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Development of an Indirect Non-Competitive Enzyme-Linked Immunosorbent Assay for the Detection of *Cronobacter muytjensii* in Infant Formula Powder

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유아용 조제분유 내 *Cronobacter muytjensii* 검지를 위한 간접 비경합 면역분석법의 개발

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국문요약

*Cronobacter muytjensii*는 유아용 조제분유(IFP)의 잠재적 위험요인으로 중요한 식품 기인성 병원균이다. 이 연구에서는 *C. muytjensii* 검지를 위한 특이적 면역글로불린 G(IgG)를 개발하고, 이 anti-*C. muytjensii* IgG를 이용하여 간접 비경쟁 효소면역측정법(INC-ELISA)을 개발하였다. 그 결과, 새롭게 개발한 INC-ELISA 방법은 *C. muytjensii*에 매우 민감하고, 순수배양 시 6.5×10^3 CFU/㎖의 검출한계와 유아용 조제분유에서 1 cell/25 g의 검출한계를 나타내었다. INC-ELISA 방법은 또한 *C. muytjensii*에 탁월한 특이성을 보이고, *Cronobacter* 속 외 11종의 다른 식품 기인성 병원균 계통과의 교차반응을 보이지 않았다. 이러한 결과는, 개발된 INC-ELISA 방법이 *C. muytjensii*에 매우 민감하고 효율적이며, 신속하고 용이한 검출을 위한 진단 키트 개발에 적용할 수 있음을 시사한다.

Key words: *Cronobacter muytjensii*, immunoglobulin G, INC-ELISA, rapid detection, infant formula powder

Introduction

The *Cronobacter* genus, previously described as *Enterobacter sakazakii*, is a novel genus within the family *Enterobacteriaceae* proposed on the basis of extensive phenotypic and genotypic characterizations (Mullane et al. 2006; Iversen et al. 2007). This genus includes seven species, *C. sakazakii*, *C. muytjensii*, *C. malonaticus*, *C. turicensis*, *C. dublinensis*, *C. universalis* sp. nov., and *C. condimenti* sp. nov. (Iversen et al. 2007, 2008; Joseph et al. 2012). *Cronobacter* spp. have been isolated from plant-based foods and food products, such as cereals, fruits, vegetables, legumes, herbs, and spices, as well as from animal-based foods, including milk, meat, fish, and the products made from the animal related sources (Muytjens et al. 1988; Iversen et al. 2004; Friedemann M, 2007; Lee et al. 2012). Furthermore, *Cronobacter* spp. including *C. muytjensii* can cause an invasive infection in humans of any age group, especially in immunocompromised elderly persons and infants, with the fatality rate between 30-80% (Iversen et al. 2008; Chap et al. 2009).

Cronobacter spp. are the most dominant foodborne pathogen, related to major outbreaks and serious infections in young and new borne babies due to the use of infant formula powder (IFP) (Himelright et al. 2002; FAO/WHO 2004; Strydom et al. 2012). Currently, the microbiological safety of IFP has become a major concern to regulatory agencies and formula

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producers because the products are used for newborn infants who have weakened immune systems and lack a competing intestinal flora (Townsend & Forsythe 2008). Therefore, developing a rapid and sensitive method for the detection of *Cronobacter* spp. would positively contribute to reduce the risk across the IFP supply chain.

Several methods have been reported for the detection of *Cronobacte*r spp. in food (Sunwoo et al. 2006; Townsend et al. 2008; Lampel et al. 2009; Huang et al. 2013). The United States Food and Drug Administration (USFDA) recommended a method for isolating and detecting *Cronobacter* (*E. sakazakii*) from IFP, however, the method requires 5 to 7 days to complete the test (USFDA 2002; Sunwoo et al. 2006). The USFDA has also developed a new detection method, featuring a real time PCR-based assay complex with selective agar that takes 2 days for the complete detection of *Cronobacter* spp. (Lampel et al. 2009). In this method, the suspended cells are isolated by centrifugation from an enrichment culture and confirmed by real-time PCR assay. Huang et al (2013) also developed species-specific primers for the detection of *C. sakazakii* and *C. dublinensis* though PCR technique, which based on DNA gyrase subunit B gene. Townsend et al. (2008) reported that 10 strains of *Enterobacter hormaechei* identified by 16s rDNA sequencing were originally identified as *C. sakazakii* by phenotyping. However, these PCR-based methods has limited application for significant technical requirements and also come with high cost of equipment and reagents.

Enzyme-linked immunosorbent assay (ELISA) has been extensively used for the rapid detection of foodborne pathogens, including *Salmonella* spp. (Brigmon et al. 1992; Kumar et al. 2008), *Escherichia coli* O157:H7 (Sunwoo et al. 2006), and *Listeria* spp. (Kim et al. 2005). The ELISA method for detection of foodborne pathogen is rapid, sensitive, simple, and less consumption. Recently, studies on the detection of *Cronobacter* in foods are being increased due to the high risk of harmful effects of these pathogens in infant food products. Therefore, developing a rapid detection method for *Cronobacter* spp. with ELISA would be very contributive to the food safety control.

On the other hand, there is also no simple and rapid method for species-specific detection of *Cronobacter* spp.. Thus, it is critically important to develop a speedy and species-specific identification method for *Cronobacter* spp. when dealing with *Cronobacter* related food safety issues. Among all *Cronobacter* spp., *C. sakazakii*, *C. muytjensii*, and *C. dubinesis* are likely to be present in various foods, such as dry powdered food, sunsik, cereal, fruit, vegetable, and seaweed (Lee et al. 2012). There are several reports related to detection method for *C. sakazakii* while only few report are available for *C. muytjensii*. (Lampel & Chen 2009; Zhang et al. 2010; Blazkova et al. 2011; Park et al. 2012; Huang et al. 2013). Therefore, we selected *C. muytjensii* for developing specific antibody and ELISA detection method. In order to increase the sensitivity and reduce the assay time for *C. myutjensii*, we developed an INC-ELISA by using a specific immunoglobulin G (IgG) and then applied the assay method in IFP for the food trial.

Materials and Methods

1. Materials

Nutrient broth (NB), peptone, and skim milk were purchased from Difco (Franklin Lakes, NJ, USA). *Enterobacteriaceae* enrichment broth (EE broth) and violet red bile glucose agar (VRBG) were from MB cell (Seoul, Republic of Korea). Sodium carbonate, sodium bicarbonate, sodium azide, potassium phosphate monobasic, potassium phosphate dibasic, sodium chloride, caprylic acid, ammonium sulfate, pre-stained SDS-PAGE marker, standard rabbit IgG, coomassie brilliant blue, and alkaline phosphatase yellow (pNPP) liquid substrate were purchased from Sigma (St. Louis, MO, USA). Phosphatase-labeled goat anti-rabbit IgG from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD, USA) and 96-well immunoplates from SPL Life Science Corporation (Pocheon-Si, Gyeonggi-Do, Republic of Korea) were used in this study. IFP used for food test was purchased from a local market in Gyeongsan-si, Republic of Korea.

2. Bacterial Strains and Culture Conditions

C. muytjensii (ATCC 51329) was used to develop an antibody in rabbit. *Bacillus cereus* (KCCM 40935), *Buttiauxella noackiae* (ATCC 51713), *C. condimenti* (LMG 26250), *C. dublinensis* (LMG 23823), *C. malonaticus* (LMG 23826), *C. muytjensii* (CDC 3523-75), *C. turicensis* (LMG 23827), *C. sakazakii* (ATCC 29544), *C. sakazakii* (ATCC 29004), *C. universalis* (LMG 26249), *Citrobacter freundi* (ATCC 8090), *Escherichia coli* (ATCC 39418), *Enterobacter aerogenes* (ATCC 15038), *Enterobacter helveticus* (LMG 23732), *Escherichia coli* O157:H7 (ATCC 43888), *Enterobacter pulveris* (LMG 24057), *Salmonella* Enterica

(ATCC13312), *Salmonella* Typhimurium (ATCC 13311), and *Yersinia enterocolitica* (KCCM 41657) were used in cross-reactivity test (Table 1). All strains were cultured in NB for 18 h at 37℃ on a shaking incubator (150 rpm).

3. Preparation of Antigen

Antigen was prepared as described by Park et al. (2012). *C. muytjensii* cells were cultured in NB at 37℃ for 18 h with shaking. The cell concentration after 18 h culturing determined by plating and counting on VRBG plate was 4.0×10^9 CFU/㎖. Then the culture (10 ㎖) of above cells concentration was inactivated by treatment with 0.5% formalin for 24 h and centrifuged (3,000 *g*, 4℃) for 30 min. The precipitate was washed three times with 10 ㎖ of 0.01 M phosphate-buffered saline (PBS) and then finally suspended in 40 ㎖ of 0.01 M PBS. The formalin-killed cells (FKC) were stored at −20℃ before being used as an antigen for the preparation of a polyclonal antibody against *C. muytjensii*.

4. Immunization

New Zealand white rabbits used in this study were raised and treated by the protocol and standard of the Canadian Council on Animal Care guidelines (2002). New Zealand white female rabbits were housed in an animal room under the conditions of 25℃, 12 h/12 h light/dark cycles and fed with standard pellet diet and water. Two months old rabbits were immunized as described by Shukla et al. (2012). Briefly, one milliliter of a mixture of *C. muytjensii* FKC and Freund's complete adjuvant (1:1, v/v) was subcutaneously injected in the back of the rabbits. Two boosting injections of the same mixture (1:1, v/v) were administered at 3 week intervals thereafter. Blood samples were collected weekly from ear vein for 14 weeks in order to monitor antibody titer.

5. Preparation and Purification of IgG

The blood samples collected from ear artery were centrifuged (10,000 *g*) for 30 min at 4℃ to separate the antisera. IgG was purified from the antisera by caprylic acid and ammonium sulfate precipitation, as described by McKinney and Parkinson (1987). The separated IgG was dialyzed in Tris-buffered saline (TBS, 0.02M, pH 7.0) for purification. To determine the purity of the IgG preparation, SDS-PAGE was performed (Laemmli UK, 1970; Park et al. 2012). For SDS-PAGE, a polyacrylamide stacking gel (4%, w/v) and separating gel (15%, w/v) were used, and IgG solutions were heated for 3 min at 100℃ in digestion buffer containing 2-mercaptoethanol (5%) in distilled water. Then, aliquots (10 ㎕) of the IgG preparations were loaded into each well. Pre-stained SDS-PAGE marker and commercial rabbit IgG were used as molecular weight marker and control, respectively. Electrophoresis was conducted at a constant voltage of 20 mA at room temperature. After SDS-PAGE, the gel was stained with coomassie brilliant blue for at least 4 h, followed by destaining overnight with a destaining solution containing 25% methanol and 7% acetic acid in distilled water.

6. Development of INC-ELISA for *C. muytjensii* Analysis

Laboratory-made rabbit anti-*C. muytjensii* IgG was used in this analysis. To determine the response curve of the developed INC-ELISA, fresh culture of *C. muytjensii* was serially diluted with 0.05 M carbonate buffer (pH 9.5) for coating on 96 well plates. These various culture dilutions were coated onto a 96-well immunoplate wells with three replication, incubated at 4℃ for overnight, and then washed three times with 0.01 M PBS (pH 7.0), followed by blocking with 200 ㎕ of 5% skim milk for 2 h at 37℃. The plate was then washed with 0.01 M PBS containing 0.05% Tween 20 (PBST). Then, rabbit anti-*C. muytjensii* IgG was added, and the plate was incubated at 37℃ for 1 h. The plate was washed again with PBST, and phosphatase-labeled goat anti-rabbit IgG was added, followed by incubation at 37℃ for 1 h. The plate was washed again with PBST, and 50 ㎕ of pNPP liquid substrate was added to each well for a 30 min enzyme-substrate reaction. The enzyme-substrate reaction was ended by adding the 50 ㎕ of NaOH solution. The yellow color produced in positively reacting wells was measured for absorbance at 405 nm using an Infinite M200 (Tecan, Seestrasse, Switzerland). Simultaneously, the numbers of *C. muytjensii* in cultures were analyzed on VRBG plate. The detection limit of the developed INC-ELISA was determined using linearity function of the data and constant standard deviation of responses to the calibration standard and samples (Hubaux and Vos, 1970).

7. Specificity of INC-ELISA Assay

Twenty bacterial strains (Table 1), including 2 strains of *C. muytjensii* (ATCC 51329 and CDC 3523-75), were used for the specificity test of the developed INC-ELISA. Cultures

Table 1. Strains used in cross-reactivity test

Bacterial species	Isolate ID	Supplier
Bacillus cereus	KCCM 40935	Korean Culture Center of Microorganisms
Buttiauxella noackiae	ATCC 51713	American Type Culture Collection
Citrobacter freundi	ATCC 8090	American Type Culture Collection
Cronobacter condimenti	LMG 26250	Belgian Coordinated Collections of Microorganisms
Cronobacter dublinensis	LNG 23823	Belgian Coordinated Collections of Microorganisms
Cronobacter malonaticus	LMG 23826	Belgian Coordinated Collections of Microorganisms
Cronobacter muytjensii	ATCC 51329	American Type Culture Collection
Cronobacter muytjensii	CDC 3523-75	University College Dublin, Ireland
Cronobacter sakazakii	ATCC 29004	American Type Culture Collection
Cronobacter sakazakii	ATCC 29544	American Type Culture Collection
Cronobacter turicensis	LMG 23827	Belgian Coordinated Collections of Microorganisms
Cronobacter universalis	LMG 26249	Belgian Coordinated Collections of Microorganisms
Enterobacter aerogenes	ATCC 15038	American Type Culture Collection
Enterobacter helveticus	LMG 23732	Belgian Coordinated Collections of Microorganisms
Enterobacter pulveris	LMG 24057	Belgian Coordinated Collections of Microorganisms
Escherichia coli	ATCC 39418	American Type Culture Collection
Escherichia coli O157:H7	ATCC 43888	American Type Culture Collection
Salmonella Enterica	ATCC 13312	American Type Culture Collection
Salmonella Typhimrium	ATCC 13311	American Type Culture Collection
Yersinia enterocolitica	KCCM 41657	Korean Culture Center of Microorganisms

of microorganisms were serially diluted to achieve final concentrations from 10^0 to 10^8 CFU/㎖ and used to check the specificity of the developed INC-ELISA. All experiments were replicated three times.

8. Application of INC-ELISA to IFP

In order to apply the developed INC-ELISA to IFP, 25 g of IFP was inoculated with different cell numbers of *C. muytjensii* (1, 12, or 120 cells). Inoculated IFP was added to the 225 ㎖ of buffered peptone water containing 1% peptone and 0.5% sodium chloride (BPW, pH 7.2), followed by pre-enrichment at 37℃ for 8 h. Then, 1 ㎖ of pre-enrichment culture was added to 50 ㎖ of EE broth, followed by enrichment at 37℃. The resulting enrichment cultures were used directly for an INC-ELISA test at enrichment culture times of 0, 6, 8, and 10 h. At the same time, the number of *C. muytjensii* cells was determined by using VRBG plate.

Results and Discussion

1. Production and Purification of Antibody

Rabbit anti-*C. muytjensii* IgG was purified from crude antisera by caprylic acid and ammonium sulfate precipitation. Purity of the IgG was confirmed by SDS-PAGE and compared with that of a commercial rabbit IgG, which was purified by fractionation and ion-exchange chromatography (Fig. 1). A strong band around 51 kDa and a light band around 25 kDa were produced by commercial rabbit IgG (lane 4 in Fig. 1), as well as IgG purified from our crude anti-serum (lanes 2 and 3 in Fig. 1). These results confirmed that the developed rabbit anit-*C. muytjensii* IgG has a high purity comparable to commercial rabbit IgG.

2. Development of INC-ELISA Assay for *C. muytjensii*

An INC-ELISA method was developed to detect pure culture of *C. muytjensii* by using rabbit anti-*C. muytjensii* IgG. Results were dose-dependent with a clear change in the absorbance curve based on the number of *C. muytjensii* cells (Fig. 2). The detection limit of INC-ELISA for *C. muytjensii* was found to be 6.5×10^3 CFU/㎖ in pure culture. This detection limit was 10 times sensitive, compared with the

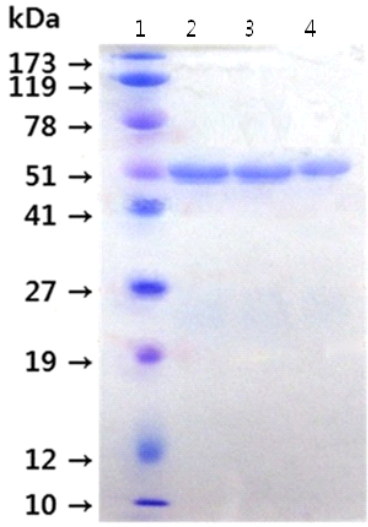

Fig. 1. SDS-PAGE patterns of purified IgG from rabbit serum. Lane 1: standard protein marker, Lanes 2 and 3: laboratory-made anti-*C. muytjensii* IgG in replicate, Lane 4: commercial rabbit IgG

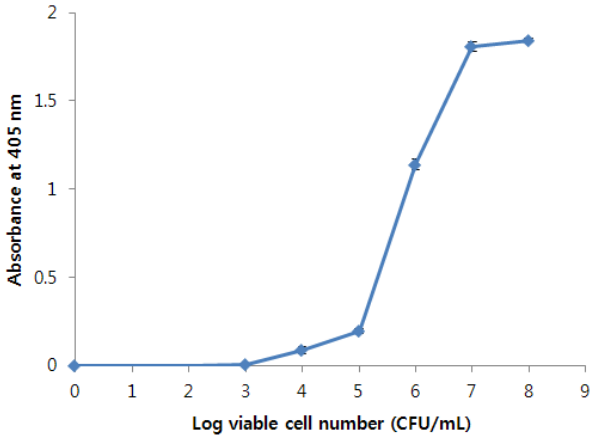

Fig. 2. Does-response curve for the detection of *C. muytjensii* by INC-ELISA. All experiments were conducted 3 times, and data represent mean±S.D.

detection limit of our previously developed sandwich ELISA (6.4×10^4 CFU/㎖) reported by Park et al. (2012). In addition, our developed immunoassay showed better detection limit than an indirect competitive ELISA for *E. sakazakii* (0.4-14.4 $\times10^5$ CFU/㎖) reported by Hochel and Skvor's (2009). Wang et al. (2012) reported a detection limit of 1.2×10^3 CFU/㎖ for *Cronobacter* spp. by using real-time PCR. Thus, it is clear that our INC-ELISA has better sensitivity compared with other antibody based reports, as well as comparable to PCR assay.

3. Specificity of Developed INC-ELISA

Nine *Cronobacter* strains and 11 foodborne pathogens as shown in Table 1 were used for cross-reactivity test. The developed INC-ELISA method showed excellent specificity with *C. muytjensii* strains (ATCC 51329 and CDC 3523-75) and no cross-reactivity with *C. sakazakii* (ATCC 29544, ATCC 29004), *C. turicensis* (LMG 23827), *C. condiment* (LMG 26250), *C. universalis* (LMG 26249), *C. malonaticus* (LMG 23826), and *C. dublinensis* (LMG 23823) (Fig. 3A.) including 11 other foodborne pathogenic strains (Fig. 3B). The INC-ELISA method developed in this study showed superior specificity toward *C. muytjensii* and no cross-reactions with other *Cronobacter* spp.. These results were consistent with previously reported study on DNA-DNA relatedness (91.9%) between *C. muytjensii* ATCC 51329 and *C. muytjensii* CDC 3523-75 (Iversen et al. 2008), whereas relatedness between *C. muytjensii* and other *Cronobacter* spp. was not higher than 56% (Iversen et al. 2008; Joseph et al. 2012). Blzakova et al. (2011) reported a method in which the PCR product of a specific gene sequence was used to target 16s rRNA from *Cronobacter* spp. In their study, amplicon labeled with digoxigenin and biotin was directly added to an immuno-chromatographic test strip composed of a nitrocellulose membrane with bound antibody against digoxigenin in the test line. In their results, all *Cronobacter* spp. displayed positive signals, whereas other non-*Cronobacter* strains displayed negative signals. Further, Zhang et al. (2010) reported a cross-priming amplification method under isothermal conditions combined with immuno-blotting analysis and used it to demonstrate genus specificity of *Cronobacter* spp.. All above reported methods are genus-specific and employ complex procedure to confirm, which could be constraints in rapid detection of species diagnosis during food safety issues. In contrast, the INC-ELISA developed in the present study was species-specific and applicable to rapid resolution of food safety issue. Stoop et al. (2009) developed six PCR primers such as Csakf/Csakr, Cmalf/Cmalr, Cturf/Cturr, Cdublf/Cdublr, Cmuyf/Cmuyr and Cgenomf/Cgenomr for *C. sakazakii, C. malonaticus*, *C. turicensis, C. dublinensis, C. muytjensii* and *C. genomo* species, respectively. It was found that in developed PCR assay, five primers (Cmalf/Cmalr, Cturf/Cturr, Cdublf/Cdublr, Cmuyf/Cmuyr and Cgenomf/Cgenomr) showed species-specificty to the target pathogens (*C. malonaticus*, *C. turicensis, C. dublinensis, C. muytjensii* and *C. genomo* species) while

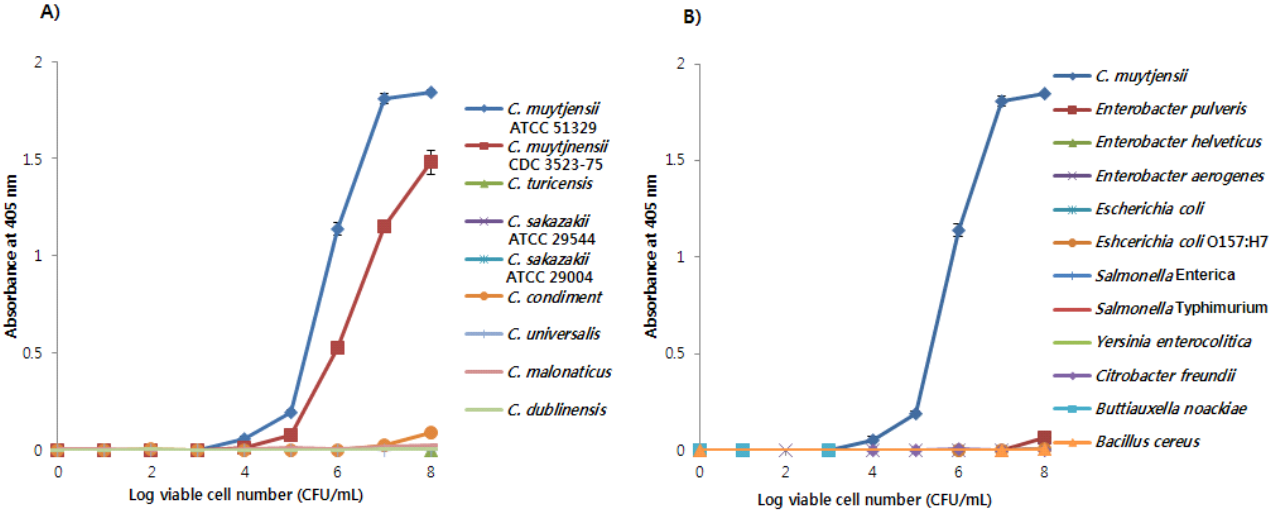

Fig. 3. (A) Cross-reactivity with other Cronobacter genus, (B) Cross-reactivity with other foodborne pathogen strains. All experiments were conducted 3 times, and data represent mean±S.D.

primer Csakf/Csakr showed specificity to two species of *Cronobacter* spp. (*C. sakazakii* and *C. malonaticus*). This may be due to close similarity of rpoB gene sequences of *C. sakazakii* and *C. malonaticus* (Stoop et al. 2009). Huang et al. (2013) designed two pairs of species-specific primers based on *gyr*B sequence which could specifically identify *C. sakazakii* and *C. dublinensis*. This PCR-based assay can identify species of *Cronobacter*, however, requires complex process with high level of handling technique and costs, compared with the developed INC-ELISA (Huang et al. 2013). On the other hand, Kim et al. (2012) reported an indirect competitive enzyme linked immunosorbent assay by using a developed anti-chicken immunoglobulin Y (IgY) with a detection limit of 10^5 CFU/㎖ in pure culture. In terms of antibody availability, IgY could be a better option than IgG. However, sensitivity needs to be more improved when IgY is used. The developed INC-ELISA using IgG is likely to be more useful, due to simplicity, rapidity, and cost saving, compared with above listed methods.

4. Application of INC-ELISA for the Detection of *C. muytjensii* in IFP

The complete assay described here for the detection of *C. muytjensii* included the following steps such as inoculation into IFP, pre-enrichment in BPW, and enrichment in EE broth. The final EE broth enrichment culture was applied to INC-ELISA at 0, 6, 8, and 10 h of enrichment period. When the number of inoculated *C. muytjensii* cells was 0, INC-ELISA showed only background signals (Fig. 4). At 6 h of enrichment, two inoculated IFP samples (12 and 120 cells/25 g) showed positive signals of 0.044±0.002 and 0.208±0.017, respectively. The sample inoculated with low cell number (1 cell/25 g) showed signals near 0 at 6 h of enrichment. At 8 h of enrichment, all three inoculated samples showed positive signals that were significantly stronger than that of the non-inoculated sample (0 cells/25 g), and their signal intensities were directly associated with the number of inoculated cells. According to these results, as few as 1 cell of *C. muytjensii* per 25 g of IFP could be detected after 8 h of pre-enrichment,

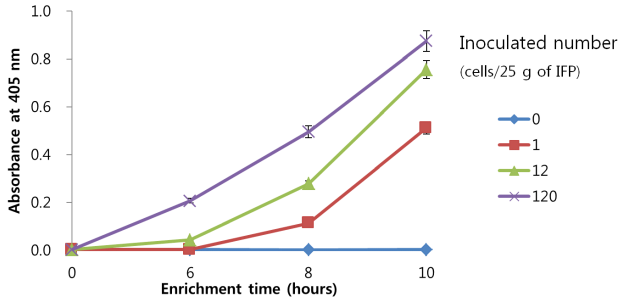

Fig. 4. Detection of *C. muytjensii* in IFP by INC-ELISA. *C. muytjensii* was inoculated into IFP, pre-enriched in BPW, and then enriched in EE broth. The final EE broth enrichment culture was applied to INC-ELISA at 0, 6, 8, and 10 h of enrichment period. All experiments were conducted 3 times, and data represent mean±S.D.

followed by 8 h of enrichment.

Detection methods including both conventional and alternative methods need pre-enrichment in BPW for recovering injured cells and the selective enrichment media for increasing the number of *Cronobacter* cells in IFP (Lampel & Chen 2009). Also, in our previous research, a sandwich ELISA was developed with a detection limit of 6.3×10^4 CFU/㎖ in spiked IFP. To increase the detection limit in IFP, the time dependent pre-enrichment and enrichment steps were included in this study. When INC-ELISA was applied in the IFP, with an inoculum level of 1 cell/25 g of IFP, the concentration of *C. muytjensii* cells increased to 10^7 CFU/㎖ after 8 h of pre-enrichment and 8 h enrichment. At this time, the optical density value was 0.11±0.03, which was similar to the value of 10^5 CFU/㎖ in pure culture. This repressed signal might be due to the matrix effect of IFP (Iversen et al. 2006). INC-ELISA developed in this study could detect *C. muytjensii* in IFP in less than 36 h, which comprises 8 h pre-enrichment, 8 h enrichment, and the remaining time for INC-ELISA test, whereas the conventional agar plating method requires 5-7 days. An improved FDA method allows for genus-specific identification of *Cronobacter* spp. in 72 h by using RT-PCR, and the confirmation of colonies were simultaneously performed by plating into Druggan-Forsythe-Iversen formulation agar (DFI formulation agar), followed by RAPID ID 32E confirmation (US FDA 2002; Lampel & Chen 2009). Mullane et al. (2006) reported a method by using cationic-magnetic-bead capture and DFI formulation agar for the detection of *E. sakazakii* with the detection limit of 1-5 CFU per 500 g of IFP, while in our developed INC-ELISA the detection limit was found to be 1 cell/25 g in IFP.

The developed INC-ELISA method in this study is species-specific and sensitive and can be used for the detection of *C. muytjensii* in IFP. In the future, the developed anti-*C. muytjensii* IgG could be a useful tool to develop a diagnostic kit for the rapid and easy detection of *C. muytjensii* in order to point out a significant way for the detection of *C. mutjensii* in food samples.

Summary

Cronobacter muytjensii is an important foodborne pathogen as a potential risk in infant formula powder (IFP). To develop a new and sensitive method for the detection of *Cronobacter* spp. in IFP, an immunoglobulin G (IgG) specific for *C. muytjensii* (formerly known as *Enterobacter sakazakii* ATCC 51329) was developed. Further, an indirect noncompetitive enzyme-linked immunosorbent assay (INC-ELISA) was developed by using the anti-*C. muytjensii* IgG. As a result, this newly developed INC-ELISA method was found very sensitive for *C. muytjensii* with detection limit of 6.5×10^3 CFU/㎖ in pure culture and 1 cell/25 g of IFP. This INC-ELISA method also displayed excellent specificity for *C. muytjensii* showing no cross-reactivity with other strains of *Cronobacter* genus and 11 other foodborne pathogenic strains. These results show that the developed INC-ELISA method was very sensitive, efficient, and rapid for the detection of *C. muytjensii*. Hence, this method could be applied to the development of diagnostic kits for the rapid and easy detection of *C. muytjensii*.

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References

Blazkova M, Javurkova B, Fukal L, Rauch P. 2011. Immuno-chromatographic strip test for detection of genus *Cronobacter*. *Biosens Bioelectron* 26:2828-2834

Brigmon RL, Zam SG, Bitton G, Farrah SR. 1992. Detection of *Salmonella enteritidis* in environmental samples by monoclonal antibody-based ELISA. *J Immunol Methods* 152:135-142

CACC. 2002. CCAC guidelines on: antibody production. The Canadian Council on Animal Care, Ottawa, Canada

Chap J, Jackson P, Siqueira R, Gaspar N, Quintas C, Park J, Osaili T, Shaker R, Jaradat Z, Hartantyo SHP, Abdullah SN, Estuningsih S, Forsythe SJ. 2009. International survey of *Cronobacter sakazakii* and other *Cronobacter* spp. in follow up formulas and infant foods. *Int J Food Microbiol* 136:185-188

FAO/WHO. 2004. *Enterobacter sakazakii* and other micro-organisms in powdered infant formula: meeting report, MRA series 6. Food and Agriculture Organization/World Health Organization. Geneva, Switzerland

Friedemann M. 2007. *Enterobacter sakazakii* in food and

beverages (other than infant formula and milk powder). *Int J Food Microbiol* 116:1-10

Gonzalez I, Martin T, Morales P, Sanz B, Hernandez PE. 1994. Detection of *Pseudomonas fluorescens* and related psychrotrophic bacteria in refrigerated meat by a sandwich ELISA. *J Food Prot* 57:710-714

Himelright I, Harris E, Lorch V, Anderson M, Jones T, Craig A, Forster T, Arduino M, Jensen B, Jernigan D. 2002. *Enterobacter sakazakii* infections associated with the use of powdered infant formula-Tennessee, 2001. *Morbidity Mortality Weekly Report* 51:298-300

Hochel I, Skvor J. 2009. Characterisation of antibodies for the immunochemical detection of *Enterobacter sakazakii*. *Czech J Food Sci* 27:66-74

Huang CH, Chang MT, Huang L. 2013. Use of the novel species-specific PCR primers targeted to DNA gyrase subunit B (*gry*B) gene for species identification of the *Cronobacter sakazakii* and *Cronobacter dublinensis*. *Mol Cell Probe* 27:15-18

Hubaux A, Vos G. 1970. Decision and detection limits for linear calibration curves. *Anal Chem* 42:849-855

Iversen C, Forsythe SJ. 2004. Isolation of *Enterobacter sakazakii* and other *Enterobacteriaceae* from powdered infant milk and related products. *Food Microbiol* 21:771-777

Iversen C, Lehner A, Mullane N, Bidlas E, Cleenwerck I, Marugg J, Fanning S, Stephan R, Joosten H. 2007. The taxonomy of *Enterobacter sakazakii*: proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter sakazakii* comb. nov. *Cronobacter sakazakii* subsp. *sakazakii*, comb. nov., *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov. and *Cronobacter genomospecies* 1. *BMC Evol Biol* 7:64

Iversen C, Mullane N, McCardell B, Tall BD, Lehner A, Fanning S, Stephan R, Joosten H. 2008. *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov. comb. nov., *C. malonaticus* sp. nov., *C. turicensis* sp. nov., *C. muytjensii* sp. nov., *C. dublinensis* sp. nov., *Cronobacter* genomospecies 1, and of three subspecies, *C. dublinensis* sp. nov. subsp. *dublinensis* subsp. nov., *C. dublinensis* sp. nov. subsp. *lausannensis* subsp. nov., and *C. dublinensis* sp. nov. subsp. *lactaridi* subsp. nov. *Int J Syst Evol Microbiol* 58:1442-1447

Iversen C, Waddington M, Farmer JJ, Forsythe SJ. 2006. The biochemical differentiation of *Enterobacter sakazakii* genotypes. *BMC Microbiol* 6:94

Joseph S, Cetinkaya E, Drahovska H, Levican A, Figueras MJ, Forsythe SJ. 2012. *Cronobacter condimenti* sp. nov., isolated from spiced meat and *Cronobacter universalis* sp. nov., a novel species designation for *Cronobacter* sp. *genomospecies* 1, recovered from a leg infection, water, and food ingredients. *Int J Syst Evol Microbiol* 62:1277-1283

Kim SH, Park MK, Kim JY, Lee YS, Yoon BS, Hwang KK, Lim YK. 2005. Development of a sandwich ELISA for the detection of *Listeria* spp. using specific flagella antibodies. *J Vet Sci* 6:41-46

Kim Y, Shukla S, Ahmed M, Son S, Kim M, Oh S. 2012. Development of chicken immunoglobulin Y for rapid detection of *Cronobacter muytjensii* in infant formula powder. *Korean J Food Sci An* 32:695-701

Kumar S, Balakrishna K, Batra HV. 2008. Enrichment-ELISA for detection of *Salmonella typhi* from food and water samples. *Biomed Environ Sci* 21:137-143

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685

Lampel KA, Chen Y. 2009. Method for the isolation and detection of *Enterobacter sakazakii* (*Cronobacter*) from powdered infant formula. *Int. J Food Microbiol* 136: 179-184

Lee YD, Park JH, Chang H. 2012. Detection, antibiotic susceptibility and biofilm formation of *Cronobacter* spp. from various foods in Korea. *Food Control* 24:225-230

McKinney MM, Parkinson A. 1987. A simple, non-chromatographic procedure to purify immunoglobulins from serum and ascites fluid. *J Immunol Methods* 96:271-278

Mullane NR, Murry J, Drudy D, Prentice N, Whyte P, Wall PG, Parton A, Fanning S. 2006. Detection of *Enterobacter sakazakii* in dried infant milk formula by cationic-magnetic-bead capture. *Appl. Environ Microb* 72:6325-6330

Muytjens HL, Roelofs-Willemse H, Jaspar GH. 1988. Quality of powdered substitutes for breast milk with regard to members of the family Enterobacteriaceae. *J Clin Microbiol* 26:743-746

Park S, Shukla S, Kim Y, Oh S, Kim SH, Kim M. 2012. Development of sandwich enzyme-linked immunosorbent

assay for the detection of *Cronobacter muytjensii* (formerly called *Enterobacter sakazakii*). *Microbiol Immunol* 56:472-479

Shukla S, Bang J, Heu S, Kim M. 2012. Development of immunoliposome-based assay for the detection of *Salmonella* Typhimurium. *Eur Food Res Technol* 234:53-59

Stoop B, Lehner A, Iversen C, Fanning S, Stephan R. 2009. Development and evaluation of rpoB based PCR systems to differentiate the six proposed species within the genus *Cronobacter*. *Int J Food Microbiol* 136:165-168

Strydom A, Cawthorn DM, Cameron M, Witthuhn RC. 2012. Species of *Cronobacter* - A review of recent advances in the genus and their significance in infant formula milk. *Int Dairy J* 27:3-12

Sunwoo HH, Wang WW, Sim JS. 2006. Detection of *Escherichia coli* O157:H7 using chicken immunoglobulin Y. *Immunol Lett* 106:191-193

Townsend S, Forsythe SJ. 2008. The neonatal intestinal microbial flora, immunity, and infections. In: Farber J, Forsythe SJ. (eds) *Enterobacter sakazakii*. ASM press. pp. 61-100

Townsend SM, Hurrell E, Caubilla-Barron J, Loc-Carrillo C, Forsythe SJ. 2008. Characterization of an extended-spectrum betalactamase *Enterobacter hormaechei* nosocomial outbreak and other *Enterobacter hormaechei* misidentified as *Cronobacter (Enterobacter) sakazakii*. *Microbiol* 3659-3567

USFDA. 2002. Isolation and enumeration of *Enterobacter sakazakii* from dehydrated infant formula. US Food and Drug Administration, Maryland, The United States of America

Wang X, Zhu CQ, Xu XL, Zhou GH. 2012. Real-time PCR with internal amplification control for the detection of *Cronobacter* spp. (*Enterobacter sakazakii*) in food samples. *Food Control* 25:144-149

Zhang YL, Zhang X, Zhang HW, Liu W, Zheng WJ, Huang XT. 2010. Rapid and sensitive detection of *Enterobacter sakazakii* by cross-priming amplification combined with immuno-blotting analysis. *Mol Cell Probe* 24:396-400

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