Outbreak of Necrotizing Enterocolitis Associated with Enterobacter sakazakii in Powdered Milk Formula

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We describe an outbreak of necrotizing enterocolitis (NEC) that occurred in the neonatal intensive care unit of our hospital. A total of 12 neonates developed NEC in June-July 1998. For two of them, twin brothers, the NEC turned out to be fatal. Enterobacter sakazakii, a known contaminant of powdered milk formula, was isolated from a stomach aspirate, anal swab, and/or blood sample for 6 of the 12 neonates. A review of feeding procedures revealed that 10 of the 12 patients were fed orally with the same brand of powdered milk formula. Enterobacter sakazakii was isolated from the implicated prepared formula milk as well as from several unopened cans of a single batch. Molecular typing by arbitrarily primed PCR (AP-PCR) confirmed, although partially, strain similarity between milk and patient isolates. No further cases of NEC were observed after the use of the contaminated milk formula was stopped. With this outbreak we show that intrinsic microbiological contamination of powdered milk formula can be a possible contributive factor in the development of NEC, a condition encountered almost exclusively in formula-fed premature infants. The use of sterilized liquid milk formula in neonatal care could prevent problems with intrinsic and extrinsic contamination of powdered milk formula.

In this report we describe for the first time a cluster of NEC associated with the isolation of E. sakazakii in patients and the use of powdered infant milk formula.

MATERIALS AND METHODS

Background. An outbreak of NEC occurred during June-July 1998 in our neonatal intensive care unit (NICU). The unit is a 16-bed tertiary referral center. In the two months of the outbreak, a cohort of 50 neonates was admitted at our NICU. Median birth weight was 2,335 g (interquartile range, 1,305 to 3,040 g), median gestational age was 35 weeks (interquartile range, 30 to 39 weeks), and median length of stay was 16 days (interquartile range, 8 to 43 days). Twenty-two (44%) neonates had a birth weight of <2,000 g.

Case definition. Bell's staging of NEC as modified by Walsh and Kliegman was used (29). Infants with stage I disease (suspected NEC) have suggestive clinical symptoms such as abdominal distention, gastric residual, emesis, and/or hematocrit but nondiagnostic radiographs. Infants with stage II disease (definite NEC) have diagnostic abdominal radiographs showing pneumatisis intestinalis. Infants with stage III disease (advanced NEC) are critically ill with impending or proven intestinal perforation.

Patient cultures. We reviewed the results of all bacterial cultures taken from the neonates during the outbreak. Surveillance cultures, consisting of an anal swab, a stomach aspirate, and a blood culture, were obtained from each NEC patient, if possible and if ordered by the pediatrician.

Environmental cultures. Taking into account the properties of the isolated microorganism and the fact that all NEC patients were orally fed, environmental sampling was focused on the milk kitchen. When formula is prepared in our milk kitchen, the powder is weighed on sterilized plates with sterilized spoons. The formula is mixed in a sterilized bowl with a sterilized blender head which is rinsed between preparations in cooked tap water. Milk solutions are prepared with chilled mineral water once a day between 9 and 11 a.m., divided into disposable bottles, and closed with disposable, gamma-irradiated teats. The bottles are stored temporarily on a special cooling table before transportation to the different pediatric wards, where they are placed immediately in the refrigerator. The milk bottles are warmed up with a dry-air bottle heater or in a microwave just before use.

During the outbreak one extra bottle of each milk formula, freshly prepared in our milk kitchen, was set aside for microbiological analysis. Furthermore, we collected samples of the mineral water used in the milk preparations and of the water used to rinse the blender head between preparations.

Microbiological methods. Anal swabs and stomach aspirates were inoculated on four agar plates: tryptic soy agar (Life Technologies, Paisley, Scotland) supplemented with 5% sheep blood, and MacConkey agar, Eiken's medium, and Lactobacillus medium for the detection of enteric organisms. In addition, a sample of powdered milk formula was taken from the kitchen and plated on the same media as used for clinical cultures.

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TABLE 1. Clinical characteristics of neonates with NEC (June-July 1998)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Date of birth (day/mo)</th>
<th>Gestational age (wk)</th>
<th>Birth wt (g)</th>
<th>Length of stay (days)</th>
<th>Type of feeding</th>
<th>Start of feeding (day/mo)</th>
<th>Date of onset (day/mo)</th>
<th>NEC stagea</th>
<th>E. sakazakii cultureb</th>
<th>Date of death (day/mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>05/05</td>
<td>27</td>
<td>850</td>
<td>120</td>
<td>Alfaré</td>
<td>19/06</td>
<td>29/06</td>
<td>I</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>21/05</td>
<td>31</td>
<td>1,930</td>
<td>64</td>
<td>Prêmatil</td>
<td>05/06</td>
<td>06/06</td>
<td>II</td>
<td>–</td>
<td>NP</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>22/05</td>
<td>27</td>
<td>995</td>
<td>42</td>
<td>Alfaré</td>
<td>26/06</td>
<td>01/07</td>
<td>III</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>4d</td>
<td>M</td>
<td>22/05</td>
<td>27</td>
<td>965</td>
<td>63</td>
<td>Alfaré</td>
<td>21/06</td>
<td>24/06</td>
<td>III</td>
<td>+*</td>
<td>23/07</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>23/05</td>
<td>29</td>
<td>815</td>
<td>74</td>
<td>Alfaré</td>
<td>11/06</td>
<td>03/07</td>
<td>II</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>08/06</td>
<td>28</td>
<td>1,200</td>
<td>89</td>
<td>Alfaré</td>
<td>12/06</td>
<td>30/06</td>
<td>III</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>10/06</td>
<td>28</td>
<td>1,100</td>
<td>80</td>
<td>Alfaré</td>
<td>16/06</td>
<td>19/06</td>
<td>III</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>14/06</td>
<td>27</td>
<td>590</td>
<td>111</td>
<td>Alfaré</td>
<td>20/07</td>
<td>23/07</td>
<td>II</td>
<td>+*</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>16/06</td>
<td>31</td>
<td>1,350</td>
<td>47</td>
<td>Alfaré</td>
<td>22/06</td>
<td>03/07</td>
<td>I</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>16/06</td>
<td>32</td>
<td>1,290</td>
<td>43</td>
<td>Alfaré</td>
<td>27/06</td>
<td>03/07</td>
<td>I</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>26/06</td>
<td>32</td>
<td>1,550</td>
<td>32</td>
<td>Prêmatil</td>
<td>06/07</td>
<td>07/07</td>
<td>I</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>03/07</td>
<td>30</td>
<td>1,500</td>
<td>32</td>
<td>Prêmatil</td>
<td>06/07</td>
<td>07/07</td>
<td>I</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

a NP, not performed; –, negative; +, positive.
b F, female; M, male.
c L, suspected disease; II, definite disease; III, advanced disease.
d Twin brother of patient 3.

Molecular typing. Molecular typing was performed by arbitrarily primed PCR (AP-PCR). Ten strains of E. sakazakii isolated in our laboratory from nine patients between 1989 and 1996 were used as control isolates. All patient, environmental, and control isolates were examined in a single assay to reduce intertest variability. Briefly, target DNA was prepared from bacteria grown overnight at 37°C on Mueller-Hinton agar (Difco Laboratories, Sparks, Md.) supplemented with 5% sheep blood. DNA samples were cultured with the BBL Septi-Chek system (Becton Dickinson, Cockeysville, Md.) by inoculation of 1 to 3 ml of milk in a 20-ml brain heart infusion broth bottle. Each environmental sample was inoculated directly on tryptic soy agar supplemented with 5% sheep blood, on MacConkey agar, and ±1 ml of the sample was inoculated in fastidious anaerobe broth (Lab M, Bury, England) for enrichment. Agar plates and broth were incubated aerobically at 37°C. All enrichments were reincubated on four agar plates after 48-h incubation. Isolates were identified as E. sakazakii by standard laboratory methods (10).

RESULTS

Patients. A total of 12 patients (24%; 55% of the neonates with a birth weight of <2,000 g) were identified to have clinical signs of NEC during June-July 1998 (Table 1). Four patients required operative treatment (stage III). For the twin brothers the NEC turned out to be fatal (patients 3 and 4).

All twelve neonates had a birth weight of <2,000 g and had been fed orally with formula milk before the development of NEC. During June-July 1998, 10 of the 12 neonates with NEC received the same semielemental formula with low osmolarity (Alfaré, produced by Nestlé, Nunspeet, The Netherlands), compared to 4 of the 38 without NEC (P < 0.0001 [Fisher’s exact test]).

During June-July 1998, 6 of the 12 neonates with NEC had positive cultures for E. sakazakii, compared to 0 of the 38 without NEC (P < 0.0001 [Fisher’s exact test]). Furthermore, 6 of the 14 neonates who received Alfaré had positive cultures for E. sakazakii, compared to 0 of the 36 who did not receive the formula (P = 0.0002 [Fisher’s exact test]). A total of 11 E. sakazakii strains from the six culture-positive NEC patients were isolated. The anal swab of patient 6 yielded Enterobacter cloacae, Escherichia coli, and Enterococcus faecalis. E. sakazakii was isolated from blood culture (patient 4), from anal swabs (patients 4, 7, 8, 9, and 11), and from stomach aspirates (patients 1, 8, and 9). In the blood culture of patient 4 and the anal swab of patient 9 two morphologically different E. sakazakii isolates were identified.

Environmental samples. Cultures of the extra prepared milk bottles from our milk kitchen revealed the presence of E. sakazakii in several Alfaré milk preparations. Cultures of milk formulas of other brands were negative or resulted in the isolation of Bacillus spp., coagulase-negative staphylococci, or Acinetobacter spp. Cultures of the mineral water and the rinsing water remained negative.

To exclude the possibility of contamination during preparation and storage, we performed cultures for unopened cans of Alfaré milk. By inoculating 3 g of powder directly in fastidious broth, E. sakazakii could be isolated from several unopened cans of one of the two batches of Alfaré milk present in our kitchen stocks (SPNAV-CT, manufactured January 1998, expiration January 2000). A total of 14 E. sakazakii strains from Alfaré milk were isolated.

Molecular typing. Molecular typing by AP-PCR was performed for 9 isolates from five patients, 14 milk isolates, and 10 control isolates (Table 2). Two patient isolates, from the anal swabs of patients 7 and 8, were not stored and thereby not available for molecular typing.
Three different profiles of *E. sakazakii* (Ia, II, and III) were found among the nine patient isolates (Fig. 1 and 2). The 14 milk isolates and four patient isolates from three patients (patients 8, 9, and 11) shared profile Ia. Profile III isolates were recovered from patients 1, 4 and 9, while profile II was found only for patient 4. The two morphologically different *E. sakazakii* isolates from the blood culture of patient 4 and the anal swab of patient 9 also had different molecular profiles, II/III and Ia/III, respectively.

Eight different profiles (Ib, IV, V, VI, VII, VIII, IX, and X) were found among the 10 control isolates. The two isolates from the same patient (C5 and C6) showed the same profile (IX). One control isolate (C7), collected in 1994 from a gastrosotomy tube of a premature infant, showed profile Ib, almost identical to the milk-related profile Ia.

**Actions.** The use of Alfaré powdered milk formula was stopped in our NICU on 10 July 1998, immediately after we suspected a possible link between Alfaré milk, *E. sakazakii*, and development of NEC. However, because our initial cultures demonstrated the presence of *E. sakazakii* only in prepared milk and not in Alfaré powder, the formula was reissued again on 20 July 1998 for the feeding of one infant (patient 8). This patient developed symptoms of NEC on 23 July 1998, and *E. sakazakii* was isolated from her stomach aspirate and anal swab. At the same time further cultures demonstrated the intrinsic contamination of Alfaré powdered milk with *E. sakazakii*. From then on, feeding with Alfaré was completely stopped.

The manufacturer’s microbiological quality control data for the batch SPNAV showed that, of the five samples analyzed, one yielded 20 coliforms/g whereas in the other four samples fewer than 1 coliform/g was found. These results fulfilled the requirements of the Codex Alimentarius (11), i.e., a minimum of four of five control samples with <3 coliforms/g and a maximum of one of five control samples with >3 but ≤20 coliforms/g. However, the manufacturer’s microbiological quality control data did not fulfill the requirements of Belgian law (3), i.e., <1 coliform/g in all control samples. This observation led to the recall of the contaminated batch SPNAV from the Belgian market.

After this incident, the production facility in Nunspeet, The Netherlands, was upgraded, appropriate hygienic measures were taken, and more stringent release norms for dietetic specialties (<0.3 coliform/g, 0 *E. sakazakii* isolates/10 g) were applied by Nestlé. In our NICU, Alfaré powdered milk formula was administered again in April 1999. Until now no further cases of NEC associated with the isolation of *E. sakazakii* were observed.

**DISCUSSION**

We described a cluster of 12 neonates with NEC treated at our NICU in June-July 1998. *E. sakazakii*, a rare pathogen known to cause severe neonatal sepsis and meningitis (1, 2, 4, 12–14, 16, 19, 22, 25, 27–28, 30) and to contaminate powdered milk formula (21), was isolated from 6 of the 12 neonates. After a review of feeding procedures, a significant association was found between the development of NEC, the consumption of a brand of powdered milk formula, and the isolation of *E. sakazakii* in neonates. *E. sakazakii* could be isolated from several unopened cans of a single batch of the implicated formula powder. Molecular typing by AP-PCR confirmed strain similarity between all milk powder isolates and three patient isolates. When the use of this formula was discontinued, the outbreak of NEC came to an end. One infant who received the implicated formula after its use was stopped developed NEC and was colonized with *E. sakazakii*. From all these elements of our cohort study, we conclude that there is a strong, if not causal, relationship between intrinsic contamination of powdered milk formula with *E. sakazakii* and the development of NEC.

The AP-PCR assay used for the molecular typing of *E. sakazakii* has high discriminatory power, as shown by the typing results for the control isolates. The results of the AP-PCR, confirmed by a ribotyping assay performed independently at the Nestlé Research Centre, Lausanne, Switzerland (data not shown), were surprising, as we expected an identical profile for all patient and milk isolates. Because Nazarowec-White and Farber already had demonstrated the presence of different genotypes of *E. sakazakii* in different samples of formula from one company (24) and because another source of a rarely
isolated organism such as \textit{E. sakazakii} coinciding with the formula source seems unlikely, we still suspect the formula to be the source of the three molecularly different patient isolates. The molecular profile Ia found in all milk isolates may possibly suggest that this was the most predominant profile present in the Alfaré milk.

It is interesting that one control isolate (C7) had the profile Ib, almost identical to the milk-related profile Ia. This strain was isolated in 1994 from a gastrostomy tube of a prematurely born girl. The infant suffered from an infection at the gastrostomy tube insertion site after she received Alfaré formula through the tube. The isolation of a closely related \textit{E. sakazakii} strain 4 years before the outbreak may point to an already long-lasting contamination problem.

Only a few outbreaks linking \textit{E. sakazakii} with contaminated milk have been reported in the literature. The first report from The Netherlands described eight cases of neonatal meningitis and sepsis due to \textit{E. sakazakii} (22). Two of the eight cases had NEC and meningitis simultaneously. \textit{E. sakazakii} was isolated from prepared milk formula, a dish brush, and a stirring spoon, but different plasmid profiles were observed for patient and environmental isolates. In Iceland meningitis caused by \textit{E. sakazakii} was reported in three cases (4). There is no mention of NEC in any of these cases. \textit{E. sakazakii} was isolated at low concentrations from the milk powder. Patient and environmental isolates had identical biotypes, antibiograms, and plasmid profiles (8). There was evidence that the formula bottles were occasionally kept at 35 to 37°C for extended periods in bottle heaters. An outbreak of \textit{E. sakazakii} in Memphis, Tennessee, involved a total of four neonates (27). Three patients had sepsis, and three had bloody diarrhea. All patients had stool colonization. \textit{E. sakazakii} isolates with the same plasmid profile were cultured from the patients, an open can of powdered milk formula, and the blender, which showed heavy growth of the organism (8).

The role of powdered milk formula in the development of NEC should not be underestimated. Milk formula can serve not only as an ideal substrate for bacterial growth but also as a source of possible pathogens, as most formula products are intrinsically contaminated. Outbreaks of NEC linked to contaminated milk formula might be missed if the isolated microorganisms are frequent nosocomial pathogens that do not arouse immediate suspicion as \textit{E. sakazakii} does. It has also been shown that confirmed NEC is 10 times as common in babies fed only formula than in those fed only breast milk (18). Until now this observation has been explained by the presence of protective immunoglobulins (immunoglobulin A) in breast milk. Alternatively, we suggest that breast milk is less frequently contaminated with pathogens that can be held responsible for the development of NEC. The frequent isolation of \textit{Enterobacteriaceae}, especially those belonging to the genus \textit{Enterobacter}, in NEC and in powdered milk formula may also suggest the involvement of orally administered contaminated formula in the development of NEC. In a study of the preantibiotic bacteriology in 125 neonates with NEC (7), \textit{Enterobacter} spp. were the most common organisms, isolated in 29% of

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1}
\caption{AP-PCR profiles of the \textit{E. sakazakii} isolates. P1 to P9, patient isolates; M1, M4, and M11, milk isolates; C1 to C10, control isolates; EC, a laboratory strain of \textit{E. cloacae}; NC, negative control; SM, molecular size marker (see Table 2 for origins of isolates).}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{Dendrogram of the AP-PCR profiles as analyzed with GelComp2 v4.1. P1 to P9, patient isolates; M1, M4, and M11, milk isolates; C1 to C10, control isolates (see Table 2 for origins of isolates).}
\end{figure}
the patients. On the other hand Muytjens et al. examined a total of 141 different powdered formulas obtained in 35 countries for the presence of members of the Enterobacteriaceae (21). Members of the genus Enterobacter were most frequently isolated: E. agglomerans was cultured from 35 formulas (25%), E. cloacae was cultured from 30 formulas (21%), and E. sakazakii was cultured from 20 formulas (14%) of the 141 formulas examined. The high thermal resistance of Enterobacter spp. in comparison to other members of the Enterobacteriaceae can possibly explain their high prevalence in powdered and prepared formula milk (23).

When a neonate develops NEC, especially when Enterobacter spp. are cultured, a careful examination of feeding procedures is mandatory. Strict hygienic measures must be taken in preparing formula milk (6). Milk bottles should never stay in a bottle heater for more than 15 min to keep the possibility of preparing formula milk (23).

The contaminated lot of Alfaré milk involved in this outbreak fulfilled the requirements of the Food and Agricultural Organization of the United Nations (11). As recommended before (20–21, 25, 27), more stringent release norms regarding microbial contamination of powdered infant milk formula need to be applied, especially in the neonatal setting. The presence of even low-grade pathogens in powdered formula cannot be allowed. The use of commercial, sterilized liquid formula can be a solution to this problem, avoiding the intrinsic powder contaminants and the potential for extrinsic contamination at the time of rehydration. However, liquid formulas are generally more expensive and require larger transport and storage facilities. Furthermore, lower quantities and different concentrations of a formula are used in neonatal care to suit specific nutritional needs. Therefore, this solution is commercially not feasible for most formula milk producers, although it could probably save children’s lives.

REFERENCES


