Genotypic and Phenotypic Analysis of *Enterobacter sakazakii* Strains from an Outbreak Resulting in Fatalities in a Neonatal Intensive Care Unit in France[▽]

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Received 25 May 2007/Returned for modification 11 July 2007/Accepted 25 September 2007

In 1994, an outbreak of *Enterobacter sakazakii* infections occurred in a neonatal intensive care unit in France from 5 May to 11 July. During the outbreak, 13 neonates were infected with *E. sakazakii*, resulting in 3 deaths. In addition, four symptomless neonates were colonized by *E. sakazakii*. The strains were subjected to 16S rRNA gene sequence analysis, genotyped using pulsed-field gel electrophoresis, and phenotyped for a range of enzyme activities. *E. sakazakii* was isolated from various anatomical sites, reconstituted formula, and an unopened can of powdered infant formula. A fourth neonate died from septic shock, attributed to *E. sakazakii* infection, during this period. However, 16S rRNA gene sequence analysis revealed that the organism was *Enterobacter cloacae*. There were three pulsotypes of *E. sakazakii* associated with infected neonates, and three neonates were infected by more than one genotype. One genotype matched isolates from unused prepared formula and unfinished formula. However, no pulsotypes matched the *E. sakazakii* strain recovered from an unopened can of powdered infant formula. One pulsotype was associated with the three fatal cases, and two of these isolates had extended-spectrum β -lactamase activity. It is possible that *E. sakazakii* strains differ in their pathogenicities, as shown by the range of symptoms associated with each pulsotype.

Enterobacter sakazakii is an opportunistic pathogen associated with the ingestion of reconstituted infant formula and is a rare cause of neonatal meningitis, necrotizing enterocolitis (NEC), and sepsis (9, 10, 11, 23). Such cases often occur among low-birth-weight preterm neonates, who are generally more susceptible to gram-negative bacterial sepsis and endotoxemia associated with NEC (1, 26). The International Commission on Microbiological Specifications for Foods (14) has ranked *E. sakazakii* as a “severe hazard for restricted populations, life-threatening or substantial chronic sequelae or long duration.” A number of reported *E. sakazakii* outbreaks have been attributed to contaminated reconstituted infant formula (4, 7, 13, 18, 31). Bowen and Braden (4) reviewed 46 cases of invasive *E. sakazakii* infections and showed a link between symptoms and birth weight but did not consider cases of NEC.

The virulence of *E. sakazakii* has been studied by Pagotto et al. (23) and Mange et al. (21), who showed the presence of enterotoxins and adhesion to brain cells, respectively. Townsend et al. demonstrated the translocation of *E. sakazakii* and other intestinal bacteria across the rat intestinal wall in response to the presence of lipopolysaccharide (28). They also demonstrated that *E. sakazakii* causes chronic-patterned inflammation in the neonatal rat brain, invades capillary endothelial brain cells, is taken up by macrophages, and induces anti-inflammatory cytokine (interleukin-10) expression in vitro

and in vivo at various levels according to strain (29). However, these publications did not report the individual case details associated with the isolates under study. Therefore, it is not possible to directly consider correlations between in vitro and in vivo studies.

This study analyzed 31 *E. sakazakii* strains isolated over a 3-month period in 1994 during a large *E. sakazakii* outbreak in a neonatal intensive care unit (NICU) in France. This paper primarily considers the genetic and phenotypic diversity of the isolates and is not an epidemiological investigation. However, where appropriate, neonatal details have been included. The strains have been identified using 16S rRNA gene sequence analysis, genotyped using pulsed-field gel electrophoresis (PFGE), and phenotyped for a range of enzyme activities. In addition, antibiograms and determination of extended-spectrum β -lactamase (ESBL) production have been undertaken.

MATERIALS AND METHODS

Background. An outbreak of NEC and invasive infections occurred during the period from 5 May to 15 July 1994 in a NICU. An internal hospital investigation, which was not published, determined that the outbreak was due to *E. sakazakii*. Details of the neonates from whom *E. sakazakii* was isolated are given in Table 1. All neonates were fed reconstituted powdered infant formula via enteral perfusion. Formula was reconstituted every 24 h and stored in the refrigerator until required. Every 4 to 6 h, syringes were filled from stored formula, and the contents were fed to infants through enteric tubes at ambient temperature.

NEC case definition. Bell's staging of NEC as modified by Walsh and Kliegman (32) was used by clinicians in the hospital. Infants with stage I disease (NEC I) have suspected NEC, with systemic signs of temperature instability, apnea, bradycardia, and lethargy. Stage II (definite NEC) is characterized by additional symptoms of absence of bowel sounds, radiological signs of intestinal dilation, ileus, and pneumatosis intestinalis. Stage III is advanced NEC, where the neonate is critically ill with metabolic acidosis, neutropenia, signs of generalized

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[▽] Published ahead of print on 10 October 2007.

TABLE 1. Clinical description of neonates

Neonate	Sex	Mode of delivery	Gestation age (wk)	Birth wt (g)	Start of feeding (days)	Onset of symptoms (days)	Symptom
A	F	NR ^a	NR	NR	NR	NR	No details
B	F	Vaginal	28	1,200	8	13	NEC II
C	F	Vaginal	29	1,380	6	18	Asymptomatic
D	M	Caesarean	32	1,800	2	17	NEC II
E	F	Caesarean	31	1,470	5	27	Asymptomatic
F (died)	M	Vaginal	28	1,000	8	28	NEC III
G	F	NR	NR	NR	NR	NR	No details
H (died)	M	Vaginal	31	1,500	1	19	Meningitis
I	F	Caesarean	31	1,570	2	12	Septicemia
J (died)	M	Caesarean	32	1,560	2	15	NEC II
K	M	Caesarean	30	1,180	6	87	NEC I
L	M	Caesarean	33	1,450	2	8	NEC II
M	F	Vaginal	29	1,200	8	16	NEC II
N	M	NR	NR	NR	NR	NR	Digestive problems
O	M	Caesarean	33	2,090	4	20	Asymptomatic
P	M	NR	NR	NR	NR	NR	Digestive problems
Q	M	Vaginal	41	1,600	2	13	Asymptomatic
R (died)	NR	NR	NR	NR	NR	NR	Sepsis

^a NR, no record.

peritonitis, and marked tenderness and distension of the abdomen. Neonates without NEC were also tested for *E. sakazakii* colonization.

Bacterial strains. In 1994, *E. sakazakii* strains were isolated from various anatomical sites of 17 neonates (Table 2). These included sputum, feces, skin, peritoneal fluid, and conjunctivae. *E. sakazakii* was also isolated from the remains of prepared formula, unfinished formula collected during the outbreak

period, and an unopened can of powdered infant formula that was collected after the end of the outbreak period. The putative *E. sakazakii* colonies were distinguished on lactose agar plates by their very mucoid aspect. The hospital identified the strains as *E. sakazakii* using API20E (BioMerieux), with gas production as the confirmatory test. The isolates were kept in long-term storage at -80°C until the studies reported here. One strain (strain 766), originally identified as *E.*

TABLE 2. PFGE profiles, biotyping, and plasmid and antibiotic profiles of patient, prepared formula, and powdered infant formula isolates

Neonate	Symptoms	Isolation date (day/mo/yr)	Isolation site	Strain no.	PFGE profile	Antibiogram ^a
A	No details	23/03/1994	Trachea	699	1	DOX
B	NEC II	25/04/1994	Trachea	703	1	DOX
		29/04/1994	Trachea	704	1	DOX
C	Asymptomatic	09/05/1994	Trachea	708	1	DOX
D	NEC II	08/06/1994	Stools	696	1	DOX
E	Asymptomatic	19/06/1994	Stools	690	1	DOX
F (died)	NEC III	07/04/1994	Peritoneal fluid	701	2	DOX
G	No details	19/04/1994	Sputum	691	2	DOX
H (died)	Meningitis	11/05/1994	Trachea	767	2	DOX, AMP, CTX, CXM (ESBL)
C	Septicemia	12/05/1994	Trachea	709	2	DOX
B	NEC II	24/05/1994	Trachea	705	2	DOX
J (died)	NEC II	07/06/1994	Trachea	695	2	DOX, AMP, CTX, CXM (ESBL)
B	NEC II	09/06/1994	Stools	706	2	DOX
D	NEC II	13/06/1994	Stools	697	2	DOX
K	NEC I	13/06/1994	Stools	702	2	DOX
L	NEC II	13/06/1994	Stools	692	2	DOX
M	NEC II	14/06/1994	Conjunctivae	694	2	DOX
		17/06/1994	Prepared formula	712	2	DOX
B	NEC II	26/06/1994	Skin	707	2	DOX
K	NEC I	27/06/1994	Stools	730	2	DOX
O	Asymptomatic	27/06/1994	Stools	711	2	DOX
D	NEC II	01/07/1994	Stools	698	2	DOX
P	Digestive problems	15/06/1994	Stools	700	3	DOX
Q	Asymptomatic	18/06/1994	Stools	693	3	DOX
		20/06/1994	End of bottle	713	3	DOX
		27/06/1994	End of bottle	714	3	DOX
		27/06/1994	Prepared formula	715	3	DOX
		11/07/1994	Infant formula	716	4	DOX
		11/07/1994	Infant formula	717	4	DOX
		11/07/1994	Infant formula	718	4	DOX
R (died)	Sepsis	04/06/1994	Blood culture	766	NA ^b	DOX, AMP, CXM, AMC

^a Resistance profile for doxycycline (DOX), ampicillin (AMP), cefotaxime (CTX), cefuroxime (CXM), and amoxicillin-clavulanate (AMC).^b NA, not applicable.

sakazakii, has been reidentified, by using 16S rRNA gene sequence analysis, as *Enterobacter cloacae*.

Bacterial identification and 16S rRNA gene cluster group analysis. The recovered bacterial strains were identified using 16S rRNA gene sequence analysis (Accugenix, Newark, DE) as described by Iversen et al. (15). The sequences were compared with *E. sakazakii* sequences to determine the 16S rRNA gene cluster group.

DNA isolation and PCR. Genomic DNA was prepared using the GenElute bacterial genomic DNA kit (Sigma) and 1.5 ml of overnight culture grown in LB broth according to the manufacturer's instructions. By following methods prescribed by Keyser et al. (19), primers Esak2 (5'-CCCGCATCTCTGCAGGAT TCTC-3') and Esak3 (5'-CTAATACCGCATAACGTCTACG-3') were used to amplify an 850-bp PCR product from a region of the *E. sakazakii* 16S rRNA gene. Lehner et al. (20) used Esakf (5'-GCTYTGTGACGAGTGGCGG-3') and Esakr (5'-ATCTCTGCAGGATTCTCTGG-3') to amplify a 929-bp PCR product, also from a region of the *E. sakazakii* 16S rRNA gene. The *ompA* gene was amplified with primers ESSF (5'-GGATTAAACCGTGAACCTTTCC-3') and ESSR (5'-CGCCAGCGATGTTAGAAGA-3'), resulting in a 469-bp product by using the PCR conditions described by Mohan Nair and Venkitanarayanan (22). The PCR protocols documented above were followed as described in each publication using 2.5 U of GoTaq Flexi DNA polymerase, 5× Green GoTaq Flexi buffer (Promega Corporation, Madison, WI), and a Genius thermocycler (FGEN05TD; Techne Ltd., Cambridge, United Kingdom). *E. sakazakii* strains NCTC 11467^T and ATCC 12868 were used as positive controls. PCR products were visualized on 1% agarose gels stained with 0.5 µg ml⁻¹ ethidium bromide.

PFGE. PFGE of *E. sakazakii* was performed by following the Pulse Net USA protocol for molecular subtyping of *Escherichia coli* O157:H7, nontyphoidal *Salmonella* serotypes, and *Shigella sonnei* (6). The gel was run at switch times of 5 to 50 s for 20 h at 6 V in a CHEF-DR II system (Bio-Rad, Hercules, CA).

The PFGE patterns were analyzed by Bionumerics software, version 3.5 (Applied Maths, Sint-Martens-Latem, Belgium). The patterns were compared and clustered by the unweighted-pair group method using arithmetic averages (UPGMA) by using the Dice coefficient. The position tolerance was set to 1.5%, and an optimization of 1.5% was applied during the comparison of PFGE fingerprint patterns. PFGE patterns were interpreted according to the criteria of Tenover et al. (27).

Antibiotic sensitivity testing. The susceptibilities of *E. sakazakii* to antimicrobial agents were determined by the disk diffusion method on Iso-Sensitest agar (catalog no. CM0471; Oxoid Ltd.) according to the British Society for Antimicrobial Chemotherapy protocol (5). The antibiotics tested were amikacin, ampicillin, cefotaxime, cefuroxime, cefpodoxime, ceftazidime, chloramphenicol, ciprofloxacin, amoxicillin-clavulanate, doxycycline, gentamicin, imipenem, piperacillin, and trimethoprim from Oxoid Ltd. UK (Basingstoke, United Kingdom). ESBL production was detected using the combination disc method as described in HPA QSOP 51 (30) using ceftazidime-clavulanic acid, cefotaxime-clavulanic acid, and cefpodoxime-clavulanic acid combination discs in comparison to individual-antibiotic ceftazidime, cefotaxime, and cefpodoxime discs according to the manufacturer's instructions (Mast Diagnostics, Bootle, United Kingdom).

Biotyping. The biotype for each strain was determined according to the Farmer et al. (8) biogrouping scheme as revised by Iversen et al. (17). Standardized biochemical test strips (API20E, ID32, APIZYM) were employed according to the manufacturer's instructions (BioMérieux UK). Additional tests of motility, acid production from sugars, gas production from glucose, and malonate utilization, the methyl red test, the Voges-Proskauer test, and the indole production test were conducted as previously described. Bacterial isolates were subcultured on tryptone soy agar (catalog no. 1.05458; Merck KGaA, Darmstadt, Germany) prior to analysis.

Capsule production. Bacterial cultures were grown overnight at 37°C on milk agar, which was composed of 3 g of agar (catalog no. LP0011; Oxoid Ltd., Basingstoke, United Kingdom) and 0.4 g of ammonium sulfate dissolved into 40 ml of distilled water. After autoclaving at 121°C for 15 min, the mixture was combined with 200 ml of warm (55°C) liquid infant formula (Premium 1, milk-based; Cow & Gate, Trowbridge, United Kingdom) and dispensed into petri dishes. Each strain was evaluated for capsule production by visual comparison with the colony morphology of *E. sakazakii* strains 1 (noncapsulated) and 2 (capsulated).

Protease activity. Skim milk powder (2%, wt/vol) was added to plate count agar (tryptone glucose yeast agar; catalog no. CM325; Oxoid Ltd.) after autoclaving to make SM-PCA. Plates were inoculated by a simple streak of the bacteria and incubated at 37°C for 72 h. A positive result was indicated by zones of clearing around bacterial growth.

Nucleotide sequence accession numbers. The GenBank accession numbers of the *E. sakazakii* isolates sequenced in this study are AM778409 to AM778415.

RESULTS

Patients. During the 3-month outbreak period, 18 neonates were infected or asymptotically colonized, and there were four deaths (Table 1). All infected neonates, except for one (neonate D), were preterm. The average birth weight was 1,461 g, and birth weights ranged from 1,000 to 2,090 g. Nine neonates had severe clinical symptoms: seven cases of NEC (one [neonate F] with abdominal perforation), one case of septicemia (neonate I), and one case of meningitis (neonate H). An autopsy of the latter neonate revealed cerebral lesions. All neonates with NEC had birth weights of <2,000 g. The neonate with meningitis had a birth weight of 1,500 g. The onset of illness was <28 days for the majority (17/18) of neonates. The exception was neonate K, who developed NEC I after 87 days and had a low birth weight (1,180 g). Three neonates who died (F, H, and J) had birth weights of 1,000 g, 1,500 g, and 1,560 g, respectively. The first dates of illness for these neonates were 28, 19, and 15 days after birth. Four other neonates (C, E, O, and Q) were colonized by *E. sakazakii* without any clinical signs, and two neonates (N and P) had moderate digestive problems.

Identification of bacterial isolates. Thirty-one strains were recovered from the original outbreak collection. No isolates were recovered for neonates I and N. All strains, except isolate 766, were confirmed as *E. sakazakii* using 16S rRNA gene sequence analysis and were assigned to 16S rRNA gene cluster group 1 (Table 2). The remaining strain (strain 766) was identified as *E. cloacae*. This strain was associated with the death of neonate R through septic shock during the general *E. sakazakii* outbreak and is considered in a separate section below.

PFGE typing of bacterial isolates. Three distinguishable *E. sakazakii* pulsotypes were isolated from neonates and reconstituted formula. A fourth pulsotype was isolated from an unopened can of infant formula (Fig. 1). The pulsotypes were numbered in chronological order.

E. sakazakii pulsotype 1 was isolated from 23 March to 19 June and included strains from two cases of NEC and two asymptomatic colonizations (Tables 1 and 2). No clinical records were provided for neonate A, from whom the first isolate of *E. sakazakii* was obtained. Six strains belonged to pulsotype 1 and were isolated from either the trachea or stool samples.

E. sakazakii pulsotype 2 was isolated from 7 April to 1 July from neonatal peritoneal fluid, sputum, trachea, stools, conjunctivae, and skin. This period overlapped with the period of isolation of pulsotype 1. A total of 16 strains belonged to pulsotype 2. These were obtained from one case of meningitis, seven cases of NEC, two asymptomatic colonizations, and unused prepared formula. Three neonates (F, H, and J) colonized by *E. sakazakii* pulsotype 2 died from NEC or meningitis. Isolates 701, 767, and 695 from these neonates were obtained from the peritoneal fluid and trachea. *E. sakazakii* pulsotype 2 strains 705, 706, and 707 were isolated from neonate B on three occasions over 2 months (24 May, 9 June, and 26 June 1994). These strains were from the trachea, stool, and skin. Their identical PFGE profiles formed a subcluster within the

FIG. 1. Dendrogram generated from PFGE profiles of *E. sakazakii* isolates ($n = 30$) by Bionumerics software, version 3.5. Clustering was done with UPGMA by using the Dice coefficient. Pulsotypes are identified on the left. The tolerance in the band was 1.5%, with an optimization of 1.5%.

pulsotype 2 cluster. Previously, *E. sakazakii* pulsotype 1 had been isolated from the trachea of this neonate on 25 and 29 April 1994. *E. sakazakii* pulsotypes 1 and 2 were isolated 3 days apart from neonate C on 9 and 12 May 1994. This neonate was asymptotically colonized, and the strains were isolated from the trachea on both occasions. *E. sakazakii* pulsotype 1 was isolated from neonate D on 8 June 1994, and pulsotype 2 was isolated from the same neonate on 13 June and 1 July 1994. On each occasion, the organism was isolated from the stool.

Pulsotype 3 was isolated over a 2-week period from 15 to 27 June. This period overlapped with both pulsotypes 1 and 2. There were five strains in pulsotype 3. Two strains were from the stools of neonates P and Q. Neonate P had a digestive problem, and neonate Q was asymptomatic. Two further strains were obtained 1 week apart (20 and 27 June 1994) from leftover formula, and on the same day (27 June 1994) from unused prepared formula.

An unopened can of powdered infant formula was sampled for bacterial contamination on 11 July. This was after the neonatal pulsotypes had been isolated. Three strains of *E. sakazakii* (strains 716, 717, and 718) were isolated with PFGE

profiles that were more than three bands different from pulsotypes 1 to 3. They were designated pulsotype 4.

Characterization of pulsotypes. Table 2 compares the phenotypic traits of the four pulsotypes. Pulsotypes 1 to 3 corresponded to biotype 13, and pulsotype 4 corresponded to biotype 5 (indole positive). APIZYM profiles did not differ between pulsotypes. All strains had high levels (4 to 5 U) of esterase-lipase and β -galactosidase activities. Moderate (1 to 2 U) levels of α -glucosidase activity were detected for all strains. No proteolytic activity was detected for pulsotype 4 strains growing on SM-PCA. In addition, pulsotype 4 strains did not produce capsulated colonies on milk agar plates. Some strains in pulsotypes 1 to 3 produced such profuse capsular material that the colonies contacted the inverted petri dish lid.

All *E. sakazakii* strains were sensitive to ciprofloxacin, amikacin, gentamicin, imipenem, piperacillin, and trimethoprim and resistant to doxycycline. All but two strains (strains 695 and 767) of *E. sakazakii* were sensitive to ampicillin, cefotaxime, and cefuroxime. Strains 695 and 767 were also resistant to cefpodoxime, ceftazidime, and chloramphenicol. Further analysis using the combination disc method recommended by

the HPA (30) demonstrated that these strains possessed ESBLs. These two strains, both pulsotype 2, were isolated in two fatal cases (neonates H and J).

E. cloacae. Strain 766 was isolated as a blood culture from neonate R, who died from septic shock. It was identified in 1994, using API20E, as *E. sakazakii*. However 16S rRNA gene sequence analysis revealed that it was *E. cloacae* and had been misidentified. Several methods for the identification of *E. sakazakii* via PCR have been described and were used in this study (19, 20, 22). PCR products of the indicative size were obtained when genomic DNAs from *E. sakazakii* strains NCTC 11467^T and ATCC 12868 were probed as previously described. However, no corresponding PCR product was obtained for strain 766. This further confirmed that strain 766 was not *E. sakazakii*. Due to the lack of α -glucosidase activity, the organism did not produce characteristic blue-green *E. sakazakii* colonies on the chromogenic *E. sakazakii* agar DFI (catalog no. CM1055; Oxoid Ltd., Basingstoke, United Kingdom). It also failed to produce any yellow pigmentation on tryptone soy agar after 48 h of incubation at 21°C. The APIZYM profile differed from that of *E. sakazakii* by the lack of detectable esterase and esterase-lipase activities. Antibio-gram profiling showed that the strain was resistant to doxycycline, ampicillin, cefuroxime, and amoxicillin-clavulanate.

DISCUSSION

This is the largest outbreak of *E. sakazakii* in a NICU, with the most deaths, that has been reported to date. Unfortunately, because of the long time between the outbreak and this study, a number of clinical details were not available to us. Therefore, this study is not an epidemiological outbreak investigation; instead, it has focused on the diversity of the isolates. Nevertheless, neonatal information regarding gestation age, birth weight, age of onset of symptoms, etc., are reported here (Table 1) and will be of use for future studies on the risk factors for *E. sakazakii* infections.

This study shows that, over the period from March to July 1994, neonates were infected by three pulsotypes of *E. sakazakii* for which no definitive sources were identified (Table 2). The first pulsotype was isolated from March to June. It was isolated from five neonates (A to E), including two cases of NEC II, no fatal cases, and two asymptomatic neonates. The two NEC II cases were cocolonized by pulsotype 2. Unfortunately, no records were available for the first case, neonate A. Pulsotype 2 was isolated from April to July. It was associated with six NEC II or NEC III cases, one suspected NEC case, one case of meningitis, and two asymptomatic neonates. There were a total of three deaths. This pulsotype was also isolated from prepared formula but not from an unopened can of powdered infant formula. Pulsotype 3 was isolated over a 2-week period in June from two neonates: neonate O, with a digestive problem, and neonate P, who was asymptomatic. It was also isolated from unused formula and two leftover formulas. Pulsotypes 1 to 3 were not isolated from unopened cans of powdered infant formula analyzed in July, but only from prepared formula on 17 and 27 June 1994 (Table 2). Pulsotype 3 was isolated from unfinished formula on two occasions (20 and 27 June 1994) and from prepared formula on 27 June 1994. No unopened cans of powdered infant formula were

tested at this time, and therefore the source of contamination remains uncertain. *E. sakazakii* was isolated from an unopened can of powdered infant formula (sample date, 11 July 1994). This was 2 weeks after the last case. These strains had a unique pulsotype. Previously, *E. sakazakii* has been isolated from infant formula preparation equipment, the environment, and the human throat (2, 12, 25). Therefore, given the ubiquity of *E. sakazakii*, unattributable sources of *E. sakazakii* could have been caregivers, cross-contamination from other neonates, the environment, and batches of reconstituted infant formula. It is not known if any neonates were fed the batch of powdered infant formula from which *E. sakazakii* pulsotype 4 was isolated (sample date, 11 July 1994), since there were no neonatal cases in July with that pulsotype.

Multiple *E. sakazakii* pulsotypes were isolated from neonates B, C, and D. Five isolates from the trachea, stool samples, and skin were obtained from neonate B over a 2-month period (25 April to 26 June 1994) (Table 2). The three pulsotype 2 strains (strains 705, 706, and 707) were from the trachea, stools, and skin, respectively. Due to their identical PFGE profiles, they formed a subcluster within the pulsotype 2 cluster (Fig. 1). This indicates that the neonate was colonized by a specific *E. sakazakii* strain from 24 May to 26 June. Neonate C was asymptomatic. Pulsotype 1 and 2 strains 708 and 709 were isolated 3 days apart (9 and 12 May 1994), both from the trachea. Neonate D suffered from NEC II. Pulsotype 1 and 2 strains 696 to 698 were isolated from stool samples between 8 June and 1 July 1994. Therefore, multiple isolates from neonates should be pulse typed; otherwise, clinical strains may not be matched with source isolates. Prompt analysis of possible sources is required, especially for substances such as powdered infant formula, since many batches may be in use over a short period of time.

The NICU feeding practices of reconstituting formula every 24 h and administering the formula over a 4- to 6-h period are not currently recommended. After the outbreak, the following internal recommendations were made: to chill the enteral feeding syringe using cryogel and to change the syringe and syringe end every 3 h. These are very similar to the 2-h maximum period between preparation and administration recommended by the FAO-WHO (9).

It should be noted that in 1994 *E. sakazakii* was not a well-recognized neonatal pathogen and was not associated with contaminated reconstituted infant formula. Therefore, the hospital investigation was not initially focused on the infant formula. The Belgian outbreak that associated *E. sakazakii* with powdered infant formula, and in which two neonates died, occurred in 1996, and the report of that outbreak was not published until 2001 (31).

All strains were doxycycline resistant. Doxycycline is a tetracycline antibiotic that is not used for this patient group and therefore has no clinical relevance. Cefotaxime and cefuroxime are commonly used first-line treatments for neonates. Therefore, the antibiograms of strains 767 and 695, from fatal cases, are of particular interest. Both these strains had ESBL activity, which may have been acquired by horizontal transfer from other *Enterobacteriaceae*, since it was absent in the other pulsotype 2 strains. There are more than 30 variants of CTX-M ESBLs, some of which have evolved from the chromosomal β -lactamases of *Kluyvera* species (3). Cefpirome is stable to the

inducible AmpC chromosomal β -lactamases of *Enterobacter* species, which give a false-positive ESBL result with other cephalosporins that are used. The use of cephalosporins is not recommended for the treatment of *Enterobacter* species infections, due to the selection of AmpC-derepressed mutants. *E. cloacae* strain 766 was also from a fatal case and was resistant to ampicillin, cefuroxime, and amoxicillin-clavulanate.

All *E. sakazakii* strains were in 16S rRNA gene cluster group 1, the largest of the four *E. sakazakii* genotypes (16). Pulsotype 2 isolates were from eight cases of NEC, one case of septicemia, and one case of meningitis. A total of three deaths were associated with this pulsotype, and therefore it appears to be more virulent than the other pulsotypes. In addition, two pulsotype 2 strains (strains 696 and 767) from neonates H and J, who died, had acquired ESBL activities. Pulsotype 4 strains, isolated from an unopened can of powdered infant formula, differed from the other pulsotypes by the lack of protease activity on SM-PCA and the absence of capsule production. Whether these phenotypes are related to virulence determinants is unknown, but the question is currently under investigation.

Strain 766 was initially identified as *E. sakazakii* by using API20E and was isolated in a fatal case of septic shock (4 June 1994) during the NICU *E. sakazakii* outbreak (Table 1). However, 16S rRNA gene sequencing revealed that it did not fall within any of the *E. sakazakii* cluster groups. Several PCR protocols for the specific amplification of *E. sakazakii* have been developed that amplify the 16S rRNA gene. More recently, *ompA*-specific PCR primers were shown to distinguish *E. sakazakii* from similar organisms (22). Our study used these published protocols and PCR probes in order to obtain a presumptive identification of strain 766 and to further understand at what level the misidentification as *E. sakazakii* may have occurred. While strains NCTC 11467^T and ATCC 12868 were correctly identified by these techniques, no PCR product was obtained from strain 766.

Discrepancies in *E. sakazakii* identification between commercial biochemical kits have been noted previously (15, 24). Therefore, 16S rRNA gene sequence analysis was used as our "gold standard" for identification. Commercially available specific *E. sakazakii* chromogenic agars are designed to detect α -glucosidase activity. Therefore, *E. cloacae* strain 766 (α -glucosidase negative) would not have been misidentified by routine use of these agars. Nor would the strain be regarded as a presumptive *E. sakazakii* strain by use of PCR probes. This further shows the need for rigorous testing and careful consideration of rapid phenotypic tests.

This investigation highlights the need for accurate isolate identification and prompt typing of neonatal and associated isolates. The source of the three pulsotypes could not definitely be identified as contaminated reconstituted infant formula. Nevertheless, the feeding practices of preparation for 24-h periods and prolonged (>2-h) administration could have enabled bacterial growth in the formula and increased the risk of infection.

ACKNOWLEDGMENTS

We thank Stacey Brown for technical assistance, Julie Rainard for translating documents, and Anna Bowen (CDC, Atlanta, GA) for helpful discussions.

We also thank Nottingham Trent University, the Centre National de la Recherche Scientifique, and the Université Paul Sabatier for financial support.

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