Food Sci. Biotechnol. Vol. 14, No. 6, pp. 875 ~ 877 (2005)

RESEARCH NOTE

Food Science and Biotechnology

© The Korean Society of Food Science and Technology

Isolation and Genotyping of *Enterobacter sakazakii* from Powdered Infant Formula Manufactured in Korea

Mi Kyung Yoo, Suk Shin Kim[1] and Sangsuk Oh*

Department of Food and Nutritional Sciences, Ewha Womans University, Seoul 120-750, Korea
[1]Department of Food and Nutrition, Catholic University, Bucheon, Gyeonggi 420-743 Korea

Abstract Presence of *Enterobacter sakazakii*, occasional pathogen of powdered infant formula causing rare, but life-threatening diseases such as neonatal meningitis, bacteremia, necrotizing enterocolitis, and necrotizing meningoencephalitis after ingestion was examined in 45 powdered infant formula products manufactured in Korea using chromogenic Druggan-Forsythe-Iversen (DFI) medium, and isolates were identified with API 20E. *Ent. sakazakii* was isolated from three products. *Ent. sakazakii* isolates were genotyped by RAPD-PCR using two random primers, and their banding patterns were compared.

Keywords: *Enterobacter sakazkaii*, powdered infant formula, DFI medium, isolation, RAPD-PCR

Introduction

Enterobacter sakazakii, a bacterium belonging to the family of Enterobacteriaceae, is a motile, non-spore forming, gram-negative, facultative anaerobe rod (1), and, until designated as a new species in 1980, was known as 'yellow pigmented *Enterobacter cloacae*' (2). It is an occasional pathogen of powdered infant formula that can cause rare, but life-threatening diseases such as neonatal meningitis, bacteremia, necrotizing enterocolitis (NEC), and necrotizing meningoencephalitis (3). Recently, the International Commission on Microbiological Specifications for Foods (ICMSF) has ranked *Ent. sakazakii* as 'Severe hazard for restricted populations, life-threatening or substantial chronic sequelae of long duration' (4), in the same ranking as with those of more familiar food- and waterborne pathogens such as *L. monocytogenes*, *C. botulinum* types A and B, and *C. parvum*. Although *Ent. sakazakii* has been isolated from a wide range of foods including cheese, meat, vegetables, grains, herbs, and spices (1), powdered infant formula is both the vehicle and the direct/indirect source of 50-80% cases of diseases induced from *Ent. sakazakii* (5). Contamination of even low levels of *Ent. sakazakii* in powdered infant formula is a risk factor, given the opportunity for multiplication of the pathogen during preparation and holding time before consumption of the formula (6). For infants not able to breast-feed, infant formula may be the only source of nutrients (7), and those at greatest risk for *Ent. sakazakii* infection are neonates (first 28 days), pre-term, low birth weight, and immunocompromised infants, and infants of HIV-positive mothers (5).

Therefore, it is of utmost importance to be able to detect contamination of the powdered infant formula by *Ent. sakazakii*. In this study, we examined the presence of *Ent. sakazakii* in powdered infant formula samples manufactured in Korea, and, to estimate the general hygienic state of infant formula, other members of the family Enterobacteriaceae were also surveyed.

*Corresponding author: Tel: 82-2-3277-3558; Fax: 82-2-3277-2862
E-mail: ssoh71@ewha.ac.kr
Received September 2, 2005; accepted September 30, 2005

Materials and Methods

Powdered infant formula (PIF) sample selection The presence of *Ent. sakazakii* and other Enterobacteriaceae members was monitored in 45 powdered infant formula samples of four domestic manufacturers. The samples were purchased from retailers located in Seoul and Gyeonggi-do.

Isolation of *Ent. sakazakii* from PIF using Druggan-Forsythe-Iversen (DFI) medium Twenty-five grams of each powdered infant formula was dissolved into 225 mL buffered peptone-water (Oxoid Ltd., Basingstoke, Hampshire, England) and incubated overnight at 37°C. Ten milliliters of pre-enriched buffered peptone-water was added to 90 mL EE broth (Oxoid Ltd.) and incubated overnight at 37°C. Incubated EE broth was then streaked onto DFI medium (Oxoid Ltd.), a chromogenic medium specific to *Ent. Sakazakii*, and incubated overnight at 37 °C. The presumptive *Ent. sakazakii* colonies, appearing entirely blue-green on the DFI medium, as well as non blue-green colonies to identify other Enterobacteriaceae, were identified using API 20E (bioMérieux, Marcy l'Etoile, France). *Ent. sakazakii* KCTC 2949 strain was used as a positive control.

DNA extraction of *Ent. sakazakii* isolates originated from PIF All isolates and *Ent. sakazakii* KCTC 2949 were grown overnight on nutrient broth (Difco, Le Pont de Claix, France) at 37°C, and streaked onto nutrient agar (Difco) plates. Approximately two loops of biomass were scrapped off the agar plates, suspended in 100 μL sterile distilled water, and boiled for 10 min. After centrifugation at 12,000×g for 10 min at 4°C, the supernatants were used as the templates for randomly amplified polymorphic DNA (RAPD)-PCR to genotype the *Ent. sakazakii* isolates. (8).

RAPD-PCR amplification Amplification was performed in 25 μL reaction volumes containing High Fidelity 10X PCR Buffer with $MgCl_2$ (Applied Biosystems, Foster City, CA, USA), 200 μM dNTP mixture (TaKaRa Biotechnology Co., Inc., Otsu, Shiga, Japan), 2.5 units High Fidelity enzyme mix (Applied Biosystems), 1 μL (10 pmole/μL) random primer (Genomine, Pohang, Gyeongsangbuk-do, Korea), and 25 ng DNA each of *Ent. sakazakii* isolates and *Ent. sakazakii* KCTC 2949. The sterile distilled water was substituted for the template as a negative control. UBC 245 (5'-CGC GTG CCA G-3') and UBC 282 (5'-GGG AAA GCA G-3') were used as the random primers of each PCR amplification (9). PCR cycling consisted of 1 cycle of 2 min at 94°C, followed by 40 cycles of 1 min at 94°C for denaturation, 1 min at 35°C for annealing (ramp time from 35 to 72°C was 2 min), 1.5 min at 72°C for polymerization, and 1 cycle of 5 min at 72°C for an additional extension (9). All amplifications were performed in a thermal cycler (Biometra, Goettingen, Germany).

Agarose gel electrophoresis Twenty microliters of the PCR products were loaded on 1.5% agarose gels containing 0.05 μg/mL ethidium bromide in 1×TBE buffer. DNA ladder (BioLabs Inc., Ipswich, MA, USA) was also loaded on gels as a molecular size marker. The PCR products were separated by electrophoresis at 50 V for 40 min. Subsequently, the gels were photographed by digital gel documentation system (Korea Bio-Tech Co., Ltd., Seongnam, Gyeonggi-do, Korea).

Results and Discussion

Isolation of *Ent. sakazakii* and other Enterobacteriaceae from PIF *Ent. cloacae* was the most frequently isolated organism from powdered infant formula. In addition to *Ent. cloacae*, *K. oxytoca*, and *C. freundii*, which are involved in the necrotization of enterocolitis, the most common gastrointestinal illness in newborns, other members of Enterobacteriaceae in the powdered infant formulas manufactured in Korea may also cause Enterobacterial infections (Table 1) (4). Enterobacterial infections are disorders of the digestive tract, and species of *Salmonella*, *Shigella*, *Klebsiella*, *Enterobacter*, *Serratia*, *Proteus*, and *Yersinia* are the groups responsible for the infections. *Klebsiella* and *Proteus* sometimes cause pneumonia, ear infections, and urinary tract infections. *Enterobacter* and *Serratia* often cause bacteremia, particularly in patients with weakened immune system. Enterobacteriaceae that have been identified in infants with necrotizing enterocolitis (NEC) are *Salmonella*, *E. coli*, *Klebsiella*, and *Enterobacter* (10).

Ent. sakazakii was isolated from 3 out of 45 powdered infant formula samples, similar to the result of Iversen C (4), who reported that *Ent. sakazakii* was isolated from 2 among 82 powdered infant formula samples. Because powdered infant formula is not a sterilized product and the newborn infant has a weak immune system, a high level of microbiological quality control of powdered infant formula must be ensured. However, results of the present

Table 1. *Ent. sakazakii* and other Enterobacteriaceae isolated from powdered infant formula (PIF) samples manufactured in Korea

Organisms	The number of PIF samples (%)
Acinetobacter baumannii/calco	1/45 (3%)
Cedecea davisae	1/45 (3%)
Citrobacter freundii	2/45 (5%)
Cryseomonas luteola	2/45 (5%)
Enterobacter amnigenus	1/45 (3%)
Enterobacter cloacae	12/45 (30%)
Enterobacter sakazakii	3/45 (7%)
Escherichia hermannii	1/45 (3%)
Escherichia vulneris	1/45 (3%)
Flavimonas oryzihabitans	1/45 (3%)
Klebsiella oxytoca	1/45 (3%)
Micrococcus spp.	1/45 (3%)
Pantoea spp.	1/45 (3%)
Pasteurella pneumotropica/haemolytica	1/45 (3%)
Shigella spp.	1/45 (3%)

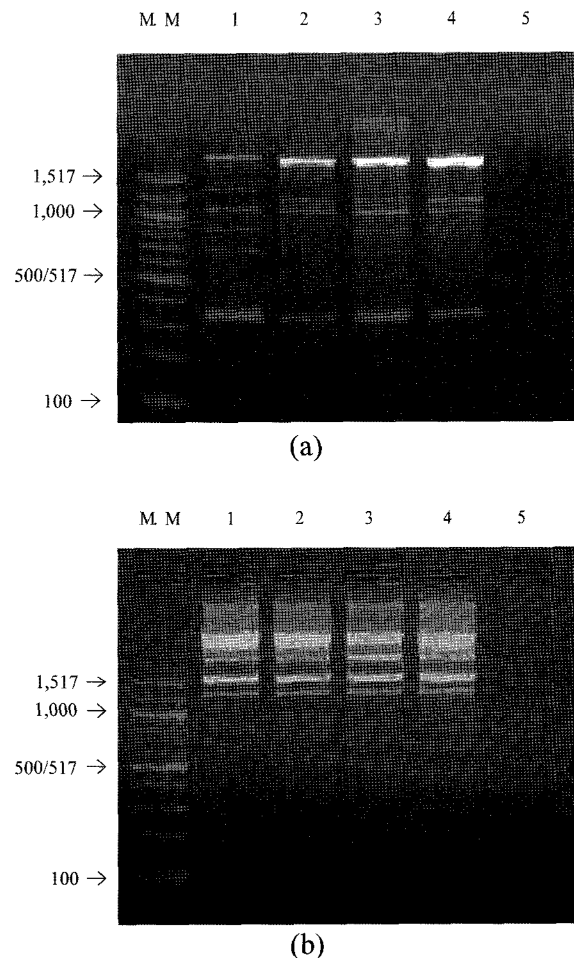

Fig. 1. RAPD-PCR patterns of *Ent. sakazakii* isolated from powdered infant formula. a: UBC 245 as a primer, b: UBC 282 as a primer; Lane M. M: DNA ladder, Lane 1: *Ent. sakazakii* KCTC 2949, Lane 2: *Ent. sakazakii* isolate from sample 1, Lane 3: *Ent. sakazakii* isolate from sample 2, Lane 4: *Ent. sakazakii* isolate from sample 3, Lane 5: negative control

study revealed that powdered infant formulas manufactured in Korea are not safe from microbiological contamination and should be monitored for the presence of *Ent. sakazakii.*

Genotyping of *Ent. sakazakii* strains isolated from PIF Among the many biochemical and molecular techniques that can be used for distinguishing isolates of a given bacterial species, RAPD is being used increasingly to type microorganisms, because, compared to other typing methods, it is rapid, relatively inexpensive, technically feasible for most laboratories, and theoretically applicable to all organisms (11). In the present study, RAPD-PCR was conducted with two different random primers, UBC 245 and UBC 282, previously described by Farber JM (9). UBC 245 gave distinguishable banding patterns, 5-10 amplified PCR bands ranging from 300 to ~2,000 bp (Fig. 1a). Banding patterns of *Ent. sakazakii* isolates from samples 1 and 3 were similar. On the other hand, UBC 282 gave indistinguishable banding patterns, about five amplified PCR bands up to 1,200 bp (Fig. 1b). The three isolates of *Ent. sakazakii* from samples were genetically homogeneous when amplified with the primer UBC 282.

Ent. sakazakii is more thermotolerant than most other Enterobacteriaceae, which makes it possible to survive heat treatments (12). D-values of *Ent. sakazakii* were 0.6 ± 0.3 min at 65°C and 0.07 min at 70°C (13). In another study, when the dried infant formula was rehydrated with water at 70°C, the *Ent. sakazakii* survivors were under the lower limit of detection (14). Therefore, to minimize the infection of *Ent. sakazakii* and other microorganisms, water must be boiled and cooled to 70°C before adding the powdered product, and the formula fed to the infants immediately after preparation.

References

1. Iversen C, Forsythe S. Risk profile of *Enterobacter sakazakii*, an emergent pathogen associated with infant milk formula. Trends Food Sci. Technol. 14: 443-454 (2003)
2. Farmer JJ, Asbury MA, Hickman FW, Brenner DJ the Enterobacteriaceae Study Group (USA). *Enterobacter sakazakii*, new species of Enterobacteriaceae isolated from clinical specimens. Int. J. Syst. Bacteriol. 30: 569-584 (1980)
3. Muytjens HL, Kollee LA. *Enterobacter sakazakii* meningitis in neonates: causative role of formula. Pediatr. Infect. Dis. J. 9: 372-373 (1990)
4. Iversen C, Forsythe S. Isolation of *Enterobacter sakazakii* and other Enterobacteriaceae from powdered infant formula milk and related products. J. Food Microbiol. 21: 771-777 (2004)
5. WHO. Questions and Answers on *Enterobacter sakazakii* in powdered infant formula. Version 4. Available from: http://www.who.int/foodsafety/publications/micro/en/qa2.pdf. Accessed Feb. 13, 2004.
6. FAO/WHO. *Enterobacter sakazakii* and other microorganisms in powdered infant formula: meeting report, MRA Series 6. Available from: http://www.who.int/foodsafety/publications/micro/en/es.pdf. Accessed Feb., 2004.
7. FDA/CFSAN. Infant Formula - Frequently Asked Questions. Available from: http://www.cfsan.fda.gov/~dms/qa-inf1.html. Accessed Jan. 9, 2003.
8. Clementino MM, De Filippis I, Nascimento CR, Branquinho R, Rocha CL, Martins OB. PCR analyses of tRNA intergenic spacer, 16S-23S internal transcribed spacer, and randomly amplified polymorphic DNA reveal inter- and intraspecific relationships of *Enterobacter cloacae* strains. J. Clin. Microbiol. 39: 3865-3870 (2001)
9. Nazarowec-White M, Farber JM. Phenotypic and genotypic typing of food and clinical isolates of *Enterobacter sakazakii*. J. Med. Microbiol. 48: 559-567 (1999)
10. Dr. Joseph F. Smith Medical Library. Enterobacterial infections. Available from: http://www.chclibrary.org/micromed/00046830.html. Accessed Apr. 18, 2005.
11. Power EGM. RAPD typing in microbiology - a technical review. J. Hosp. Infect. 34: 247-265 (1996)
12. Nazarowec-White M, Farber JM. Incidence, survival, and growth of *Enterobacter sakazakii* in infant formula. J. Food Prot. 60: 226-230 (1997)
13. Edelson-Mammel SG, Buchanan RL. Thermal inactivation of *Enterobacter sakazakii* in rehydrated infant formula. J. Food Prot. 67: 60-63 (2004)
14. Buchanan RL. Resistance-Thermal and other. Available from: http://www.fda.gov/ohrms/dockets/ac/03/slides/3939s1_Buchanan.ppt. Accessed Mar. 24, 2004.