Improvement of *Enterobacteriaceae* enrichment broth by supplementation with sodium citrate for detection of *Cronobacter sakazakii* using real-time PCR

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Abstract *Enterobacteriaceae* enrichment (EE) broth, a selective enrichment medium for *Cronobacter sakazakii* detection, has been shown to contain polymerase chain reaction (PCR) inhibitors. Therefore, a modified enrichment broth was developed, wherein possible PCR inhibitors, brilliant green and bile salts, were substituted with sodium citrate. Investigations were conducted to determine whether real-time PCR detection of *C. sakazakii* in powdered infant formula and dried vegetables with this modified medium improved compared with conventional culture methods. The detection rate of the modified EE broth was significantly higher ($p < 0.05$) than that of the conventional EE broth for both types of food. For dried pumpkin samples, real-time PCR after culturing in modified EE broth yielded significantly higher detection rates and selectivity than those achieved using conventional culture in Druggan-Forsythe-Iversen agar. These results show that real-time PCR after enrichment in the modified EE broth may be an effective screening method for detection of *C. sakazakii*.

Keywords: *Cronobacter sakazakii*, PCR inhibitor, sodium citrate, EE broth, real-time PCR

Introduction

Although *Cronobacter sakazakii* can cause health problems in all age groups, certain groups, such as infants, have been reported to be more vulnerable to infection than other groups (1). *Cronobacter* spp. have been isolated from a variety of foods, such as powdered infant formula (PIF), dry infant food, herbs, spices, desiccated agriculture, and marine products (2,3). Contamination of PIF, milk powder, and weaning foods can occur through several routes (2). Extrinsic contamination could occur because of unhygienic storage of the reconstituted PIF, while intrinsic contamination can occur in cases where the bacterium was isolated from unopened cans of PIF (3).

Several *Cronobacter* detection methods ranging from chromogenic detection media to molecular culture-independent techniques have been developed (4,5). The conventional culture detection method requires long durations of 5-7 days. Therefore, a rapid detection method, i.e., real-time PCR, which utilizes simultaneous amplification of target genes and capture of fluorescence, has been developed (5). This method, applied after enrichment, enables detection of the target microorganism within 3 h, shortening the entire detection process to 1-2 days and thereby significantly reducing time and labor requirements.

Real-time PCR for detection of *C. sakazakii* has already been verified by Chon *et al.* (7). However, several research groups have indicated that inhibition of PCR can occur during the detection of pathogens from selective medium, resulting in higher Ct values and false positive results compared with those from non-selective buffered peptone water (BPW) or phosphate-buffered saline (PBS) (7,8). Thus, PCR inhibitors, which hinder accurate quantification and detection, are likely to be present in selective enrichment broth.

In this study, a new enrichment broth was developed to replace possible PCR inhibitors with sodium citrate. The detection rate and sensitivity of the conventional *Enterobacteriaceae* enrichment (EE) broth and the new modified EE (mEE) broth were compared in PIF and dried vegetables, and the new broth was verified with artificial inoculation in food samples with conventional culture methods and real-time PCR.

Materials and Methods

Bacteria *C. sakazakii* strain ATCC 29544 was prepared by culturing in tryptic soy broth (TSB; Oxoid, Basingstoke, UK) overnight at 37°C. The overnight culture of *C. sakazakii* cells was serially diluted with

PBS (pH 7.2), and 100 µL of the dilution was plated on nutrient agar (NA; Difco, Becton Dickinson, Sparks, MD, USA). The plates were then incubated at 37°C overnight, and the number of colony-forming units (CFU) of *C. sakazakii* was counted. To confirm enrichment capacity, bacterial strains in Table 1 were obtained from the culture collection of the US Food and Drug Administration (College Park, MD, USA).

Preparation of enrichment broths The EE broth (Oxoid) was prepared according to the manufacturer's recommendations. The mEE broth was prepared with D-glucose (Oxoid), bacteriological peptone (Oxoid), disodium hydrogen phosphate anhydrous (Junsei Chemical Co. Ltd., Tokyo, Japan), and potassium dihydrogen phosphate (Sigma-Aldrich, St. Louis, MO, USA) with the concentrations shown in Table 2. Distilled water containing the above ingredients was autoclaved at 121°C for 15 min and cooled before the experiment. The BPW (AES Laboratoire, Combourg, France) was used for the primary enrichment, while Druggan-Forsythe-Iversen (DFI) *C. sakazakii* chromogenic agar (Oxoid) was used as selective agar.

Growth of pure *Cronobacter* cultures in enrichment broths For the analysis of *C. sakazakii* growth, 10–20 cells for each of the eight *C. sakazakii* isolates from environmental milk and three *C. sakazakii* isolates from clinical cases were inoculated into 10 mL of EE or mEE broth and cultured at 37°C for 24 h. To determine cell numbers, the enriched cultures were 10-fold serially diluted, and 0.1 mL of each dilution was spread on duplicate DFI agar plates and incubated at 37°C for 24 h followed by colony counting.

DNA extraction and real-time PCR Template DNA used in real-time PCR was extracted by boiling with PrepMan Ultra Reagent (Applied Biosystems, Foster City, CA, USA). One milliliter of each broth was transferred to centrifuge tubes and centrifuged at 16,000×g for 3 min, and the supernatants were aspirated and discarded. The cell pellets were resuspended in 200 µL PrepMan Ultra Reagent, thoroughly pipetted, and boiled for 10 min. After cooling at room temperature for 2 min, the tubes were centrifuged at 16,000×g for 3 min, and the supernatants were collected in new tubes and kept at 20°C for later use as DNA templates.

The extracted DNA was added to a 20 µL mixture of *MMS* gene primers and an *MMS* gene probe, and real-time PCR was conducted as described earlier (5,9). The Ct values below 38 were considered as positive reactions.

Real-time PCR detection of *C. sakazakii* cells enriched in EE and mEE broths Less than 100 *C. sakazakii* cells were inoculated in 10 mL EE or mEE broth and enriched for 24 h at 37°C for real-time PCR of pure cultures. Less than 100 *C. sakazakii* cells were inoculated in 25 g of PIF or dried pumpkin, with triplicate samples. Next, 225 mL of BPW was added, and samples were cultured at 37°C for 24 h. One hundred microliters of the enriched sample was inoculated into 10 mL EE or mEE broth and cultured at 37°C for 24 h. DNA extraction and real-

Table 1. Comparison of the compositions of mEE broth and commercial EE broth

Functions	Formula (g/L)	
	EE broth (Oxoid)	mEE broth
Buffers		
-Disodium hydrogen phosphate anhydrous	6.45	6.45
-Potassium dihydrogen phosphate	2.0	2.0
Growth promoters		
-Glucose	5.0	5.0
-Peptone	10.0	10.0
Competing flora inhibitors		
-Ox bile purified	20.0	
-Brilliant green	0.0135	
-Sodium citrate		10.0

time PCR were conducted accordingly.

Detection of *C. sakazakii* in artificially contaminated food products

PIF and dried pumpkin were used to determine the differences in detection capabilities between EE and mEE broths. All samples were purchased from a local grocery store in Seoul, Korea and were found to be free of *C. sakazakii* by standard culture methods. A plate count of the background flora of each food type was conducted as described earlier (9–11).

C. sakazakii ATCC 29544 was used in experimental inoculation testing. An overnight culture of the inoculum was serially diluted, and 1 mL of each dilution was then evenly inoculated into 500 g of food bulk samples by pipetting to generate partial positives and negatives for statistical comparisons after division into subsamples. The inoculated bulk samples were divided into 20 subsamples of 25 g each. Two additional food samples (25 g each) were used as positive and negative controls. A positive control was prepared by spiking approximately 10⁷ CFU/mL of *C. sakazakii* KCTC 2949 in the 25 g food sample. One milliliter of sterilized PBS was inoculated in 25 g of the food sample as a negative control.

Statistical analysis The differences in detection rates between EE and mEE broths were statistically analyzed with InStat software (Version 3.05; GraphPad, La Jolla, CA, USA). Results of the culture method and real-time PCR were also analyzed using Fisher's exact tests. Differences with *p* values of less than 0.05 were considered significant.

Results and Discussion

C. sakazakii cells from different origins (environmental milk and clinical samples) were enriched in EE or mEE broth, and viable cells were counted on DFI agar. The enriched cell count in mEE broth was slightly higher than that in EE broth; however, this difference was not statistically significant (Table 2). Thus, mEE broth was capable of

Table 2. Comparison of the EE and mEE broths for the growth of pure *Cronobacter sakazakii* and non-*C. sakazakii* cultures

	ID	Source	Growth after 24 h enrichment (log CFU/mL)	
			EE broth	mEE broth
<i>Cronobacter sakazakii</i>	FSM 145	Environmental milk	9.8±0.18	9.18±0.04
	FSM 262	Environmental milk	7.74±0.37	9.06±0.08
	FSM 265	Environmental milk	7.89±0.58	9.07±0.32
	FSM 270	Environmental milk	10.06±0.02	9.09±0.12
	FSM 272	Environmental milk	8.65±0.07	9.06±0.08
	FSM 292	Environmental milk	8.78±0.00	8.93±0.04
	FSM 295	Environmental milk	9.98±0.01	9.27±0.05
	FSM 300	Environmental milk	8.92±0.11	8.98±0.28
	2.82	Human	9.68±0.01	8.63±0.21
	2.4	Human	8.97±0.52	8.72±0.17
Non- <i>C. sakazakii</i> <i>Enterobacteriaceae</i>	1.83	Human	8.30±0.00	8.24±0.34
	<i>E. cloacae</i>	Environmental milk	8.45±0.64	8.78±0.74
	<i>E. vulneris</i>	Environmental milk	8.19±0.41	9.03±0.82
	<i>E. agglomerans</i>	Environmental milk	7.85±0.2	8.37±0.13
	Mean±SD (log CFU/mL) ¹⁾		8.31±0.4A	8.78±0.46A

¹⁾Different letters (A, B) within a row indicate significant differences ($p<0.05$)

Table 3. Comparison of Ct values for detection of *C. sakazakii* in pure culture and in powdered infant formula (mean±SD; $n=3$); the smaller the Ct value, the better is the detection limit

	Pure culture		Powdered infant formula		Dried pumpkin	
	EE	mEE	EE	mEE	EE	mEE
Ct values	>40	17.82±0.22	>40	16.28±0.43	>40	24.25±0.55

successfully enriching the target cells.

Real-time PCR amplification of templates extracted from the two broths from three different samples (pure culture, PIF, and dried pumpkin) is presented in Table 3. DNA extracted from the EE broth presented Ct values over 40 (false negative results) in all three samples in the real-time PCR analysis, whereas that extracted from the mEE broth presented smaller Ct values (true positive results). Data by Chon *et al.* (7) showed that more positive reactions were present at real-time reactions of nonselective enrichment in distilled water than selective enrichment in EE broth. The consistent false negative results of the EE broth prove the presence of PCR inhibitors in the EE broth. When bile salts and brilliant green in the EE broth were replaced with sodium citrate, *C. sakazakii* was accurately detected by real-time PCR with low Ct values (Table 3). Thus, these data showed that the bile salts and brilliant green inhibited the PCR assay, unlike sodium citrate.

A comparison of the numbers of *C. sakazakii*-positive samples between the standard culture method and real-time PCR in artificially inoculated PIF and dried pumpkin is shown in Table 4. Negative reactions were observed in the negative controls for all food samples in both broths for both detection methods. However, positive controls showed different results; while positive reactions were observed following conventional methods and real-time PCR of samples cultured in the mEE broth, samples cultured in the EE broth showed positive results in the conventional culture method but false negative results in real-time PCR for all four trials, further verifying

the presence of PCR inhibitors in the EE broth.

No significant differences between the EE and mEE broths were observed for the conventional culture method ($p>0.05$), irrespective of the food matrix and count of background microflora (Table 4). However, significant differences were observed between the real-time PCR results for both broths. Moreover, in both PIF and dried pumpkin samples enriched in the EE broth, the positive results in the real-time PCR did not align with the positive results in the conventional culture method (data not shown); therefore, the positive results for real-time PCR of EE broth samples were all considered to be false positives. The positive controls in the EE broth also displayed false negative results by real-time PCR in all four trials.

Real-time PCR showed higher detection capabilities than conventional culture methods ($p<0.05$) for two artificially inoculated food samples for both the broths (Table 4). In PIF samples, real-time PCR of the mEE broth detected 27 out of 40 samples, while that of the EE broth detected 18 out of 40 samples, all of which were false positives. In addition, the culture method detected 21 out of 40 samples for the mEE broth. In dried pumpkin samples, the difference was even more significant; real-time PCR of the mEE broth detected 38 out of 40 samples, while that of the EE broth detected only 9 out of 40 samples (all false positives). For EE broth samples, the culture method detected only 3 out of 40 samples.

For dried pumpkin samples having more than 5.8 log CFU/g of background flora, *C. sakazakii* could be missed in detection with conventional culture methods because of the masking effect (Fig. 1);

Table 4. Comparison of the numbers of *C. sakazakii*-positive samples between standard culture methods and real-time PCR in artificially inoculated powdered infant formula and dried pumpkin (number of positive samples/tested total samples)

Samples	Background flora ¹⁾	Trials	Inoculation level (CFU/500 g)	Enrichment broth-Culture media		Enrichment broth-PCR	
				EE-DFI ²⁾	mEE-DFI	EE-RTi	mEE-RTi
Powdered infant formula	<1±0.0	1	15	7/20	9/20	10/20*	13/20
		2	30	15/20	13/20	8/20	14/20
		Total ^{a3)}		22/40A	21/40A	18/40A	27/40B
Dried pumpkin	5.8±0.2	1	15	2/20 ^a	1/20 ^a	6/20	18/20
		2	30	1/20 ^a	5/20 ^a	3/20	20/20
		Total ^a		3/40A	6/40A	9/40A	38/40B

¹⁾Each value is the mean log CFU/g±SD
²⁾EE, *Enterobacteriaceae* enrichment broth; mEE, modified *Enterobacteriaceae* enrichment broth; DFI, Brilliance *E. sakazakii* agar (Druggan-Forsythe-Iversen); RTi, real-time PCR
³⁾Different letters (A, B) within a row indicate significant differences (*p*<0.05)

Fig. 1. Representative image of DFI agar showing the growth of *C. sakazakii* inoculated in dried pumpkin after enrichment at mEE broth (A) compared to traditional EE broth (B). White arrow indicates typical *C. sakazakii* blue-green colonies on DFI agar while the black arrow indicates background flora of dried pumpkin. Background flora significantly reduced after enrichment in mEE broth, allowing easy confirmation of the contamination.

however, the mEE broth was able to significantly reduce the background flora, allowing clearer detection of *C. sakazakii* on DFI agar. Nonetheless, real-time PCR in combination with enrichment in the mEE broth could successfully detect all positives, and this approach was demonstrated to be the best method to detect *C. sakazakii* in food samples.

The concentration of sodium citrate was optimized in preliminary experiments (data not shown). The addition of sodium citrate to the mEE broth is known to have inhibitory effects on gram-positive microorganisms (12,13). Therefore, some selective media, such as gram-negative broth, thiosulfate citrate bile salts sucrose agar, and deoxycholate citrate agar, contain sodium citrate for this purpose. Sodium citrate showed better results than brilliant green and ox bile in inhibiting background flora of food samples while successfully enriching *C. sakazakii* cells for detection with real-time PCR.

The improved medium was also more convenient to prepare than the EE broth. While the EE broth (Oxoid) requires distributing the medium into 100 mL volumes in 250 mL flasks, heating at 100°C for

30 min, and rapid cooling under running water, the mEE broth can be formulated in large quantities as well as conveniently autoclaved at 121°C for 15 min.

In summary, the mEE broth facilitated rapid and accurate detection of *C. sakazakii* in PIF and dried pumpkin using real-time PCR while suppressing the background flora present in the food samples. Validation of the mEE broth in other food samples known to be contaminated with *C. sakazakii* should be conducted. Also, further research is needed to test the ability of the mEE broth to recover stressed (desiccated) *C. sakazakii* cells and other poorly growing *Cronobacter* strains.

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Disclosure The authors declare no conflict of interest.

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