

Development of Chicken Immunoglobulin Y for Rapid Detection of *Cronobacter mytjensii* in Infant Formula Powder

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Abstract

The present study was aimed to produce a chicken polyclonal antibody against *Cronobacter mytjensii* and to develop an immunoassay for its detection. Purification of anti-*C. mytjensii* IgY from egg yolk was accomplished using various methods such as water dilution and salt precipitation. As a result, sodium dodecyl sulfate-polyacrylamide gel electrophoresis produced two bands around 30 and 66 kDa, corresponding to a light and a heavy chain, respectively. Indirect competitive enzyme-linked immunosorbent assay (IC-ELISA) was performed to determine the effectiveness of the chicken IgY against *C. mytjensii*. The optimum conditions for detecting *C. mytjensii* by indirect ELISA and checkerboard titration of the antigen revealed an optimum average absorbance at the concentration of 18 µg/mL, having ca. 10⁸ coated cells per well. The anti-*C. mytjensii* IgY antibody had high specificity for *C. mytjensii* and low cross-reactivity with other tested pathogens. In this assay, no cross-reactivity was observed with the other genera of pathogenic bacteria including *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Staphylococcus aureus*, *Bacillus cereus*, *Enterobacter aerogenes*, *Salmonella* Enteritidis and *Listeria monocytogenes*. In addition, detection of *C. mytjensii* in infant formula powder showed a low matrix effect on the detection curve of IC-ELISA for *C. mytjensii*, with similar detection limit of 10⁵ CFU/mL as shown in standard curve. These findings demonstrate that the developed method is able to detect *C. mytjensii* in infant formula powder. Due to the stable antibody supply without sacrificing animals, this IgY can have wide applications for the rapid and accurate detection of *C. mytjensii* in dairy foods samples.

Key words: enzyme-linked immunosorbent assay, egg yolk, *Cronobacter mytjensii*, IgY, pathogen detection, infant formula powder

Introduction

Enterobacteriaceae are Gram-negative bacilli that normally inhabit the intestine of animals. Although most of the species are harmless, some of them produce exotoxins. Particularly, *Cronobacter mytjensii* is one of the newly classified microorganisms from *Enterobacter sakazakii* group (Iversen and Forsythe, 2003). *C. mytjensii* is usually found in powdered milk and is an opportunistic pathogen that causes meningitis, septicemia and necrotizing enterocolitis in neonates and small children (Iversen and Forsythe, 2003). *C. mytjensii* is an extremely harmful microorganism and is associated with a mortality rate of 40-80% among infants (Lai, 2001). *C. mytjensii* has been isolated from plant foods and food ingredients such

as cereal, fruits, vegetables, legume products and animal origin foods like milk, meat and fish, which poses a serious threat to the food and medical industries (Friedmann, 2007).

C. mytjensii is usually detected on tryptic soy agar, and its colonies are developed as a yellow pigment. However, Druggan-Forsythe-Iversen medium has been recently used to detect *C. mytjensii* (Bar-Oz *et al.*, 2001; Iversen and Forsythe, 2003). Various other methods such as DNA hybridization, polymerase chain reaction, immunomagnetic beads and immunoenzymatic assays have been used to detect *C. mytjensii* in various food matrices (Schneid *et al.*, 2006). The enzyme-linked immunosorbent assay (ELISA) has been shown to be a sensitive technique for the detection of several infectious agents (Voller and Bidwell, 1976). ELISAs are considered as useful tools to detect and quantify the immunological reactions and have several advantages such as rapidity, potent mobility, better convenience, higher sensitivity and efficiency for

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detecting the pathogens at low detection limit (Liu *et al.*, 2010). In contrast, nowadays, the demand for immunoglobulin (IgY) is increasing not only for immunological purposes but also for therapeutic applications (Carlander *et al.*, 2003). Chicken egg yolk is a valuable and inexpensive source of polyclonal antibodies, and large amount of IgY can be extracted from the eggs of immunized chickens (Kovacs-Nolan and Mine, 2004). Previously, a polyclonal antibody against bbb-cyclocitral has been isolated from chicken eggs (Munene, 2004).

To the best of our knowledge, no several studies have been conducted so far on the production of a polyclonal antibody against *C. mytjensii* and its detection by using an ELISA. Therefore, the principal objectives of this study were to prepare a specific polyclonal antibody (IgY) against *C. mytjensii* and to develop an ELISA in order to determine the effectiveness of developed anti-*C. mytjensii* IgY in infant formula powder.

Materials and Methods

Reference strains and selective media

The bacterial pathogens such as *Cronobacter mytjensii* ATCC51329, *Escherichia coli* O157:H7 ATCC43895, *Enterobacter aerogenes* ATCC15038, *Salmonella* Typhimurium ATCC13311, *Salmonella* Enteritidis ATCC4931, *Staphylococcus aureus* ATCC10832, *Bacillus cereus* ATCC14579 and *Listeria monocytogenes* KCTC3596 were collected from the American Type Culture Collection and the Korean Collection for Type Culture. *C. mytjensii*, *E. coli* O157:H7 and *E. aerogenes* were grown in tryptic soy broth (TSB; Difco, Franklin Lakes, NJ, USA), whereas *Bacillus* spp., *Salmonella* spp. and *S. aureus* were grown in nutrient broth (NB, Difco, USA).

Animals

Animal use protocol was in accordance with international standard on the care and use of experimental animals (Canadian Council on Animal Care, 1993). Brown Leghorn chickens (25 wk old) were used for the experimental purpose. Animals were fed with standard pellet diet and housed under the following standard conditions: temperature (25°C), humidity (55%) and light/dark cycles (12 h/12 h).

Reagents and materials

Goat anti-chicken IgY-horseradish peroxidase (HRP) and chicken IgY (positive control in purity test) were provided by Santa Cruz Biotechnology (USA) and Promega

(USA), respectively. Freund's complete and incomplete adjuvants were obtained from Sigma-Aldrich (USA). All chemicals including 3,3',5,5'-tetramethylbenzidine (TMB), sodium carbonate and sodium hydrogen carbonate were purchased from Sigma-Aldrich. Phosphate-buffered saline (PBS, pH 7.0), consisting of 138 mM NaCl, 1.5 mM KH₂PO₄, 7 mM Na₂HPO₄ and 2.7 mM KCl, and carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃) were prepared in the laboratory for this study.

Antigen preparation

For antigen preparation, *C. mytjensii* ATCC51329 was cultivated in TSB at 37°C for 18 h with shaking. The cells were harvested after cultivation by centrifugation at 3,000 g for 10 min. The collected cells were washed thrice with 0.01 M PBS (pH 7.0), mixed with equal volume of 0.01 M PBS (pH 7.0) and fragmented by sonication (Sonics & Materials Inc., USA). The cellular material was removed by centrifugation at 3,000 g for 10 min, and the supernatant (*C. mytjensii* whole cell lysate) was collected (Park *et al.*, 2012) to measure the protein concentration using the Bradford method (Bradford, 1976).

Immunization

To produce chicken anti-*C. mytjensii* IgY, brown Leghorn chickens were immunized intramuscularly with *C. mytjensii* whole cell lysate (0.5 mL) using an equal volume of Freund's complete adjuvant as described previously (Lee *et al.*, 2002) with some modifications. Chickens were injected at four different sites (0.25 mL per site) of the leg muscle. Three booster injections with Freund's incomplete adjuvant were given at two weeks interval following the first injection. After one month of immunization, the eggs laid were collected daily for 30 d and stored at 4°C. Egg yolk was separated, pooled and stored at -20°C prior to purification step.

Purification and preparation of egg yolk antibody

In the present study, four different methods were used for the purification of the egg yolk IgY, which were compared from each other for better purification efficacy.

1. In the first method, the purification of chicken anti-*C. mytjensii* IgY was performed by water dilution and salt precipitation methods (Hansen *et al.*, 1998). The egg yolk was separated, washed with distilled water, diluted 10 times with 3 mM HCl, adjusted to give the desired pH 5.0 with 10% acetic acid and then kept at 4°C for 6 h. Following the centrifugation at 10,000 g at 4°C for 15 min, the supernatant was collected. Further, ammonium

sulfate was added to the supernatant in the ratio of 6:4 (supernatant:ammonium sulfate) and stirred for 15 min. The pellet was again collected by centrifugation at 10,000 g for 15 min at 4°C. Finally, the pellet containing the IgY was collected and dissolved in 0.01 M PBS (pH 7.0) with an equal quantity of egg yolk's initial volume.

2. In the second method, the purification of chicken anti-*C. mytjensii* IgY was performed according to the developed procedure of Akita and Nakai (1992). Briefly, egg yolk was separated from the white proportion of the egg and diluted with distilled water (acidified with 0.1N HCl, predetermined to give the desired pH 5.0). The supernatant was collected after centrifugation of the egg yolk at 10,000 g for 60 min at 4°C. Further, collected supernatant and saturated ammonium sulfate were mixed (6:4), and this mixture was centrifuged at 10,000 g for 60 min at 4°C. The pellet obtained was washed with 14% sodium sulfate and centrifuged at 10,000 g for 60 min. The precipitate containing IgY was obtained and dissolved in 0.01 M PBS with a quantity of half of the initial volume.

3. In the third modified-water dilution method, the separated yolk was diluted nine-times with distilled water and acidified with 0.1 N HCl to adjust the pH at 5.0 (Akita and Nakai, 1993). The supernatant was collected after centrifugation of the egg yolk at 10,000 g for 25 min at 4°C. Further, to the supernatant, 19% sodium sulfate was added slowly, and the mixture was kept at room temperature for 2 h. The supernatant was discarded after centrifuging at 10,000 g for 25 min at 4°C, and the precipitate containing the IgY was dissolved in 0.01 M PBS (pH 7.0) with an equal quantity of egg yolk's initial volume.

4. The fourth method was adopted from Wallmann *et al.* (1990). Briefly, egg yolk was separated from the white proportion of the egg, diluted five-times with distilled water and incubated at -20°C for 72 h. The supernatant was collected after centrifugation at 2,800 g for 20 min at room temperature. Then, 0.27 g/mL ammonium sulfate was added to the supernatant and allowed to stir at room temperature for 2 h. The centrifugation step was re-performed at 2,800 g for 20 min at room temperature. Finally, a precipitate containing IgY was obtained and dissolved in 5 mL PBS/egg yolk mL.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The purity of various IgY preparations was checked using SDS-PAGE (Atto, Japan) under reduced conditions

as described by Laemmli (1970) with some modifications. A polyacrylamide stacking gel (4%, w/v) and a separating gel (15%, w/v) were used to separate the distinct proteins into bands. Briefly, the samples were mixed with digestion buffer containing 5% 2-mercaptoethanol (v/v) and then heated for 3 min at 100°C. Electrophoresis was conducted at a constant voltage of 20 mA at room temperature. Purity and recovery of chicken anti-*C. mytjensii* IgY were monitored at various stages by SDS-PAGE.

Measurement of antibody titer by ELISA

Formalin treated whole *C. mytjensii* cells were diluted in 0.01 M carbonate buffer (pH 9.6) to adjust 10^7 cells/mL. A 100 μ L of the cell suspension (10^7 cells/mL) was coated onto a 96-well microtiter plate (Maxisorp, CA) for 24 h at 4°C, after which the plate was washed three times with 0.01 M PBS (pH 7.0) and then blocked with 200 μ L of 5% (w/v) skim milk for 1 h at 37°C. The plate was then washed three times with 0.01 M PBS containing 0.05% (v/v) Tween 20 (PBST). An appropriately diluted primary antibody (chicken anti-*C. mytjensii* IgY) solution (100 μ L) was added to the wells, followed by incubation at 4°C for 24 h. The plate was rewashed with PBST, and then 100 μ L of an appropriately diluted secondary antibody (goat anti-chicken IgY-HRP) solution was added, followed by incubation at 37°C for 1 h. After that, the plate was rewashed five times with PBST, and 50 μ L of TMB was added to each well, followed by incubation at 37°C for 30 min. In order to stop the reaction, 50 μ L of 1 N hydrochloric acid (HCl) was added to each well, and then absorbance was measured at 450 nm using an ELISA plate reader (Tecan, Infinite M200, Switzerland). Each experiment was performed in triplicates.

Assay format

The indirect competitive enzyme linked immunosorbent assay (IC-ELISA) method was adopted for this assay with slight modifications (Engvall and Perlmann, 1971). Briefly, formalin treated whole *C. mytjensii* cells were coated onto a 96-well microtiter plate, after which the plate was washed three times with 0.01 M PBS (pH 7.0) and then blocked with 200 μ L of 5% (w/v) skim milk for 1 h at 37°C. The plate was then rewashed with PBST. An appropriately diluted primary antibody (chicken anti-*C. mytjensii* IgY) solution (50 μ L) was mixed with cultured *C. mytjensii* cells (50 μ L). From the mixture, a 100 μ L solution was added in each well, and the plate was incubated at 37°C for 1 h. The plate was again washed with PBST, and then an appropriately diluted solution (100

μL) of secondary goat anti-chicken IgY-HRP was added to each well. After the plate was incubated at 37°C for 1 h, the plate was rewashed with PBST, and $50\ \mu\text{L}$ of TMB was added to each well, followed by incubation at 37°C for 30 min. In order to stop the reaction, $50\ \mu\text{L}$ of 1 N HCl was added to each well, and then absorbance was measured at 450 nm using an ELISA plate reader. Each experiment was performed in triplicates.

Optimization and construction of standard curve for IC-ELISA

Optimization of IC-ELISA assay was conducted by using checkerboard test. An IC-ELISA requires the competition between IgY and *C. mytjensii*. Briefly, for optimization, the plate was coated with primary antibody (chicken anti-*C. mytjensii* IgY) at various concentrations of $0.018\ \mu\text{g}/\text{mL}$, $0.18\ \mu\text{g}/\text{mL}$, $1.8\ \mu\text{g}/\text{mL}$, $18\ \mu\text{g}/\text{mL}$ and $180\ \mu\text{g}/\text{mL}$. Then, *C. mytjensii* at various concentrations of 10^5 - 10^8 cells/mL was added for the reaction to occur at 4°C for overnight, and values were determined by measuring the absorbance at 450 nm. In order to construct standard curve for the developed IC-ELISA, various parameters such as antibody concentrations, blocking buffer, incubation time and temperature were optimized. Finally, the standard curve was constructed on the basis of optimal conditions.

Specificity test

The cross-reactivity of developed antibody was evaluated by IC-ELISA using the following pathogenic strains such as *E. coli* O157:H7 (ATCC43895), *E. aerogenes* (ATCC15038), *S. Typhimurium* (ATCC13311), *S. Enteritidis* (ATCC4931), *S. aureus* (ATCC10832), *B. cereus* (ATCC14579) and *L. monocytogenes* (KCTC3596).

Detection of *C. mytjensii* in infant formula powder

After adding pure cultures of *C. mytjensii* to the infant formula powder, the concentration of *C. mytjensii* was determined using the IC-ELISA technique developed in this study. Briefly, a 25 g of infant formula powder was mixed with 225 mL of TSB at pH 7.0. Pure cultures of *C. mytjensii* were cultured in TSB at 37°C for 18 h. A 1% serially diluted *C. mytjensii* pure culture was added into infant formula powder solution, and the IC-ELISA method was employed to determine the concentration of *C. mytjensii* in infant formula powder. In parallel, an equal volume of TBS buffer mixed with serially diluted the pure culture of *C. mytjensii* served as a positive control, whereas only TBS served as a negative control.

Results and Discussion

Measurement of antibody titer

The chicken anti-*C. mytjensii* IgY was harvested from chicken egg yolk. Fig. 1 shows the immune response against *C. mytjensii* whole cell lysate. Measurement of the antibody titer for 11 wk resulted in the high antibody titer value, starting to significant increase from 4th wk, and the highest activity of IgY was found on 9th wk which was measured by ELISA (Fig. 1). Calzado *et al.* (2001) reported that the IgY level was highest at 4 wk and remained stable for up to 7 wk.

SDS-PAGE analysis

Purification of IgY from egg yolk was performed by four different methods such as pure water dilution and salt precipitation methods (Akita and Nakai, 1992; Akita and Nakai, 1993; Wallmann *et al.*, 1990; Hansen *et al.*, 1998). Purification of anti-*C. mytjensii* IgY was confirmed by SDS-PAGE analysis using purified chicken IgY as control. The most effective method to purify the IgY was found to be the water dilution method. By using all purification methods, two strong bands around 30 and 66 kDa were produced (Fig. 2). Akita and Nakai (1992) method produced the highest yield without non-specific proteins (lane 2 in Fig. 2) and had no adverse effects on IgY immunoactivity (data not shown). Therefore, further experiments were conducted using this purified antibody (IgY) by the method of Akita and Nakai (1992).

Optimal conditions for detecting *C. mytjensii*

The optimal purified antibody concentration and the antigen coating concentration were assessed by using the

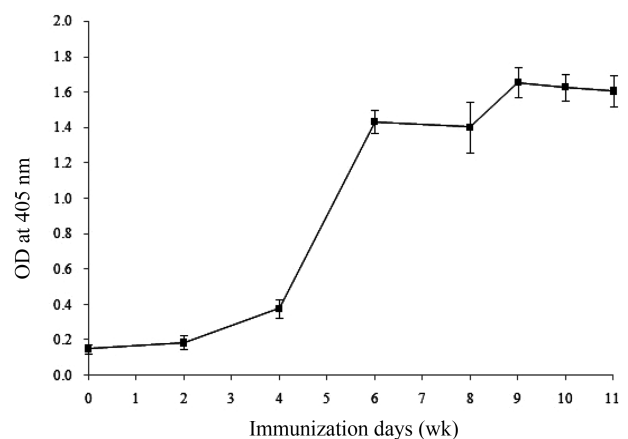


Fig. 1. Production profile of immunoglobulin Y in the eggs of immunized chickens.

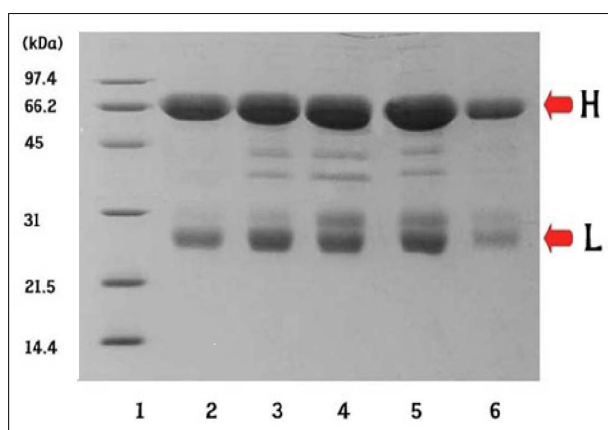


Fig. 2. SDS-PAGE patterns of purified IgY from egg yolk. Lane 1, low-range standard marker; lane 2, Akita and Nakai (1992); lane 3, Hansen *et al.* (1998); lane 4, Akita and Nakai (1993); lane 5, Wallmann *et al.* (1990); lane 6, chicken IgY (control); H, heavy chain; L, light chain.

checkerboard test. A final concentration of 0.5% formalin was added to the *C. mytjensii* culture medium, followed by overnight incubation at 4°C. Coating concentrations of 10^5 - 10^8 cells/well and 0.018-180 µg/mL of purified IgY was used in the ELISA. As results, 100-1000 fold IgY dilution (1.8-18 µg/mL) and 10^7 - 10^8 coated cells exhibited significant absorbance (Fig. 3a and 3b). Previously, 40-fold and 1000-fold diluted *Salmonella gallinarum* and *Enterobacter mytjensii* antibodies have been used for the detection of *Salmonella* and *Enterobacter* by Prakash and Suryanarayana (2005), respectively. In our study, IgY dilution was slightly different from the antibody dilutions used by Prakash and Suryanarayana (2005). However, whole cell coating concentrations were similar with those of Prakash and Suryanarayana (2005).

Furthermore, to optimize the secondary antibody (goat anti-chicken IgY-HRP) concentration, the secondary antibody solutions of 0.032 µg/mL, 0.04 µg/mL, 0.053 µg/mL and 0.08 µg/mL were made with 5% non-fat dry milk. Fig. 4 shows that the binding of the secondary antibody to the antigen-antibody complex (*C. mytjensii*-IgY) was higher at 0.08 µg/mL, compared to other antibody concentrations. However, the antibody concentrations of 0.04 µg/mL or 0.053 µg/mL were considered economical.

Fig. 5 shows the *C. mytjensii* ELISA standard curve, and the optimal conditions for IC-ELISA to detect *C. mytjensii* has been described in Table 1. In brief, coating antigen (10^8 formalin killed cells of *C. mytjensii*/well) was coated at 37°C for 1 h. An appropriate response was found with the secondary antibody at the concentration of 0.04 µg/mL at 37°C for 1 h, and the enzyme substrate reaction was continued at 37°C for 30 min.

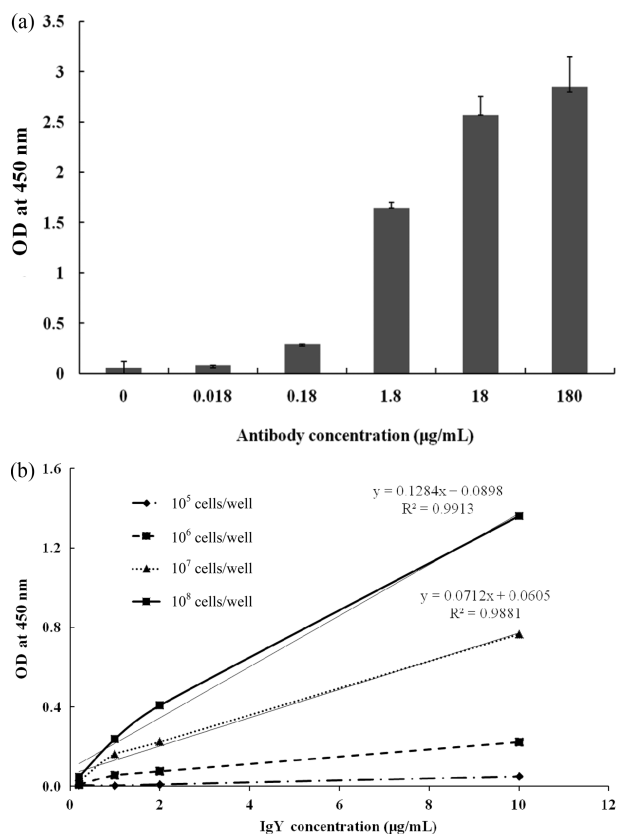


Fig. 3. Checkerboard titration curve.

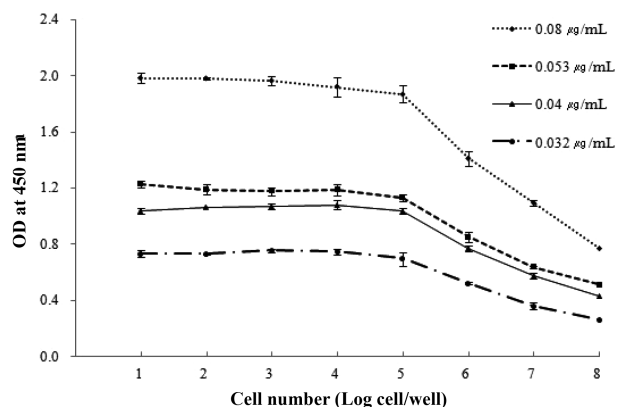


Fig. 4. Effect of secondary antibody (goat anti-chicken IgY-HRP) concentration.

IgY specificity test

The specificity of the developed chicken anti-*C. mytjensii* IgY was evaluated using a variety of foodborne pathogens including *E. coli* O157:H7, *S. Typhimurium*, *S. Enteritidis*, *S. aureus*, *B. cereus* and *L. monocytogenes*. The results revealed that IC-ELISA was found sensitive only against *C. mytjensii* with detection sensitivity of 10^5 cells/mL and showed no cross-reactivity with other tested bacteria (Fig. 6). In our study, these values were found to be useful for comparing the detection limit of *Crono-*

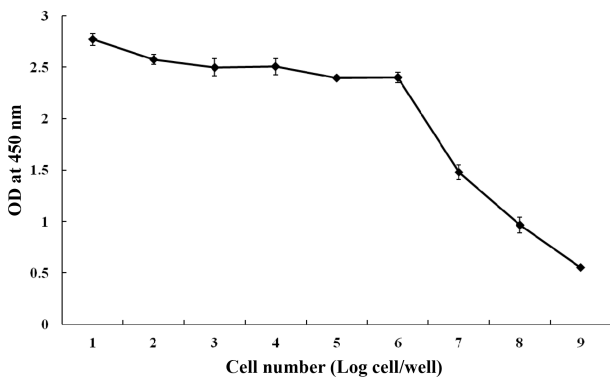


Fig. 5. IC-ELISA standard curve for *Cronobacter mytjensii*.

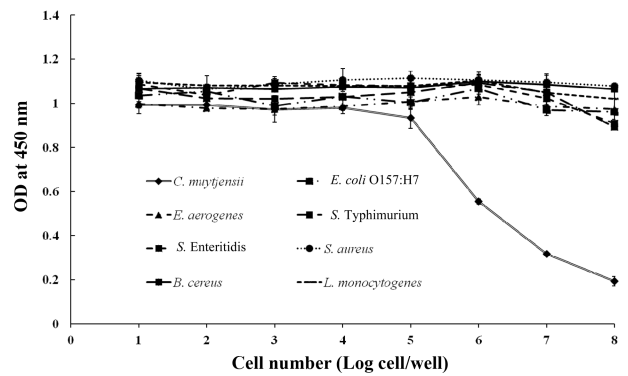


Fig. 6. Cross-reactivity with other genus of pathogenic bacteria.

bacter using sandwich ELISA. Park *et al.* (2012) also observed similar findings for *C. mytjensii* by using the developed sandwich ELISA. In addition, Baumgartner *et al.* (2009) reported 2.9×10^7 CFU/g as the detection limit of *Cronobacter* using a pulsed field electrophoresis assay method. Hochel and Skvor (2009) also reported similar findings on the lack of cross-reactivity when they tested the developed immunoassay with other bacterial species. Thus, this study confirmed that the developed immunoassay for *C. mytjensii* is sensitive and specific to *C. mytjensii* and can detect this organism within 24 h, as compared to conventional culture-dependent methods which require 5 to 7 days for *Cronobacter* detection.

Detection of *C. mytjensii* in infant formula powder

In this study, we developed an immunoassay technique for the detection of *C. mytjensii* in commercial food products such as infant formula powder. Fig. 7 shows the detection curve for *C. mytjensii* in infant formula powder using IC-ELISA. The results for the detection of *C. mytjensii* in infant formula powder showed a low matrix effect as compared with the detection curve of Fig. 5. Although signals obtained by using infant formula powder did show this minor matrix effect, the response curve (Fig. 7) still showed dose dependent characteristics, similar to the standard curve obtained without using infant formula powder (Fig. 5). Park *et al.* (2012) also reported

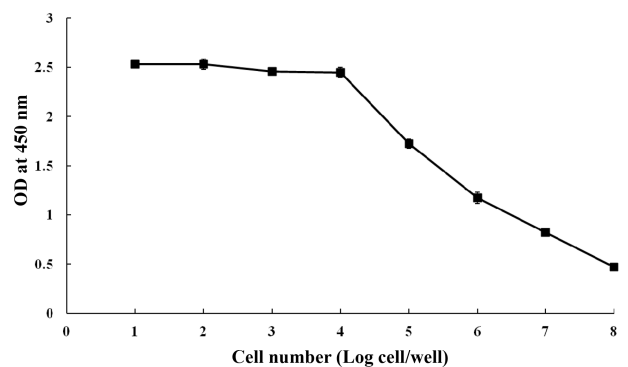


Fig. 7. Detection of *Cronobacter mytjensii* in infant formula powder by using IC-ELISA.

similar results when they developed the sandwich ELISA for the detection of *C. mytjensii* in infant formula powder.

The developed antibody (chicken anti-*C. mytjensii* IgY) was found to be very specific to *C. mytjensii* and was able to detect the *C. mytjensii* in pure culture and commercial food sample (infant formula powder) with the detection limit of 10^4 - 10^5 CFU/mL (Figs. 5 and 7). This result confirms the potential efficacy of our immunological assays for the purpose of developing new and effective pathogen detection methods. Hence, it is concluded that the developed ELISA may provide a better alternative than conventional identification methods for detecting *C. mytjensii* not only in infant formula powder

Table 1. Optimal condition for the *Cronobacter mytjensii* detection by using IC-ELISA

Item	Conditions
Antigen coating	Formallin killed cells of <i>C. mytjensii</i> (10^8 cell/well) at 37°C for 1 h (or 4°C, overnight)
Blocking condition	5% Skim milk at 37°C for 1 h
Primary antibody	IgY (18 µg/mL)
Secondary antibody	Goat anti-chicken IgY-HRP (0.04 µg/mL) at 37°C for 1 h
Substrate	3,3',5,5'- Tetramethylbenzidine at 37°C for 30 min
Washing solution	0.01 M PBS (containing 0.14 M NaCl) + 0.05% Tween 20

but also in other food samples.

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