG were obtained from the Belgian Coordinated Collections of Microorganisms (BCCM, Belgium). Other strains were bought from the Korean Culture Center of Microorganisms (KCCM, Korea) and American Type Culture Collection (ATCC, Manassas, VA, USA). Strains used in the study were as follows: *C. muytjensii* (CDC 3523-75) obtained from the Centre for Food Safety, University College Dublin, Ireland, *C. sakazakii* (ATCC 29004), *C. sakazakii* (ATCC 29544), *C. condimenti* (LMG 26250), *C. malonaticus* (LMG 23826), *C. dublinensis* (LMG 23823), *C. turicensis* (LMG 23827), *C. muytjensii* (ATCC 51329), *C. muytjensii* (CDC 3523-75), *C. universalis* (LMG 26249), *Bacillus cereus* (KCCM 40935), *Franconibacter helveticus* (LMG 23732), *Buttiauxella noackiae* (ATCC 51713), *Enterobacter aerogenes* (ATCC 15038), *Franconibacter pulveris* (LMG 24057), *Salmonella choleraesuis* (ATCC 13312), and *Salmonella* Typhimurium (ATCC 13311). All tested strains were grown in nutrient broth (NB) at 150 rpm for 18 h under optimal temperature for the strain. NB and skim milk were obtained from Difco (USA). Violet red bile glucose (VRBG) agar was obtained from MB Cell (USA). Sodium chloride, sodium azide, potassium phosphate monobasic, potassium phosphate dibasic, ammonium sulfate, caprylic acid, n-octyl-β-D-glucopyranoside (OG), ethylenediaminetetraacetic acid (EDTA), cholesterol, polyvinyl-pyrrolidone (PVP), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium dodecyl sulfate (SDS), tris(hydroxymethyl) aminomethane (Tris), formalin, sodium acetate, Tween 20, hydroxylamine hydrochloride, and casein were obtained from Sigma (USA). Ninety-six-well plates were obtained from SPL Life Science (Korea). The goat anti-rabbit immunoglobulin G (IgG) and unstained protein ladder (standard protein marker) were obtained from Thermo (USA).

N-Succinimidyl-*S*-acetylthioacetate (SATA), 1,2-bis (diphenylphosphino)ethane (DPPE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylglycerol (DPPG) were obtained from Avanti Polar Lipids (USA). Sulforhodamine B (SRB) was obtained from Pierce (USA). Disposable culture tubes (12 mm × 78 mm) were obtained from Kimble & Chase (USA). Polycarbonate membrane filters (0.4 μm, 0.8 μm) were purchased from Whatman (UK). Nitrocellulose membranes (Hiflow plus) were purchased from Merck Millipore (USA).

Animal Care Ethics

The animal use protocol was reviewed by the committee members of Yeungnam University and approved by the Korea Food and Drug Administration, Republic of Korea (Animal Ethics License No. 2013-012 and 2012-010).

Preparation of Immunogen and Immunization

Sonicated cell protein (SCP) of *C. sakazakii* (ATCC 29544) and formalin-killed cells (FKC) were used as immunogens, respectively. *C. sakazakii* was grown in NB for 18 h to a concentration of 10^9 CFU/ml. Cells of *C. sakazakii* were separated by centrifugation (3,000 ×*g*, 4°C, 30 min) and washed three times with 0.01 M phosphate-buffered saline (PBS) containing 1.5 M NaCl and 0.1% NaN_3, and then suspended in 0.01 M PBS to adjust the *C. sakazakii* cells to 10^9 CFU/ml. For preparation of SCP, cells of *C. sakazakii* were sonicated under 40 kHz for 5 min, and then the supernatant was collected by centrifugation at 10,000 ×*g* for use as an immunogen. For preparation of the FKC, cells of *C. sakazakii* were treated with 0.5% formalin for 24 h and separated by centrifugation (3,000 ×*g*, 4°C, 30 min), after which the cells were washed three times with 0.01 M PBS and then suspended in 0.01 M PBS to use as an immunogen.

In order to produce antibody against *C. sakazakii*, the first injection consisted of mixed immunogen and Freund's complete adjuvant in equal volumes, whereas the second and third injections consisted of mixed immunogen and Freund's incomplete adjuvant in equal volumes. The immunogen was injected into the backs of New Zealand white rabbits at four sites at a dose of 0.25 ml/site. Blood samples were collected every week after the first injection until the 18th week to monitor the titer of developed antiserum.

Preparation and Purification of IgG

Blood samples were stored for 12–18 h at 4°C before centrifugation (10,000 ×*g*, 4°C, 30 min). IgG was separated from the antiserum as described by Shukla *et al*. [25] and Song *et al*. [29]. That is, antiserum (0.5 ml) was added to double volumes of 60 mM sodium acetate buffer (pH 4.0) and gently mixed with 55 μl of caprylic acid for 30 min. After centrifugation (10,000 ×*g*, 30 min), the supernatant was collected and filtered through a glass wool-padded syringe. The filtered supernatant was added to

the same volume of cold-saturated ammonium sulfate and gently shaken for 30 min. Following centrifugation at 10,000 ×*g* for 30 min, the pellet was collected and dissolved in 0.02 M Tris buffer saline (TBS), containing 1.5 M NaCl and 0.1% NaN_3, pH 7.0, for dialysis in the same buffer. The purity of prepared anti-*C. sakazakii* IgG was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 4% (w/v) polyacrylamide stacking gel and 15% (w/v) separating gel under 20 mA of electrophoresis at room temperature. The titer of anti-*C. sakazakii* IgG was tested by indirect noncompetitive enzyme-linked immunosorbent assay (INC-ELISA).

Preparation of Dye-Encapsulated Liposome

DPPE-acetylthioacetate, which was formed with DPPE and SATA, DPPC, DPPG, SRB, and cholesterol were used for liposome preparation by following the method described by Shukla *et al*. [26], and the prepared liposome solution was kept at 4°C in the dark before use. The particle size of liposomes was checked by a Zetasizer (UK) in 0.01 M HEPES buffer (pH 7.5).

Deprotection of –SH Groups from Liposomes

Deprotection of –SH groups from liposomes was performed by adding 30 µl of hydroxylamine hydrochloride (0.5 M), prepared by using 0.1 M HEPES buffer containing 0.025 µM EDTA (pH 7.8), to 300 µl of liposome suspension at a volume ratio of 1:10 [29]. The mixture was then incubated under N_2 flushing for 1 min and left on a shaker for 2 h at room temperature in order to complete the reaction [29].

Conjugation of IgG with SH-Containing Liposome

The anti-*C. sakazakii* IgG-tagged liposomes were developed as described by Shukla *et al*. [24] and Song *et al*. [29]. In the present preparation, 1 mg/ml of developed rabbit anti-*C. sakazakii* IgG was used to be conjugated onto SH-containing liposome. The anti-*C. sakazakii* IgG-tagged liposome was dialyzed at 4°C in the dark using 0.02 M TBS for overnight. Antibody conjugation with liposomes was confirmed by fluorescence-based liposome immunoassay [29].

Preparation of Immunochromatographic Strip

The immunochromatographic strip was developed by the method described by Shukla *et al*. [26]. Nitrocellulose membrane (8 cm × 10 cm) was wetted with 10% (v/v) methanol in 0.01 M PBS (pH 7.0) for 15 min at room temperature under orbital shaking at 70 rpm, and then the membrane was dried under vacuum (15 psi) for 2 h at 30°C. The IgG coating process was carried out using an automatic sample injector (Camag, Switzerland). The developed rabbit anti-*C. sakazakii* 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 buffer (pH 7.0) containing 0.5% PVP and 0.02% casein. The membrane was then dried overnight under vacuum (15 psi) at 30°C, after which the membrane was cut into 0.5 cm × 8 cm size test strips and kept at 4°C until use.

Binding Abilities of *C. sakazakii* toward Developed Immunoliposome

The binding abilities of *C. sakazakii* to the developed immunoliposome were checked by fluorescence-based liposome immunoassay [29]. Rabbit anti-*C. sakazakii* IgG, goat anti-rabbit IgG, and *C. sakazakii* were separately coated onto a 96-well plate for 2 h at 37°C, while 0.01 M TBS was used as a negative control. Next, 5% skim milk in 0.01 M PBS (pH 7.0) was added as the blocking buffer, and then the plate was washed several times with 200 µl of 0.01 M PBS (pH 7.0). Anti-*C. sakazakii* IgG-tagged liposomes were subsequently added to the plate, which was then incubated for 1 h at 37°C. After washing the plate using 200 µl of 0.01 M PBS with 0.05% Tween 20, 280 µl of 30 mM OG was added to lyse the liposomes. The resulting fluorescence from SRB was measured at the excitation and emission wavelengths of 550 nm and 585 nm, respectively.

Detection of *C. sakazakii* Using Developed Immunochromatographic Strip Assay

C. sakazakii cells grown at 37°C for 18 h were serially diluted with 0.85% NaCl (w/v) for further use. Stock immunoliposome solution was diluted to 1:5 with 0.02 M TBS buffer (pH 7.0) with the osmolality of 387 mmol/kg. The immunochromatographic strip was dropped in a mixture of 50 µl of diluted culture and 50 µl of immunoliposome in a disposable culture tube (12 mm × 78 mm) and then reacted for 15 min to allow the liquid to flow up. The color intensity of the test line and the control line of the test strip was checked visually. The presence of purple color in both lines indicated the presence of *C. sakazakii*, whereas no color in the test line and a purple color in the control line were interpreted as the absence of *C. sakazakii*.

Cross-Reactivity Test of Developed Immunochromatographic Strip Assay

The specificity of the developed immunochromatographic strip assay was tested with other *Cronobacter* species and foodborne pathogens. Tested strains were cultured for 18 h at 37°C with shaking and then serially diluted for further use. The test procedure was the same as described earlier under the section of assay development.

Results and Discussion

Characterization of Developed Anti-*C. sakazakii* IgG

The titers of anti-*C. sakazakii* IgG are shown in Fig. 1. For the antibody developed with FKC and SCP, respectively, the titer kept increasing after booster injections at week 3 and week 6. Anti-*C. sakazakii* IgG developed using SCP of *C. sakazakii* showed a higher titer than the IgG developed with FKC of *C. sakazakii*. The produced IgG was separated from crude antisera with caprylic acid and ammonium

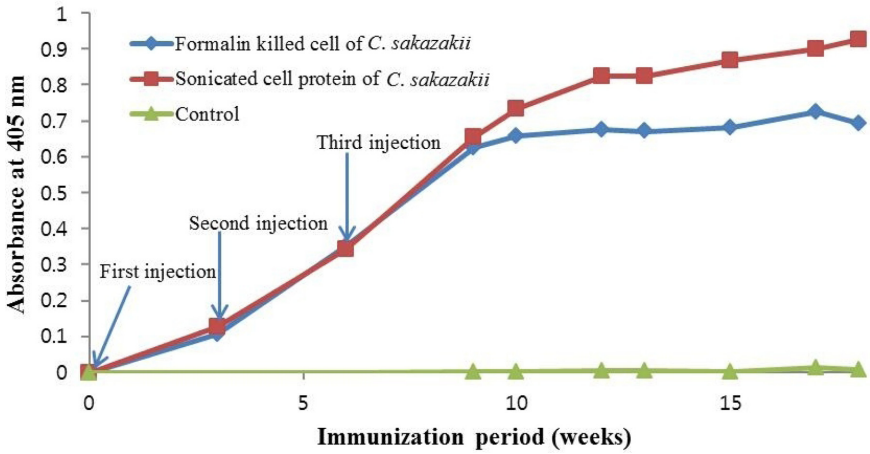

Fig. 1. Titer of anti-*C. sakazakii* IgG produced by two different immunogens.

sulfate precipitation, and its purity was tested by SDS-PAGE. A commercial rabbit IgG, purified by ion-exchange chromatography, was used for comparision (Fig. 2). Two bands at 51 kDa and 25 kDa were observed from the commercially available rabbit IgG (lane 2 in Fig. 2) and anti-*C. sakazakii* IgG (lanes 3 and 4 in Fig. 2), respectively. Previously, an anti-*C. muytjensii* IgG separated by caprylic acid and ammonium sulfate precipitation also showed good purity and was successfully used to develop an INC-ELISA for detecting *C. muytjensii* [29]. These results confirm that the rabbit anti-*C. sakazakii* IgG had high purity comparable to the commercial rabbit IgG and could be used for developing an immunoassay for the detection of *C. sakazakii*.

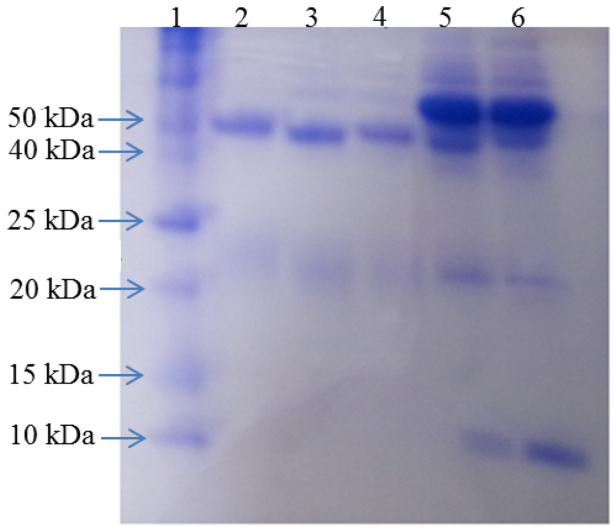

Fig. 2. SDS-PAGE of developed rabbit anti-*C. sakazakii* IgG.
Lane 1: standard protein marker; lane 2: commercial rabbit IgG; lane 3: purified anti-*C. sakazakii* IgG against FKC of *C. sakazakii*; lane 4: purified anti-*C. sakazakii* IgG against SCP of *C. sakazakii*; lane 5: antiserum against FKC of *C. sakazakii*; lane 6: antiserum against SCP of *C. sakazakii*.

Anti-*C. sakazakii* IgG against FKC of *C. sakazakii* showed species specificity despite slight cross-reactivity at a high concentration (10^8 CFU/ml) of *C. turicensis* (Table 1). The anti-*C. sakazakii* IgG developed with SCP of *C. sakazakii* showed higher titer (Fig. 1) and better species specificity than anti-*C. sakazakii* IgG against FKC of *C. sakazakii* (Table 1). Therefore, anti-*C. sakazakii* IgG against SCP of *C. sakazakii* was selected to develop an immunochromatographic strip assay for detecting *C. sakazakii*.

Characterization of Developed Immunoliposome

A variety of liposomes can be distinguished and classified based on their structure and size, including small unilamellar vesicles (20 nm to 200 nm), large unilamellar vesicles (100 nm to 1.0 μm), giant unilamellar vesicles (1.0 μm to 200 μm), and multi-vesicular vesicles (1.6 μm to 10.5 μm) [11, 19]. As shown in Table 2, the average size of produced SRB-encapsulated liposomes was 229 nm. Liposome vesicles were produced using multiple ultrasonic vibrations and extrusions, whereas SRB was enclosed in the liposome vesicles as a fluorescent dye. Liposomes formed a uniform size (229 nm of diameter) after passing through 0.8 μm and 0.4 μm polycarbonate filters. The developed liposomes were determined to carry 5.02×10^{-13} μmol of SRB per vesicle based on calculation of the inner volume [3, 10]. The particle concentration of liposomes was 4.64×10^{11} particles/ml based on a SRB concentration of 100 mM inside the liposomes. As shown in Fig. 3, the fluorescence signal ratio (signal after OG treatment/signal before OG treatment)

Table 1. Cross-reactivity of anti-*C. sakazakii* IgG produced against different immunogens.

Strains used for cross-reactivity	Anti-*C. sakazakii* IgG developed with SCP	Anti-*C. sakazakii* IgG developed with FKC
Cronobacter sakazakii (ATCC 29544)	+	+
Cronobacter sakazakii (ATCC 29004)	+	+
Cronobacter condimenti	-	-
Cronobacter dublinensis	-	-
Cronobacter malonaticus	-	-
Cronobacter muytjensii (ATCC 51329)	-	-
Cronobacter muytjensii (CDC 3523-75)	-	-
Cronobacter universalis	-	-
Cronobacter turicensis	-	+
Bacillus cereus	-	-
Buttiauxella noackiae	-	-
Enterobacter aerogenes	-	-
Franconibacter helveticus	-	-
Franconibacter pulveris	-	-
Salmonella enterica subsp. *enterica*	-	-
Salmonella Typhimurium	-	-

Table 2. Characterization of SRB-encapsulated liposome.

Mean diameter	229 nm
Inner volume of liposome assuming a bilayer thickness of 4 nm	5.02×10^{-12} µl
SRB concentration	100 mM
Amount of SRB/liposome	5.02×10^{-13} µmol
Concentration of liposome	4.64×10^{11} particles/ml

showed that the developed immunoliposome has excellent encapsulation ability, which makes it suitable for developing a *C. sakazakii* detection method. Similarly, two different SRB-encapsulated liposomes (sizes of 223 nm and 206 nm) were developed for detecting *Salmonella* and *C. muytjensii*, respectively [27, 29]. Ho *et al.* [12] also used methyl blue-encapsulated liposomes with a size of 236 nm to develop an immunoliposome-based strip assay for detecting *Salmonella*.

Binding Abilities of *C. sakazakii* toward Developed Immunoliposome

The binding abilities of *C. sakazakii* to the developed immunoliposome were checked by fluorescence-based liposome immunoassay. The results showed that the developed immunoliposome did not react with anti-*C. sakazakii* IgG-tagged liposomes, producing a negligible fluorescence signal (B in Fig. 4). The developed immunoliposomes were tightly bound to *C. sakazakii*, producing a very high fluorescence signal (D in Fig. 4). Moreover, the developed immunoliposome reacted with goat anti-rabbit IgG, producing a fluorescence signal (C in Fig. 4). The binding ability of the developed immunoliposome was measured based on antibody attached to the liposome

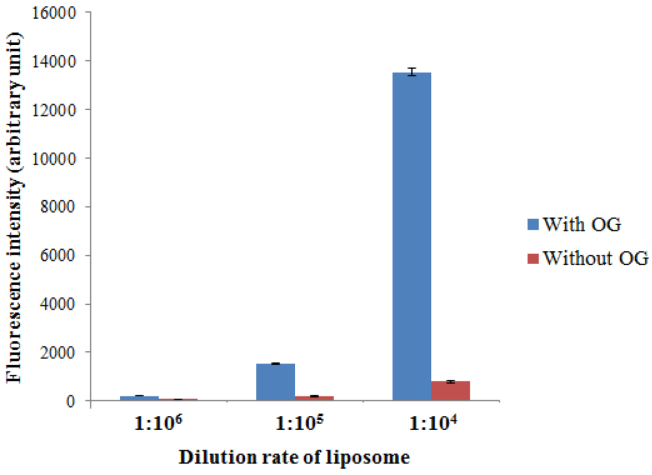

Fig. 3. Fluorescence signal of developed immunoliposomes with/without a detergent.

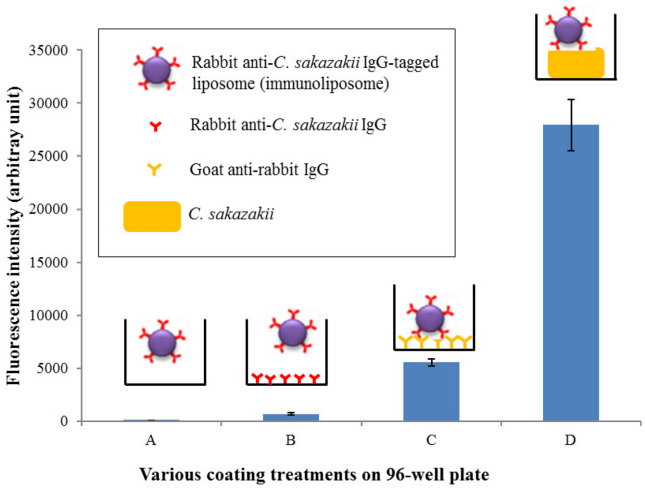

Fig. 4. Interaction of developed anti-*C. sakazakii* IgG-tagged liposome with various treatments.
A: Control (TBS buffer); B: rabbit anti-*C. sakazakii* IgG; C: goat anti-rabbit IgG; D: *C. sakazakii*.

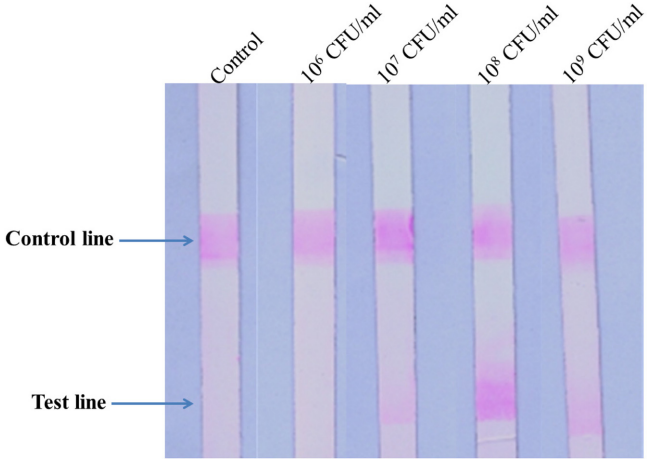

Fig. 5. Detection of *C. sakazakii* (ATCC 29544) using the immunochromatographic strip.

surface. Shukla *et al*. [27] reported an anti-*Salmonella* IgG-tagged liposome showing excellent specificity toward *Salmonella*. The same result was reported by DeCory *et al*. [7], who applied an anti-*Escherichia coli* O157:H7 antibody-tagged immunoliposome for *E. coli* O157:H7 detection. The binding abilities of the immunoliposome with *C. sakazakii* and goat anti-rabbit IgG suggest the strong possibility of applying immunoliposomes to strip test detection for *C. sakazakii*.

Detection Limit and Specificity of Developed Immunochromatographic Strip Assay in Pure Culture

As shown in Fig. 5, the developed immunoliposome produced signals on the test line and the control line of the positive test strip, as well as on the control line of the negative control test strip. The detection limit of the immunochromatographic strip assay was 10^7 CFU/ml. Cross-reactivity of the immunochromatographic strip assay with other *Cronobacter* species was shown in Fig. 6. The results showed that the developed immunochromatographic strip assay was species-specific with no cross-reactivity with other *Cronobacter* species. Usually, conventional methods need 18−24 h of enrichment and isolation culture on selective media such as Druggan-Forsythe-Iversen agar and VRBG agar. The presence of typical morphological colonies on selective media constitutes only gives a presumptive identification of *Cronobacter* species, which require further confirmatory test by biochemical methods or molecular methods like polymerase chain reaction. Previously, Shukla *et al*. [27] reported an immunochromatographic test strip based on liposomes for detecting *Salmonella* Typhimurium, which showed a limit of detection of 10^6–10^7 CFU/ml. Blazkova *et al*. [2] also developed an immunochromatographic test strip for detecting *Cronobacter* species by amplicons labeled with digoxigenin and biotin with good detection limits, but the method complexity could limit the application of the test strip. Chen *et al*. [6] also reported an immunochromatographic strip (coated with anti-digoxigenin monoclonal antibody) combined with a 16S rRNA probe with a limit of detection of 10^7 CFU/ml in pure culture. Recently, Song *et al*. [28] reported an immunofluorescence

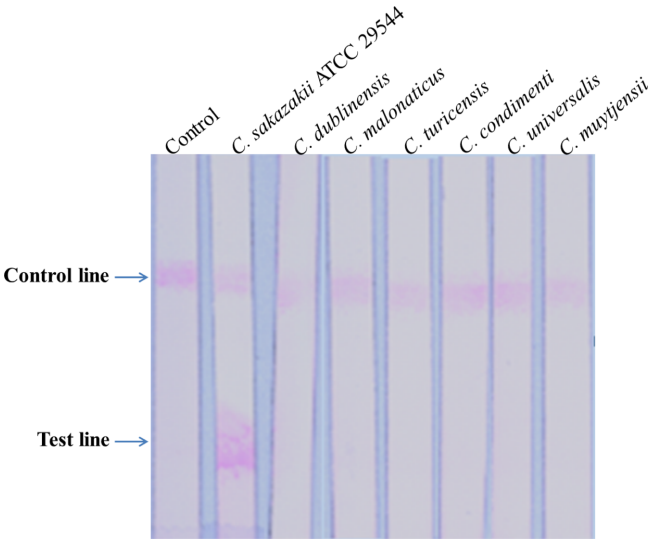

Fig. 6. Cross-reactivity of the developed immunochromatographic strip assay with other *Cronobacter* species.

strip for detecting *E. coli* O157:H7 based on the reaction of fluorescent bacteria and the 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 semiquantitative was upgraded to 10^5 CFU/ml by using fluorescence scan reader [28]. This method gives a prospective for upgrading the current developed immunochromatographic strip assay using fluorescent dye-encapsulated liposomes. When compared with these previously reported methods, our developed immunoliposome-based strip assay is simple, inexpensive, and rapid for the on-site detection of *C. sakazakii*, which can generate results in 15 min and requires no sample pretreatment and less expensive instruments.

In the present study, rabbit anti-*C. sakazakii* IgG was developed and applied for the immunochromatographic strip assay. The developed anti-*C. sakazakii* IgG showed good purity and specificity and reacted with only *C. sakazakii* among the 16 tested strains. This immunochromatographic strip assay could obtain results in 15 min, and the detection limit of the test was 10^7 CFU/ml in pure culture. Future studies are ongoing in order to reduce the detection limit of the developed method for the detection of *C. sakazakii*.

Acknowledgments

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