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Genotyping Based on Polymerase Chain Reaction of *Enterobacter sakazakii* Isolates from Powdered Infant Foods

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Abstract This study was undertaken to classify *Enterobacter sakazakii* isolates from 13 powdered infant formula products, 25 powdered weaning diet products, and 33 weaning diet ingredients on polymerse chain reaction (PCR) methods. The numbers of the isolates from 1 powdered infant formula product, 7 powdered weaning diet products, and 6 weaning diet ingredients were 1, 14, and 8, respectively. The contaminated ingredients were 1 rice powder, 2 millet powders, 2 vegetable powders, and 1 fruit and vegetable premix. PCR with the primer of repetitive extragenic palindromic element (REP-PCR) and random amplification of polymorphic DNA(RAPD) were effective in discriminating among the isolates, but tRNA-PCR and PCR with the primer of 16S-23S internal transcribed spacer (ITS-PCR) were not. Some of *E. sakazakii* isolates from vegetable powders, fruit and vegetable premix, and millets powders were classified into the clonal groups based on the DNA patterns in the REP-PCR and RAPD analysis. A close genetic relationship among the isolates from some of the powdered weaning diet products and the rice powder was also detected in the cluster analysis based on the DNA patterns in RAPD.

Keywords: *Enterobacter sakazakii*, infant formula, weaning diet, polymerase chain reaction (PCR)

Introduction

Enterobacter sakazakii is recognized as a pathogen causing life-threatening infections in infants. This microorganism causes meningitis, septicemia, and necrotizing enterocolitis in infants. Premature infants, low-birth-weight infants, and those aged <28 days are considered to be more susceptible to the infections than older infants. The infections frequently result in death (1). Some epidemiological studies of clinical cases have found that powdered infant formula products contaminated with *E. sakazakii* were linked to the infections (2).

The powdered infant formula products and the powdered weaning diet products whose major ingredients are milk and cereal, respectively, are not sterile products, because the powder is exposed to the environment such as dust, insects, and humans during the manufacture in factories. In addition, the powdered weaning diet products have greater chances of microbial contamination from their ingredients, such as cereal powder, fruit powder, vegetable powder, and other supplements which are combined by dry mixing. Quality control of the weaning diet ingredients and sanitation during production of the powdered weaning diet products should be carefully monitored in order to prevent contamination and multiplication of *E. sakazakii* (3).

E. sakazakii belongs to the family Enterobacteriaceae and the genus *Enterobacter* and is a Gram-negative rod which is motile due to peritrichous fimbria. The bacteria produce a characteristic yellow pigment on agar, which enables differentiation of *E. sakazakii* from *Enterobacter cloacae*. *E. sakazakii* isolates from food, clinical case, and environment have been grouped based on antibiogram, phenotypic characteristics including biochemical reactions, ribotyping, random amplification of polymorphic DNA (RAPD), pulsed-field gel electrophoresis (PFGE), and amplified fragment length polymorphism (4-6). Drudy *et al.* (4) reported that RAPD was useful to discriminate among *E. sakazakii* isolates from infant formula. The best typing procedures to differentiate among *E. sakazakii* isolates from clinical cases and infant formula were suggested to be RAPD and PFGE followed by ribotype, biotype, and antibiograms (6). Recently, the DNA sequence analysis of 16S rDNA gene revealed that *E. sakazakii* stains consisted of heterogeneous members of bacteria. It was suggested that the strains should be classified into several species in the newly-named genus *Cronobacter* (5,7).

In this study, we isolated and identified *E. sakazakii* from powdered weaning diet products, weaning diet ingredients, and powdered infant formula products. We analyzed molecular typing patterns of the isolates using polymerase chain reaction (PCR) with the primer of tRNA intergenic spacer (tRNA-PCR) (8), PCR with the primer of 16S-23S internal transcribed spacer (ITS-PCR) (9), and PCR with the primer of repetitive extragenic palindromic element (REP-PCR) (10), and RAPD method.

Materials and Methods

Powdered weaning diet products, weaning diet ingredients, and powdered infant formula products Twenty-five powdered weaning diet products for the babies more than 6-month-old and 13 powdered infant formula products for the babies less than 12-month-old were purchased from grocery stores. Thirty-three weaning diet ingredients were obtained from the company which manufactured powdered weaning diet products.

Microorganisms *Enterobacter aerogenes* KCCM 12177

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was purchased from Korean Culture Center of Microorganisms. *E. cloacae* KCTC 2361 and *E. sakazakii* KCTC 2949 were purchased from the Biological Resources Center in Korea.

Isolation and identification of *E. sakazakii* *E. sakazakii* was isolated from powdered weaning diet products, weaning diet ingredients, and powdered infant formula products, following the procedure described by U.S. Food and Drug Administration (FDA) (11). The samples of powdered weaning diet products and powdered infant formula products in amounts of 100, 10, and 1 g were reconstituted into 900, 90, and 9 mL of sterile distilled water, respectively, in triplicate. The samples of weaning diet ingredients were reconstituted into sterile buffered peptone water (12). The dispersed solutions were incubated at 35°C for 24 hr for pre-enrichment of *E. sakazakii.* The cultures (10 mL) were transferred into 90 mL of EE broths (11) and the mixtures were incubated at 35°C for 24 hr. The cultures were inoculated on violet red bile glucose agar (Oxoid, Cambridge, UK) and incubated at 35°C for 24 hr. The mucous purple-colored colony was selected and transferred onto Tryptic soy agar (Oxoid), which was incubated at 25°C for 48 hr. The yellow-pigmented colony was selected for identification. API 20E (bioMerieux, Marcy-l'Etoile, France), cytochrome oxidase, motility test, and OF-glucose fermentation/oxidation test (13) were used for identification.

DNA isolation Tryptic soy agar (Oxoid) was inoculated and incubated at 37°C for 24 hr. The colonies were suspended in saline solution. The turbidity of cell suspension was adjusted to McFarland 4. The cell suspension (83 μL) was placed in a microtube and centrifuged at 7,000×g for 5 min. The supernatant was removed and the pellet was resuspended into of 5% Chelex resin (Bio-Rad, Hercules, CA, USA) suspension (200 μL) and 10 mg/mL proteinase K (10 μL) was added. The mixture was stirred briefly, incubated at 56°C for 30 min with several vortex, and then at 100°C for 8 min. The mixture was centrifuged at 7,000 ×g for 5 min. The supernatant (100 μL) containing DNA was taken and stored in freezer.

tRNA-PCR and ITS-PCR The primers used in tRNA-PCR were T5A (5'-AGTCCGGTGCTCTAACCAACTGAG-3') and T3B (5'-AGGTCGCGGGTTCGAATCC-3') (8). The tRNA-PCR synthesizes DNA spacer between tRNA genes. The primers used in ITS-PCR were L1 (5'-CAAG GCATCCACCGT-3') and G1 (5'-GAAGTCGTAACAAGG-3') (9). The primer sequences were designed based on conserved DNA region in the genes of 16S rRNA and 23S rRNA. The ITS-PCR synthesizes the DNA spacer between the two genes. The DNA samples (1 mL) and 12.5 pmol primers were added to the PCR premix (Bioneer, Daejeon, Korea) and the reaction mixture was made up to 20 mL with sterile distilled water. The PCR premix contained 1 U Taq polymerase, 5 nmol NTP, 200 nmol Tris-HCl (pH 9.0), 800 nmol KCl, and 20 nmol $MgCl_2$. The reaction consisted of denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and elongation at 72°C for 1 min, and then elongation at 72°C for 10 min. The resultant reaction mixture was electrophoresed using 2% agarose gel in Tris-borate buffer.

REP-PCR The primers in REP-PCR were REP1R-Dt (5'-IIINCGNCGNCATCNGGC-3') (N=A, T, C or G; I=iosine) and REP2R-Dt (5'-NCGNCTTATCNGGCCTAC-3'), respectively (10). REP-PCR synthesizes DNA between repetitive extragenic palindromic element (14). Ten pmol primer, 25 mM $MgCl_2$ (1.2 μL), and DNA (1 μL) were added to the PCR Premix and the reaction mixture was made up to 20 μL with sterile distilled water. The reaction condition consisted of denaturation of genomic DNA at 95°C for 7 min, 35 cycles of denaturation at 90°C for 30 sec, annealing at 40°C for 1 min, and elongation at 65 for 1 min, and then elongation at 65°C for 10 min. The resultant reaction mixture was electrophoresed using 2% agarose gel in Tris-borate buffer.

RAPD The primers used in RAPD were OPC-13 (5'-AAGCCTCGTC-3') and OPC-19 (5'-GTTGCCAGCC-3') (Operon, Cologne, France). Eight pmol primer, 25 mM $MgCl_2$ (1.2 μL), and DNA (1 μL) were added to the PCR premix and the reaction mixture was made up to 20 μL with sterile distilled water. The reaction condition consisted of denaturation at 94°C for 7 min, 45 cycles of denaturation at 94°C for 1 min, annealing at 37°C for 1 min, elongation at 72°C for 1 min and then elongation at 72°C for 4 min. The resultant reaction mixture was electrophoresed using 2% agarose gel in Tris-borate buffer.

Cluster analysis Clustering pattern of the *E. sakazakii* isolates based on the combined DNA patterns from the RAPD analysis with the primers OPC-13 and OPC-19 was analyzed using unweighted pair-group method of arithmetic averages in SAS version 9.1. Average distances between clusters were described in the dendrogram obtained using Tree procedure in the SAS.

Results and Discussion

Isolation and identification of *E. sakazakii* Thirteen powdered infant formula products, 25 powdered weaning diet products, and 33 samples of weaning diet ingredients as shown in Table 1 were examined for *E. sakazakii* by using pre-enrichment, selective enrichment in EE broth, and sequential selection of colonies on violet red bile glucose agar and tryptic soy agar. The 51 isolates forming yellow-pigmented colonies on tryptic soy agar were selected for further study. Identification of the isolates was based on biochemical reactions using API 20E. Finally, 23 isolates were identified as *E. sakazakii.* The remaining 28 isolates which were not identified as *E. sakazakii* included 15 isolates of *Pantoea* spp. as well as other isolates of *Leclercia adecarboxylata*, *Citrobacter braakii*, *E. cloacae*, and *Escherchia hermannii.*

The percentages of the detected samples of powdered infant formula products, powdered weaning diet products, and weaning diet ingredients were calculated as 7, 28, and 18%, respectively (Table 1). The average most probable numbers (MPN)/kg of the detected samples of 1 powdered infant formula product, 7 powdered weaning diet products, and 6 weaning diet ingredients were 3.6, 6.2, and 5.0, respectively. The detected samples of weaning diet ingredients included 1 pregelatinized rice powder, 2

Table 1. Isolation of *E. sakazakii* from powdered infant formula products, powdered weaning diet products, and weaning diet ingredients

Sample classification	No. of examined sample	No. of detected sample	MPN[1]/kg of detected sample	No. of *E. sakazakii* isolates
Powdered infant formula	13	1	3.6	1
Powdered weaning diet	25	7	6.2±3.4	14
Weaning diet ingredient	33	6	5.0±2.3	8
Pregelatinized rice powder	4	1	9.2	2
Pregelatinized millet powder	3	2	3.6±0.0	2
Vegetable powder	8	2	3.6±0.0	2
Fruit and vegetable premix	1	1	6.1	2
Fruit powder	5	0	-	0
Milk powder	2	0	-	0
Meat powder	2	0	-	0
Egg yolk powder	1	0	-	0
Soy protein isolate	1	0	-	0
Sugar	3	0	-	0
Mineral	3	0	-	0
Total	71	14	5.5±2.9	23

[1]Most probable numbers.

Table 2. Biochemical identification and genotyping of *E. sakazakii* isolates based on API 20E, tDNA-PCR, REP-PCR, and RAPD with the primers of OPC-13 and OPC-19

Isolate	Source	API 20E (%Id)	tDNA-PCR	REP-PCR	RAPD[1]	
					OPC-13	OPC-19
A-1	Weaning diet 1	98.4	T-1	R-1	O13-1	O19-1
A-2	"	98.4	T-1	R-1	O13-2	O19-2
B-1	Weaning diet 2	98.4	T-1	R-2	O13-3	O19-3
B-2	"	98.1	T-2	R-3	O13-4	O19-4
C-1	Weaning diet 3	98.4	T-1	R-2	O13-3	O19-5
C-2	"	98.4	T-1	R-1	O13-5	O19-6
D-1	Rice powder	98.4	T-1	R-1	O13-5	O19-7
D-2	"	98.4	T-1	R-2	O13-3	O19-8
E	Vegetable powder 1	98.4	T-2	R-4	O13-6	O19-9
F	Vegetable powder 2	98.4	T-1	R-5	O13-7	O19-10
G-1	Fruit and vegetable premix	98.4	T-2	R-4	O13-6	O19-9
G-2	"	51.9	T-3	R-6	O13-8	O19-11
H	Millet powder 1	51.9	T-3	R-6	O13-8	O19-11
I	Millet powder 2	51.9	T-3	R-6	O13-8	O19-11
J-1	Weaning diet 4	98.4	T-1	R-7	O13-9	O19-12
J-2	"	98.4	ND[2]	R-7	O13-9*	O19-12*
J-3	"	98.4	ND	R-7	O13-9*	O19-12*
K-1	Weaning diet 5	98.4	T-1	R-8	O13-10	O19-13
K-2	"	98.4	ND	R-8	O13-10*	O19-13*
L-1	Weaning diet 6	98.4	T-1	R-9	O13-11	O19-14
L-2	"	98.4	T-1	R-9	O13-11	O19-14
M	Weaning diet 7	51.9	T-1	R-10	O13-12	O19-9
N	Infant formula	98.4	T-1	R-11	O13-13	O19-15

[1]*Results not shown in Fig. 3.
[2]Not determined.

pregelatinized millet powders, 2 vegetable powders, and 1 fruit and vegetable premix, which showed the MPN/kg of 9.2, 3.6, 3.6, and 6.1, respectively. *E. sakazakii* was not isolated from fruit powders, milk powders, meat powders, egg yolk powder, and soy protein isolate, sugars, and minerals. The numbers of *E. sakazakii* isolates obtained from the detected samples of the powdered infant formula product, the powdered weaning diet products, and the

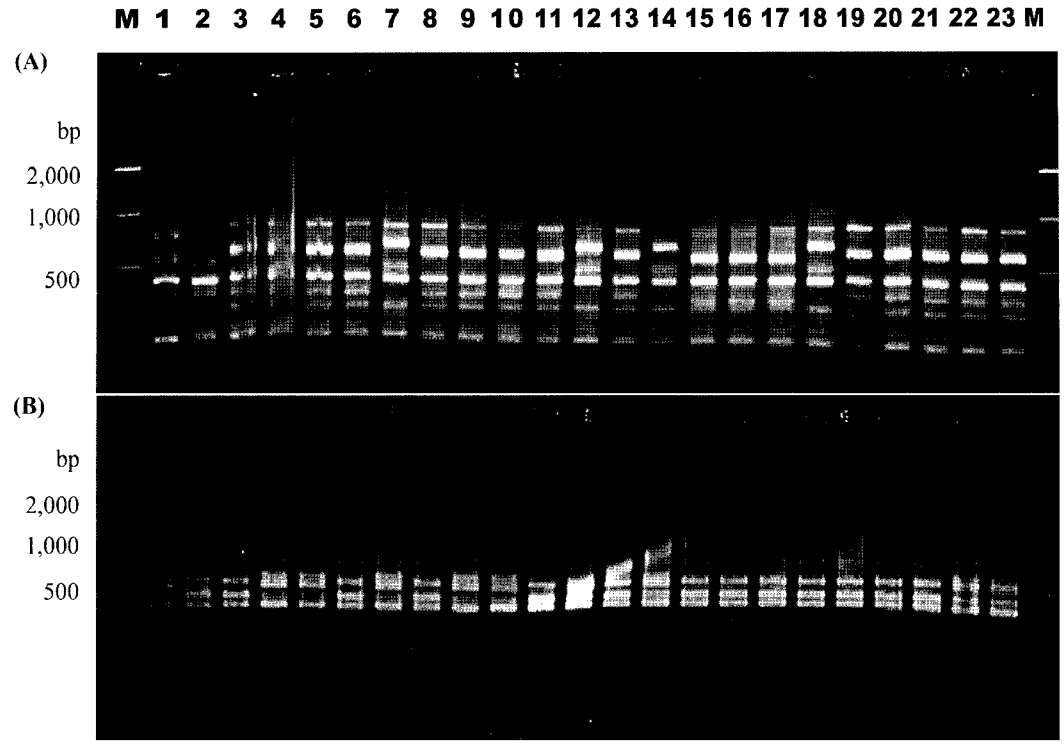

Fig. 1. tRNA-PCR (A) and ITS-PCR (B) of *E. sakazakii* isolates from powdered weaning diet products, weaning diet ingredients, and powdered infant formula products. M, DNA marker; 1, *E. aerogenes* KCCM 12177; 2, *E. cloacae* KCTC 2361; 3, *E. sakazakii* KCTC 2949; 4, A-1; 5, A-2; 6, B-1; 7, B-2; 8, C-1; 9, C-2; 10, D-1; 11, D-2; 12, E; 13, F; 14, G-1; 15, G-2; 16, H; 17, I; 18, J-1; 19, K-1; 20, L-1; 21, L-2; 22, M; 23, N.

weaning diet ingredients were 1, 14, and 8, respectively (Table 1).

The numbers of isolates with %Id in API 20E of 98.4, 98.1, and 51.9% were 18, 1, and 4, respectively (Table 2). *E. sakazakii* isolates with %Id of 98.4% in API 20E were positive for β-galactosidase, arginine dihydrolase, ornithine decarboxylase, acetoin production, fermentation and oxidation of glucose, mannose, inositol, rhamnose, sucrose, melibiose, and arabinose, NO_2 production, motility, MacConkey medium growth, OF-glucose fermentation and oxidation and negative in lysine decarboxylase, citirate utilization, H_2S production, urease, trytophane deaminase, indole production, gelatinase, sorbitol, cytochrome oxidase, and N_2 gas reduction. The isolates with %Id of 98.1% were positive in ornithine decarboxylase and the isolates with %Id of 51.9% were positive in citrate utilization and negative in oxidation and fermentation of inositol, which were different from the characteristics of the isolates with %Id of 98.4%.

tRNA-PCR and ITS-PCR The DNA polymorphism in the tRNA-PCR of the isolates was investigated (Fig. 1A). The DNA patterns in the tRNA-PCR were different in numbers and sizes of bands with several common bands among the type strains of *E. aerogenes* KCCM 12177, *E. cloacae* KCTC 2361, and *E. sakazakii* KCTC 2949. The DNA patterns in the tRNA-PCR of *E. sakazakii* KCTC 2949 and the isolates were relatively similar to each other, and consisted of 6 to 7 DNA bands distributed between 100 and 900 bp. Based on the DNA patterns in the tRNA-PCR, the isolates were classified into 3 groups from T-1 to T-3 as shown in Table 2. Although *Enterobacter* species could be discerned using the tRNA-PCR, it was an inferior method to differentiate among *E. sakazakii* isolates. The DNA patterns in the ITS-PCR consisted of 3 to 4 DNA bands, which were of the sizes between 400 and 800 bp and showed similar patterns among *E. aerogenes* KCCM 12177, *E. cloacae* KCTC 2361, and *E. sakazakii* KCTC 2949, and the isolates (Fig. 1B). The results showed that the ITS-PCR was able to differentiate neither among *E. sakazakii* strains nor among *Enterobacter* species.

REP-PCR and RAPD The DNA patterns in the REP-PCR showed 9 to 14 DNA bands and were distinctive, easily discernable, and widely variable among the *E. sakazakii* isolates (Fig. 2), which were classified into 11 groups from R-1 to R-11 (Table 2). The distinctive DNA patterns in the REP-PCR made it easy to classify the isolates into the clonal groups and suggested that the groups seemed to have independent genetic make-ups regarding the distribution of repetitive extragenic palindromic elements in the genome. The RAPD analysis with OPC-13 primer yielded 4 to 7 DNA bands for each isolates and showed a couple of common DNA bands in the size range of 500 and 700 bp (Fig. 3A) among all the isolates. The isolates were classified into 13 groups from O13-1 to O13-13 (Table 2). The RAPD analysis with OPC-19 primer (Fig. 3B) showed 5 to 9 discernible DNA bands for each isolates and enabled classification of the isolates into 15 groups from O19-1 to O19-15 (Table 2). There was a DNA band of 680 bp common among all the isolates. The RAPD with OPC-19 primer enabled classification the isolates into more groups and seemed to be more discriminating than the REP-PCR and the RAPD analysis with OPC-13.

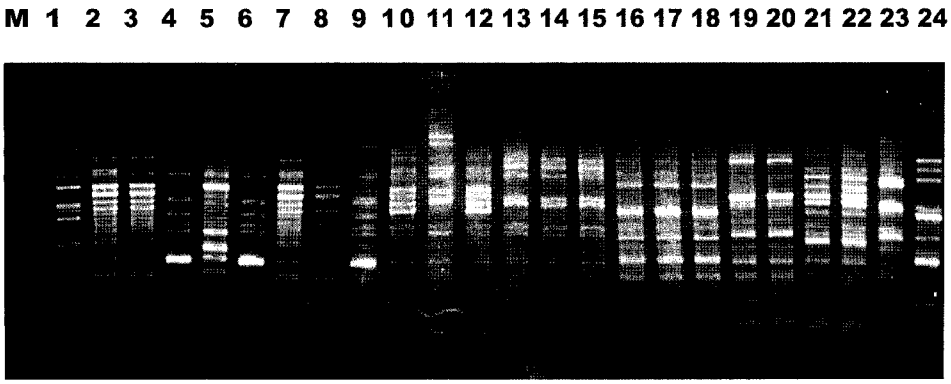

Fig. 2. REP-PCR of *E. sakazakii* isolates from powdered weaning diet products, weaning diet ingredients, and powdered infant formula products. M, DNA marker; 1, *E. sakazakii* KCTC 2949; 2, A-1; 3, A-2; 4, B-1; 5, B-2; 6, C-1; 7, C-2; 8, D-1; 9, D-2; 10, E; 11, F; 12, G-1; 13, G-2; 14, H; 15, I; 16, J-1; 17, J-2; 18, J-3; 19, K-1; 20, K-1; 21, L-1; 22, L-2; 23, M; 24, N.

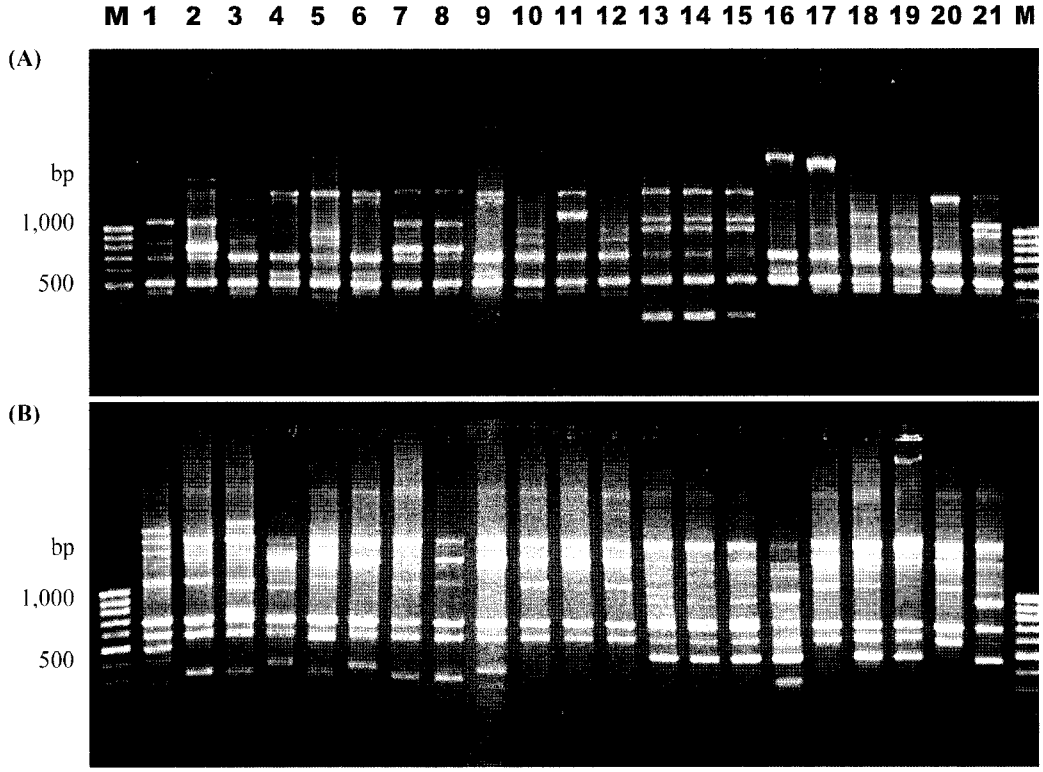

Fig. 3. RAPD analyses of *E. sakazakii* isolates from powdered weaning diet products, weaning diet ingredients, and powdered infant formula products. A, OPC-13; B, OPC-19. M, DNA marker; 1, *E. sakazakii* KCTC 2949; 2, A-1; 3, A-2; 4, B-1; 5, B-2; 6, C-1; 7, C-2; 8, D-1; 9, D-2; 10, E; 11, F; 12, G-1; 13, G-2; 14, H; 15, I; 16, J-1; 17, K-1; 18, L-1; 19, L-2; 20, M; 21, N.

According to the REP-PCR in Fig. 2, the isolates A-1, A-2, C-2, and D-1 from the weaning diets 1 and 3 from and the rice powder and the isolates B-1, C-1, and D-2 from the weaning diets 2 and 3 and the rice powder were into the clonal groups R-1 and R-2, respectively (Table 2). RAPD analysis with the primer OPC-13 (Fig. 3A) enabled classification of the isolates B-1, C-1, and D-2 from the weaning diets 2 and 3 and the rice powder and the isolates C-2 and D-1 from the weaning diet 3 and the rice powder into the clonal groups O13-3 and O13-5, respectively (Table 2). Cluster analysis (Fig. 4) based on RAPD analyses with the primers of OPC-13 and OPC-19 (Fig. 3) showed the group of isolates C-1, and D-2 from the weaning diet 3 and the rice powder showed average distances lower than 0.5 in cluster analysis. These results showed that the isolates from the powdered weaning diet products and the rice powder were genetically closely linked each other and thus suggested that the isolates from the powdered weaning diet products might have been originated from the rice powder.

Both REP-PCR (Fig. 2) and RAPD analyses (Fig. 3) enabled classification of the isolates E and G-1 from the vegetable powder 1 and the fruit and vegetable premix and the isolates G-2, H, and I from the fruit and vegetable premix and the millet powders 2 and 3 into the clonal groups, respectively (Table 2). These results suggested that some of the weaning diet ingredients might have been cross-contaminated each other or contaminated from the same sources. The isolates J-1, J-2, and J-3 from the weaning diet 4 and the isolates K-1 and K-2 from the

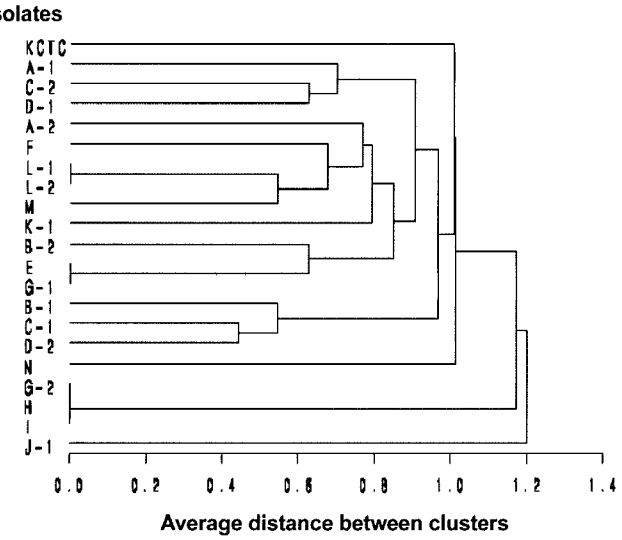

Fig. 4. Cluster analysis of *E. sakazakii* isolates based on the DNA patterns from RAPD analyses with the primers of OPC-13 and OPC-19.

weaning diet 5, and the isolates L-1 and L-2 from the weaning diet 6 were classified into the clonal groups (Table 2), respectively, which suggested that the isolates might have resulted from either multiplication of a bacterial cell during the manufacture of the powdered weaning diet products and their ingredients at factories or during the experiment to isolate *E. sakazakii* from the powdered weaning diet products in this study.

Kandhai *et al.* (14) reported that environmental samples from various food factories manufacturing milk powder, chocolate cereal, potato flour, pasta, and households contained *E. sakazakii*, suggesting its widespread presence in the environment. *E. sakazakii* has also been isolated from milk powders, cheese products, infant foods, minced beef, sausage meat, and vegetables (15). An industry survey reported that starch was most frequently contaminated among the ingredients used for powdered infant formula (3). Jung and Park (16) reported that *E. sakazakii* strains were isolated from unprocessed agricultural products, such grain, laver, vegetables but not from fruits.

The present study showed that the powdered weaning diet products might be more susceptible than powdered infant formula products to contamination with *E. sakazakii* from their ingredients as shown in Table 1. Contamination of powdered weaning diet products might occur during dry mixing of their powdered ingredients, since there was no pasteurization process after dry mixing. However, the ingredients for the powdered infant formula products are usually reconstituted into either water or milk and then the mix is pasteurized to prevent cross-contamination from the ingredients before spray drying.

Yoo *et al.* (17) isolated three *E. sakazakii* isolates from 25 g of samples of 3 infant formula products among 45 products manufactured in Korea, which showed detection rate of 6.7%. The percentage of the detected products shown in Table 1 seemed to be higher than those reported in the previous studies (3,16,17). This may be due to the total amount (333 g) of the examined samples in this study.

The present study showed that tRNA-PCR and ITS-PCR were not useful to discriminate *E. sakazakii* strains. Clementino *et al.* (9) showed that *E. sakazakii*, *E. aerogenes*, and *E. cloacae* could be differentiated using tRNA-PCR, but the strains of *E. cloacae* could not be discriminated. Welsh and McClelland (8) showed that the fingerprints obtained from tRNA-PCR were largely conserved within the species. The fingerprinting with tRNA-PCR did not uncover differences between strains but between species of streptococci. Clementino *et al.* (9) reported that ITS-PCR made it possible to discern among *Enterobacter* species as well as among *E. cloacae* strains. However, Daffonchio *et al.* (18) reported that all the species of *Bacillus cereus* showed practically identical fingerprints in ITS-PCR.

It was reported that REP-PCR could be used to discriminate eubacteria, particularly Gram-negative bacteria at the levels of species and strains (19). REP-PCR primers were designed complementary to interspersed palindromic repetitive sequences. PCR amplifies differently sized DNA fragments consisting of unique DNA sequences lying between these palindromic repeats. Georghiou *et al.* (20) identified hospital outbreak-associated strains of *E. aerogenes* by using REP-PCR. They concluded that genomic fingerprinting with REP-PCR might be useful techniques for the epidemiological typing of *E. aerogenes*, providing with ease, speed, and broad species applicability. Stumpf *et al.* (21) suggested that at the strain level, REP-PCR was a better method to differentiate *E. cloacae* than ERIC-PCR. However, *Clostridium botulinum* could be differentiated at the group level by using REP-PCR (9). In our study, REP-PCR resulted in various DNA patterns distinctive among the *E. sakazakii* isolates. Thus, classification of the isolates into the clonal groups based on REP-PCR was simple and straightforward and the number of the clonal groups from REP-PCR was comparable with those from RAPD.

Previous studies showed that RAPD could discriminate *E. sakazakii* at the strain level (4,6,17). However, RAPD was known to have shortcomings in the lack of reproducibility due to the short length of its primer and the low annealing temperature in PCR. We could have improved it by keeping the reaction mixtures cold in ice and moving them into heating block just after its temperature reached to 94°C (results not shown). Drudy *et al.* (4) used RAPD method to differentiate 51 *E. sakazakii* environmental and food isolates. There were 3 major clusters of 17 *E. sakazakii* isolates and remaining 34 isolates. They maintained that RAPD was a useful molecular tool providing rapid analysis of isolates in a local context. Nazarowec-White and Farber (6) reported that RAPD and PFGE were the most discriminatory typing schemes for *E. sakazakii*, followed by ribotyping, biotyping, and antibiograms. Our study also showed that *E. sakazakii* isolates could be classified using RAPD. RAPD with the pertinent primers and REP-PCR could be good methods for discrimination of *E. sakazakii* at the strain level.

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