The phenotypic and genotypic characterization of *Enterobacter sakazakii* strains from infant formula milk

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ABSTRACT

Enterobacter sakazakii is an emerging foodborne pathogen associated with severe diseases in neonates. Infant formula milk (IFM) has been identified as one of the major contaminated sources and a transmission vehicle. To determine the phenotypic and genotypic characterization of this pathogen, 22 *E. sakazakii* strains isolated from IFM by an FDA-recommended method and PCR on the α -glucosidase gene were subtyped by random amplified polymorphic DNA (RAPD)-PCR, enterobacterial repetitive intergenic consensus (ERIC)-PCR, and antibiotic resistance patterns. At a similarity threshold of 80%, 16 ERIC-PCR fingerprint types were identified with a discriminatory power (D) of 0.933, and 18 RAPD-PCR types were identified with D of 0.973. Resistance to 9 antibiotics tested by disk diffusion assay revealed 6 antibiotic resistance patterns with D of 0.749. The comparison of characterization indicated that RAPD-PCR and ERIC-PCR have high discriminatory power showing genetic diversity of *E. sakazakii* isolates, and ERIC-PCR patterns showed a closer correlation than RAPD-PCR patterns to phenotypic characterization and the brands of IFM. Overall, the ERIC-PCR typing method could be used for tracing sources of *E. sakazakii* isolates in the food chain.

Key words: *Enterobacter sakazakii*, enterobacterial repetitive intergenic consensus (ERIC)-PCR, random amplified polymorphic DNA (RAPD)-PCR, antibiotic resistance test

INTRODUCTION

The presence of *Enterobacter sakazakii* in infant formula milk (IFM) has been associated with outbreaks of a rare form of infant meningitis, necrotizing entero-

colitis, and bacteremia (Muyltjens et al., 1983; Clark et al., 1990; Gallagher and Ball, 1991; Centers for Disease Control and Prevention, 2002; Iversen and Forsythe, 2003; Gurtler et al., 2005; Caubilla-Barron et al., 2007; Iversen et al., 2008). Many efforts have been devoted to searching for sources of *E. sakazakii* infections, and IFM is considered one of the major sources of contamination (Muyltjens et al., 1983; Biering et al., 1989; Van Acker et al., 2001; Iversen et al., 2004; Caubilla-Barron et al., 2007). However, *E. sakazakii* has also been isolated from a wide range of foods and ingredients (Iversen et al., 2004; Friedemann, 2007; Shaker et al., 2007; Kim et al., 2008). Although not all outbreaks of *E. sakazakii* have been attributed to ingestion of contaminated IFM, *E. sakazakii* poses a serious risk of infection for immature neonates, and the microbiological safety of IFM is of great concern (Iversen and Forsythe, 2007). To minimize the possibility of IFM contamination, both raw materials and the production environments must be constantly monitored. Phenotypic analyses are unreliable because of the unstable expression of the corresponding markers (Olive and Bean, 1999). For this reason, DNA-based molecular subtyping methods such as random amplified polymorphic DNA (RAPD)-PCR and enterobacterial repetitive intergenic consensus (ERIC)-PCR have been applied as useful tools to facilitate surveillance and to trace routes from a source to an infected individual caused by *Staphylococcus aureus*, *Escherichia coli*, *E. sakazakii*, *Campylobacter coli*, and *Campylobacter jejuni* (Clark et al., 1990; Ramanantsoa et al., 1997; Nazarowec-White and Farber, 1999; Meacham et al., 2003; Mouwen et al., 2005). In addition, the ERIC-PCR typing method—a potential tool for studies of epidemiology or genetic diversity—is fast, easy to operate, and inexpensive (Jurkovic et al., 2007; Maiti et al., 2007; Xie et al., 2008). Recently, reported methods include ribotyping, pulsed-field gel electrophoresis (PFGE), and RAPD-PCR, which facilitate tracing disease outbreaks from clinical sources to the contaminated batch of IFM or to the manufacturing environment (Drudy et al., 2006). To date, no studies

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Table 1. Primers used in the study

Primer ¹	Sequence (5' → 3')	Targeted region	Reference
EsAgf	tgaaagcaatcgacaagaag	<i>glu A</i> gene	Lehner et al. (2006)
EsAgr	actcattaccctcctgatg	<i>glu A</i> gene	Lehner et al. (2006)
ERIC1R	atgtaagctcctggggattcac	Fingerprint pattern	Versalovic et al. (1991)
ERIC2	aagtaagtgactgggtgagcg	Fingerprint pattern	Versalovic et al. (1991)
RAPD	cgcgtgccag	Fingerprint pattern	Nazarowec-White and Farber (1999)

¹ERIC = enterobacterial repetitive intergenic consensus; RAPD = random amplified polymorphic DNA.

have focused on ERIC-PCR fingerprinting for typing of *E. sakazakii*, and ERIC-PCR patterns have not been compared with patterns from other typing methods such as RAPD-PCR, antibiotic resistance, or biochemical API-20E (bioMérieux, Craponne, France) profiles.

In present study, we primarily aimed to characterize *E. sakazakii* isolates from IFM using ERIC-PCR, RAPD-PCR, and antibiotic resistance patterns. In addition, to provide some useful information for tracing sources of *E. sakazakii* isolates, we compared characterizations by the different methods to determine the correlation between genotypic and phenotypic characterization.

MATERIALS AND METHODS

Bacterial Isolates

The type strain *Enterobacter sakazakii* ATCC29544 and 22 *E. sakazakii* isolates originally isolated from IFM and powdered milk were obtained from Guangdong Provincial Key Laboratory of Microbiology Culture Collection and Application (Guangzhou, China). All isolates were isolated and identified by the FDA-recommended method (FDA, 2002) and were then streaked for α -glucosidase activity on Druggan-Forsythe-Iversen chromogenic agar (selective for *E. sakazakii*; Oxoid, Basingstoke, UK) and a nonselective medium containing peptone (7 g), yeast extract (3 g), NaCl (5 g), agar (15 g), 5-bromo-4-chloro-3-indolyl- α -D-glucopyranoside (X- α -Glc, 0.15 g), and H₂O (1,000 mL), and incubated at 37°C for 24 h.

Biochemical Identification and Further Confirmation by PCR

Enterobacter sakazakii isolates were identified using the oxidase test and the API-20E biochemical confirmation system (bioMérieux) according to the manufacturer's instructions. To further confirm the biochemical identification, PCR amplification was performed targeting the α -glucosidase gene using the primers shown in Table 1 and generated a product of 1,680 bp. The amplification reaction mixture and thermal cycling conditions were described previously (Lehner et al., 2006).

DNA Extraction

Genomic DNA was extracted from 1.0 mL of Luria-Bertani (Huankai, Guangzhou, China) broth containing 10⁸ cfu/mL of *E. sakazakii* using an extraction kit (Sangon, Shanghai, China). The concentration and purity of DNA samples were determined spectrophotometrically, and DNA samples were stored at −20°C.

ERIC-PCR Amplification

The ERIC-PCR amplification was performed according to the methods of Ye et al. (2008) using 2 primers (Table 1), with minor modifications. The amplification products (8 μ L) were subjected to electrophoresis in 1.0% (wt/vol) agarose gels at 100 V for 1.5 h and then photographed on a UV transilluminator. Gel images were analyzed with Bionumerics software 4.0 (Applied Maths, Sint Martens-Latem, Belgium). Variations in band intensity were ignored. The index of discriminatory ability (*D*) was calculated based on the probability that 2 unrelated strains from the test population would be placed into different typing groups (Hunter and Gaston, 1988). To determine related fingerprinting types, pattern similarities were calculated using the Dice coefficient and clustered by the unweighted pair group method with arithmetic averages (UPGMA). Isolates with a fingerprint similarity index $\geq 80\%$ were considered to have closely related ERIC pattern types.

RAPD-PCR Amplification

The primer used in this study is presented in Table 1. A 25- μ L reaction mixture consisted of 50 ng of DNA template, 10 $\times$ buffers (2.5 μ L), 2.5 U of *Taq* polymerase (Fermentas, Vilnius, Lithuania), 200 μ mol/mL of each deoxynucleoside triphosphate (dNTP), 0.5 μ mol/mL primer, and water to bring the volume to 25 μ L. The RAPD-PCR was performed as follows: 95°C for 5 min, 36°C for 1 min, 72°C for 4 min, 35 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 4 min. The amplification products (8 μ L) were then subjected to electrophoresis in 1.5% (wt/vol) agarose gels at 100 V for 1.5 h and were later photographed under a UV

transilluminator. The RAPD-PCR patterns were analyzed as described above. Likewise, the pattern types of isolates with a fingerprint similarity index $\geq 80\%$ were considered as the closely related RAPD-PCR pattern type or related sources.

Antibiotic Resistance Test

To test the antibiotic resistance of the isolated strains and provide guidance for clinical treatment of *E. sakazakii* infections, the disk diffusion assay was carried out following the instructions of the Clinical and Laboratory Standards Institute (CLSI, 2005). The antibiotic susceptibility test disks (Oxoid) included ampicillin (10 μ g), cephalotin (30 μ g), gentamicin (10 μ g), kanamycin (30 μ g), vancomycin (30 μ g), erythromycin (15 μ g), norfloxacin (10 μ g), tetracycline (30 μ g) and chloramycetin (30 μ g). Colonies of *E. sakazakii* isolates incubated on Luria-Bertani agar at 37°C for 12 to 16 h were suspended in 2 mL of Mueller-Hinton broth (Oxoid) to obtain suspensions with an absorbance of 0.08 to 0.10 at 625 nm, and the inoculated plates were dried for 5 to 10 min. Then, 4 or 5 antibiotic disks were placed on the surfaces of the plates, which were incubated at 37°C for 12 to 16 h under aerobic conditions. The diameters of the inhibition zones were measured and interpreted according to NCCLS guidelines for *Enterobacteriaceae* (*Staphylococcus* spp. for erythromycin; CLSI, 2005; Kim et al., 2008).

RESULTS

Isolation and Identification of *E. sakazakii* Strains

All but 4 of the *E. sakazakii* strains isolated from IFM by the FDA-recommended method produced entirely blue-green colonies on the Druggan-Forsythe-Iversen chromogenic agar. Interestingly, these 4 strains produced blue-green colonies on nonselective chromogenic medium. The API-20E biochemical profiles identified all isolates as *E. sakazakii* with percentage identification values ranging from 97.5 to 99.9% (Table 2). The 22 strains of *E. sakazakii* gave positive results for PCR of the α -glucosidase gene.

ERIC-PCR Fingerprint Patterns and Genomic Cluster Analysis

Each isolate produced banding patterns consisting of 5 to 11 amplified products, ranging in size from 200 to 2,500 bp. The 22 strains formed 3 major clusters with a discriminatory power (D) of 0.933 (Figure 1). In cluster A, there were 6 strains that had a similarity of 50.8 to 90%. In addition, *E. sakazakii* strain numbers ES3

Table 2. The API-20E (bioMérieux, Craponne, France) profiles and sources or brands of infant formula milk (IFM) of *Enterobacter sakazakii*

Isolate	IFM brand or source	API-20E profile
ES1	A	3307373
ES2	B	3307173
ES3	C	3307373
ES4	C	3307373
ES5	D	3305373
ES6	E	3307173
ES7	F	3307173
ES8	F	3307373
ES9	F	3307373
ES10	F	3307373
ES11	F	3307373
ES12	G	3307373
ES13	G	3307373
ES14	I	3307373
ES15	I	3307373
ES16	I	3307173
ES17	J	3307373
ES18	J	3307373
ES19	K	3307373
ES20	K	3307373
ES21	K	3307373
ES22	L	3307373
ES23	ATCC29544	3305373

and ES4 had the same API-20E profiles and antibiotic resistance patterns. Cluster B contained 7 strains with identity of 54.4 to 94.1%. Seven strains were included in cluster C. With a threshold of 80% similarity, 5 strains (ES9, ES10, ES19, ES20, and ES21) had closely related ERIC-PCR patterns. In addition, isolates ES19 and ES20 had identical API-20E profiles and antibiotic resistance patterns and came from the same brand of IFM. Likewise, isolates ES8, ES18, and ES13 in cluster A had closely related ERIC-PCR fingerprinting patterns.

RAPD-PCR Patterns and Genomic Cluster Analysis

The patterns of the 8 to 14 amplified products, ranging in size from 200 to 2,500 bp from RAPD-PCR were grouped into 6 major clusters with D of 0.973 (Figure 2). Cluster I contained 4 strains (ES1, ES2, ES12, and ES13) with similarity from 39.1 to 75%. Six strains were grouped in cluster II and 2 strains (ES10 and ES16) with similarity of 81.8% had closely related RAPD-PCR types. Cluster III contained 4 strains, and ES20 and ATCC29544 had the closely RAPD-PCR patterns. Likewise, RAPD-PCR patterns of 2 strains (ES20 and ES21) had similarity of 81.5%. The remaining strains had diverse RAPD patterns with similarities of $<75\%$.

Antibiotic Resistance Patterns

All of the *E. sakazakii* strains were resistant to vancomycin but susceptible to at least 5 antibiotics (tetra-

Figure 1. Antibiotic resistance patterns (a to f) for *Enterobacter sakazakii* isolates (ES1 to ES23) from infant formula milk. AMP = ampicillin; TE = tetracycline; VA = vancomycin; C = chloramphenicol; NOR = norfloxacin; KF = cephalotin; CN = gentamicin; K = kanamycin; E = erythromycin. S = susceptible; R = resistant; I = intermediate.

cycline, ampicillin, chloramphenicol, kanamycin, gentamicin). Two isolates were resistant to cephalotin. All but 2 strains were moderately susceptible to erythromycin. Six antibiotic resistance types were observed with *D* of 0.749 (Figure 3). We also found 2 strains (ES9 and ES21) that were resistant to multiple antibiotics (vancomycin and norfloxacin).

Comparison of Phenotypic and Genotypic Characterizations

To provide some useful information correlated to brands of IFM contaminated by *E. sakazakii*, the phenotypic characterization in Table 2 was compared with the characterization in Figure 1, Figure 2, and Figure 3. Isolates ES19 and ES20 had identical phenotypic characterization and closely related ERIC-PCR patterns, but these 2 isolates showed unrelated RAPD-PCR patterns. Isolates ES7, ES8, ES9, ES10, and ES11 from brand F had identical API-20E test profiles; 4 out of these 5 strains were grouped into cluster B by ERIC-PCR fingerprinting but showed different antibiotic resistance patterns. In addition, ES20 and ATCC29544 had closely related RAPD-PCR patterns, but showed different phenotypic characterization and ERIC-PCR patterns.

DISCUSSION

Enterobacter sakazakii infections in preterm neonates and infants have been epidemiologically associated with consumption of contaminated IFM (Biering et al., 1989; Centers for Disease Control and Prevention, 2002; Gurtler et al., 2005; Wu et al., 2006; Caubilla-Barron

et al., 2007). *Enterobacter sakazakii* has several unusual physiological characteristics such as biofilm formation, and thermal, osmotic, desiccation, and antibiotic resistance (Gurtler et al., 2005; Wu et al., 2006). Consequently, *E. sakazakii* appears to have an unusual ability to survive when exposed to dry or hot conditions. In this study, 22 *E. sakazakii* strains were isolated from IFM, indicating that they could survive desiccation for long periods. Antibiotic resistance tests suggested that all *E. sakazakii* isolates were resistant to vancomycin, as was observed by Guillaume-Gentil et al. (2005). In addition, 20 of 22 strains were moderately resistant to erythromycin, but only 2 strains were resistant to cephalotin. The antibiotic resistance patterns could provide guidance for clinical treatment of *E. sakazakii* infections. All the isolates were susceptible to ampicillin, which is inconsistent with the report of Kim et al. (2008). Kim et al. (2008) investigated various food samples including dried shrimp and fish. The misuse or overuse of ampicillin in aquaculture may contribute to a high rate of ampicillin resistance in that environment. Although the rate of resistance to other antibiotics was not high, the potential for acquiring antibiotic resistance by overusing antibiotics in the processing or production of raw materials still exists. Further research is needed

Figure 2. Dendrogram obtained from cluster analysis (Dice similarity coefficient; unweighted pair-group average method) of random amplified polymorphic DNA (RAPD)-PCR patterns of 23 *Enterobacter sakazakii* isolates (ES1 to ES22 and *E. sakazakii* ATCC29544).

Figure 3. Dendrogram obtained from cluster analysis (Dice similarity coefficient; unweighted pair-group average method) of enterobacterial repetitive intergenic consensus (ERIC)-PCR patterns of 23 *Enterobacter sakazakii* isolates (ES1 to ES22 and *E. sakazakii* ATCC29544).

on the mechanisms of antibiotic resistance or desiccation in *E. sakazakii*.

The ERIC-PCR and RAPD-PCR patterns were used to determine the genetic diversity of *E. sakazakii* from IFM in this study. In addition, correlations between phenotypic and genotypic characterization were observed in some strains. Two strains (ES19, ES20) from the same brand (K) of IFM had closely related ERIC-PCR patterns. Moreover, these strains showed the same API-20E biochemical test profiles and antibiotic resistance patterns. Strains ES20 and ES21 from the same brand (K) also had the same API-20E test profiles and were grouped into the same ERIC-PCR cluster (B) and RAPD-PCR cluster (III), but had different antibiotic resistance patterns. Nonetheless, these strains (ES19, ES20, and ES21) might be closely related phylogenetically. Two strains (ES3 and ES4) from IFM with the same manufacturer had the same API-20E test profiles and identical antibiotic resistance patterns and were distributed into the same cluster C by ERIC-PCR fingerprinting, indicating a close relationship.

Five strains (ES7, ES8, ES9, ES10, and ES11) from the same brand (F) had the same API-20 test profiles,

and 4 out of the 5 strains were grouped into cluster B by ERIC-PCR fingerprinting but showed different antibiotic resistance patterns. We inferred that the 5 strains might have close phylogenetic affinity and the sources of contamination may be the factory environment or raw materials. Although they had different RAPD-PCR patterns, 2 strains (ES3 and ES4) from brand C showed the same antibiotic resistance patterns and were distributed into cluster C of ERIC-PCR patterns. Isolates ES12 and ES13 from brand G were distributed into cluster A of ERIC-PCR fingerprinting and had same API-20E profiles and antibiotic resistance patterns.

The RAPD-PCR method has been widely used to type bacteria, but several problems have been reported for RAPD-PCR assays that contribute to a lack of reproducibility and standardization (Welsh and McClelland, 1990; Meunier and Grimont, 1993; Arbeit et al., 1994; Olive and Bean, 1999). In this current study, although RAPD-PCR had slightly higher discriminatory power than ERIC-PCR, ERIC-PCR showed a good correlation with phenotypic characterization such as the brands of IFM, antibiotic resistance profiles, and API-20E profiles and could be used for tracing sources of *E. sakazakii* within the food chain.

Independent molecular methods, including fluorescent amplified fragment length polymorphism (f-AFLP), automated ribotyping, full-length 16S rRNA gene sequencing, and DNA-DNA hybridization, were employed to clarify the taxonomic relationship of 210 strains currently described as *E. sakazakii*, and amendments to the classification of these organisms have been proposed (Iversen et al., 2007). Consequently, further studies are necessary to confirm their taxonomic positions. In addition, with the clarification of housekeeping genes of *Enterobacter* species and *E. sakazakii*, multilocus sequence typing may be used to trace the origins or sources of *E. sakazakii* isolates and identify *E. sakazakii* strains.

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