**Enterobacter sakazakii** in Dried Infant Formulas and Milk Kitchens of Maternity Wards in São Paulo, Brazil

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**ABSTRACT**

This study was the first conducted in Brazil to evaluate the presence of *Enterobacter sakazakii* in milk-based powdered infant formula manufactured for infants 0 to 6 months of age and to examine the conditions of formula preparation and service in three hospitals in São Paulo State, Brazil. Samples of dried and rehydrated infant formula, environments of milk kitchens, water, bottles and nipples, utensils, and hands of personnel were analyzed, and *E. sakazakii* and *Enterobacteriaceae* populations were determined. All samples of powdered infant formula purchased at retail contained *E. sakazakii* at <0.03 most probable number (MPN)/100 g. In hospital samples, *E. sakazakii* was found in one unopened formula can (0.3 MPN/100 g) and in the residue from one nursing bottle from hospital A. All other cans of formula from the same lot bought at a retail store contained *E. sakazakii* at <0.03 MPN/100 g. The pathogen also was found in one cleaning sponge from hospital B. *Enterobacteriaceae* populations ranged from 10¹ to 10⁵ CFU/g in cleaning aids and <5 CFU/g in all formula types (dry or rehydrated), except for the sample that contained *E. sakazakii*, which also was contaminated with *Enterobacteriaceae* at 5 CFU/g. *E. sakazakii* isolates were not genetically related. In an experiment in which rehydrated formula was used as the growth medium, the temperature was that of the neonatal intensive care unit (25°C), and the incubation time was the average time that formula is left at room temperature while feeding the babies (up to 4 h), a 2-log increase in levels of *E. sakazakii* was found in the formula. Visual inspection of the facilities revealed that the hygienic conditions in the milk kitchens needed improvement. The length of time that formula is left at room temperature in the different hospitals while the babies in the neonatal intensive care unit are being fed (up to 4 h) may allow for the multiplication of *E. sakazakii* and thus may lead to an increased health risk for infants.

Bacteria of the genus *Enterobacter* are considered opportunistic pathogens that rarely causes illness in healthy individuals (14, 25). However, *E. sakazakii* is of concern both to public health and food industries (13) and has been implicated in several outbreaks and sporadic cases of illness involving mainly neonates. The infection has a high case fatality rate of 40 to 80%, and survivors often suffer from severe neurological disorders (26). A complete review of *E. sakazakii* was published previously (18).

*E. sakazakii* belongs to the family *Enterobacteriaceae* (EB) and is not part of the normal microflora of the human or animal gastrointestinal tract (12). Little is known about the ecology, taxonomy, and virulence of *E. sakazakii*. Although the reservoir and mode of transmission of this microorganism have not been clearly identified, powdered infant formula (PIF) has been implicated in a number of outbreaks of neonatal meningitis (4, 8, 19, 40, 43). Because of its capacity to accumulate trehalose (7), *E. sakazakii* has the ability to survive for long periods of time in foods of low water activity and can form biofilms (23).

Several researchers have examined the microbiological quality of dehydrated infant formulas, including the incidence of *E. sakazakii* (22, 31, 32, 37). However, information on pathogen contamination of PIF sold in developing countries and on the incidence of diseases related to the consumption of contaminated PIF is scarce. In studies conducted in hospitals in developed countries during outbreaks of *E. sakazakii* infection, equipment used for formula rehydration was an important source of the pathogen (3, 5).

The objective of this work was to evaluate for the first time the population and prevalence of *E. sakazakii* and EB in retail PIF, in hospital environments, on utensils, on the hands of formula handlers, and in dried and rehydrated infant formula from milk kitchens from three maternity wards in São Paulo, Brazil. The behavior of the isolated strains was also evaluated using the hang time conditions (conditions during the period when rehydrated formula is administered to an infant) in the different maternity wards.

**MATERIALS AND METHODS**

**Sampling.** A total of 186 milk-based PIFs specifically made for infants 0 to 6 months of age were examined. Retail samples (150 cans) representing 50 lots from two manufacturers (30% from manufacturer X and 70% from manufacturer Y) were bought from April to September in São Paulo city. The other 36 cans of formula represented 12 lots that were being used in milk kitchens in maternity wards in the São Paulo city region and were collected on the day of the visits.
Other samples (256) were collected from different sources. Swab samples were collected from different locations (3M, St. Paul, Minn.). Each sample comprised four 100-cm² areas. Upon arrival at the laboratory, 100 ml of Letheen broth was added to the environmental sample sponges, which were then stomached for 1 min. Cleaning aids (sponges and brushes) used in the milk kitchens were sampled by washing them in 100 ml of Letheen broth. Empty bottles and rubber nipples were sampled by washing them in 30 ml of 0.85% NaCl solution (LabSynth, São Paulo, Brazil), and workers’ hands were sampled by washing them in 100 ml of the same solution (9). At the hospitals, water, freshly prepared formula, 5-h cold-stored formula, and formula leftovers were aseptically transferred to sterile laboratory bottles. All samples were kept in insulated boxes and transported within 3 h to the laboratory where they were analyzed. Each hospital was visited once each month for four consecutive months, from May 2005 to March 2006.

**E. sakazakii determination in dried infant formula (MPN technique).** All media were obtained from Oxoid (Basingstoke, UK), except when stated otherwise. Three portions of dried formula (100, 10, and 1 g) were aseptically taken from each can. The ISO method (TC 34/SC 5N) (21) was followed using Drag-gan-Forsythe-Iversen (DFI) agar instead of Enterobacter sakazakii isolation agar, and the most-probable-number (MPN) technique (42) was used.

Samples were decimally diluted with buffered peptone water (BPW), homogenized, and then incubated overnight at 37°C. Aliquots (0.1 ml) from each flask were transferred to 10 ml of modified lauryl sulfate tryptose containing 10 µg/ml vancomycin (InLab-HCL Purex) and incubated at 45°C for 24 h. The broths were streaked onto DFI agar plates and incubated at 37°C for 24 h. Presumptive E. sakazakii colonies were streaked onto tryptone soya agar (TSA) and incubated at 37°C for 24 h. Identification was confirmed by α-glucosidase production (Rosco Diagnostica, Taastrup, Denmark) and by melibiose and sucrose fermentation (R&F Laboratories, Downers Grove, Ill.). The levels of E. sakazakii were estimated by using the MPN table (6).

**E. sakazakii determination in rehydrated infant formula (MPN technique).** Three portions of rehydrated formula (10, 1 and 0.1 ml) were added to enrichment broth (1:10) and processed using the same procedure as described for the dried formula.

**E. sakazakii determination in other samples (presence or absence).** Homogenized aliquots of 10 ml of each sample were transferred to 90 ml of BPW, which was incubated overnight at 37°C and processed as described for the formula samples.

Identification of isolates was confirmed using the BAX PCR System and the Ribroprinter (DuPont Qualicon, Wilmington, Del.). Strains were kept at −70°C for further use.

**EB enumeration.** Portions of 10 g of dried or 10 ml of rehydrated infant formula were added to 40 ml of 0.85% NaCl and homogenized. For all other samples, 1-ml samples were added to 9 ml of the same diluent. Decimal dilutions were prepared and plated onto Petrifilm EB count plates (3M) and incubated at 37°C for 24 h. Characteristic colonies were counted, and populations reported as CFU per gram or milliliter.

**Subtyping of E. sakazakii.** Strains of E. sakazakii were typed using pulsed-field gel electrophoresis (PFGE) using the restriction endonuclease XbaI based on the protocol developed for *Listeria monocytogenes* by the Centers for Disease Control and Prevention (CDC) (15). The universal standard was used as a marker for PFGE experiments (20).

**In vitro growth in rehydrated formula.** Each of the three strains isolated in this study and the control strain (CDC 7006) were inoculated into tryptone soya broth (TSB) and incubated at 37°C for 24 h. Decimal dilutions were prepared, and 10⁶ CFU/ml was transferred to fresh TSB, which was incubated at 37°C for 10 h until the stationary phase was reached (data not shown). Aliquots were then inoculated into 100 ml of formula rehydrated with sterile distilled water and prepared according to the manufacturer’s instructions to achieve 10⁵ CFU/ml. TSB flasks (100 ml) also were inoculated as control. Flasks were incubated at 25°C (room temperature in the neonatal intensive care unit [NICU]), and samples were taken every hour for up to 10 h and then after 24 h. Aliquots were decimal diluted, poured plated onto TSA, and incubated at 37°C for 24 h. Experiments were repeated three times.

**Analysis of growth data.** Growth data were adjusted to the Baranyi and Roberts (2) equations through the use of Baranyi’s DMFIT program. The following bacteria growth kinetic parameters were estimated: growth rate per hour (k), lag time (λ), and generation time (k = ln 2/g).

**Statistical analysis.** A mixed model (fixed and random effects) was used to test the mean of comparisons between the different treatments (28). This model was used because of the longitudinal design and the independence between the times of analysis, which is not a valid presupposition. The PROC MIXED procedure of the software SAS 9.0 was used for the analysis.

**RESULTS AND DISCUSSION**

**Microorganisms.** The populations of *E. sakazakii* and EB in 185 (99.5%) of the dried infant formula samples analyzed were <0.03 MPN/100 g and <5 CFU/g, respectively. One sample contained 5 CFU/g EB and 0.3 MPN/100 g *E. sakazakii*. *E. sakazakii* was not isolated from any of the five other cans of the same production lot. Results similar to those found in this study have been reported by other groups. Muytjens et al. (30), Block et al. (5), and Restaino et al. (35) did not find *E. sakazakii* in the dried infant formula samples they examined. Other researchers have isolated the pathogen but at lower frequencies and levels (4, 29, 31, 32, 38). In contrast, Santos et al. (37), using the BAX-PCR system, found that 12 of 86 dried infant formula samples examined in Brazil were positive for *E. sakazakii*. However, these researchers did not actually isolate viable organisms from the formula. Results similar to those of Santos et al. were found in Indonesia (11), and a higher frequency of positive samples was reported in Mexico (40).

The levels of EB found in the samples collected from the milk kitchens are presented in Table 1. Twelve samples of rehydrated formula contained populations of EB greater than 10² CFU/ml, and all of these samples came from fresh-ly prepared bottles from hospital C. Samples from the plastic jar used in this hospital to transfer the formula to the feeding bottles contained EB levels ranging from 4.0 × 10³ to 4.8 × 10⁴ CFU on the first three sampling dates. The bench where the formula was prepared and distributed produced EB levels on the first two sampling dates of 30 CFU/
TABLE 1. Levels of Enterobacteriaceae in samples collected in milk kitchens of three hospital maternity wards in São Paulo, Brazil

<table>
<thead>
<tr>
<th>Enterobacteriaceae population</th>
<th>Environmental samples</th>
<th>Utensil samples</th>
<th>Cleaning aid samples</th>
<th>Water samples</th>
<th>Operator hand samples</th>
<th>Leftover formula samples</th>
<th>Rehydrated formula samples</th>
<th>Total samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–10</td>
<td>22 (91.6)</td>
<td>89 (94.7)</td>
<td>8 (33.3)</td>
<td>12 (100)</td>
<td>26 (96.3)</td>
<td>4 (44.4)</td>
<td>48 (80.0)</td>
<td>209 (83.6)</td>
</tr>
<tr>
<td>10–10^2</td>
<td>1 (4.2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>10^3–10^4</td>
<td>1 (4.2)</td>
<td>2 (2.1)</td>
<td>2 (8.3)</td>
<td>0</td>
<td>1 (3.7)</td>
<td>0</td>
<td>2 (3.3)</td>
<td>8 (3.2)</td>
</tr>
<tr>
<td>10^2–10^5</td>
<td>0</td>
<td>1 (1.1)</td>
<td>7 (29.2)</td>
<td>0</td>
<td>0</td>
<td>5 (55.6)</td>
<td>2 (3.3)</td>
<td>15 (6.0)</td>
</tr>
<tr>
<td>10^5–10^6</td>
<td>0</td>
<td>2 (2.1)</td>
<td>5 (20.9)</td>
<td>0</td>
<td>0</td>
<td>8 (13.4)</td>
<td>15 (6.0)</td>
<td></td>
</tr>
<tr>
<td>&gt;10^6</td>
<td>0</td>
<td>0</td>
<td>2 (8.3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (0.8)</td>
<td></td>
</tr>
</tbody>
</table>

*a* For rehydrated formula samples and water, units are CFU per milliliter; for environmental samples, units are CFU per square centimeter; for all other samples (brushes, sponges, cups, jars, spoons, bottles, sieves, and blenders), units are CFU per item.

*b* Includes both freshly prepared formula (36 samples) and formula stored in the refrigerator for 5 h (24 samples).

cm² and 10² CFU/cm², respectively. On the first visit, EB was found in samples from the blender (1.1 × 10² CFU), whereas on the second visit the rubber nipples contained 7 × 10² CFU per nipple (data not shown).

Cleaning aids (sponges and brushes) from all three hospitals harbored EB populations ranging from 2.6 × 10² to 5.4 × 10⁵ CFU per item. Cleaning sponges and brushes from hospital B were positive for EB on only two of the four occasions, whereas EB was found in hospitals A and C on every visit. Other than cleaning sponges and brushes from hospitals A and B, all other samples contained EB levels at either <10 CFU/ml per item or <2.5 CFU/cm² for environmental samples.

The hands of milk kitchen workers were, except on one occasion, “clean,” i.e., they contained <10 CFU of EB per hand. The one contaminated sample (10² CFU) was from one of the workers from hospital C on the third sampling day (Table 1).

*E. sakazakii* was found in 2 (0.8%) of the 256 samples from the milk kitchens: a cleaning sponge from hospital B and a sample of leftover formula from hospital A. EB populations in these samples were 2.2 × 10⁴ CFU per sponge and 1.8 × 10³ CFU/ml, respectively. PFGE and ribotyping of the three *E. sakazakii* isolates revealed no genetic relationship between them.

The conditions of formula preparation, kitchens, and distribution were evaluated in the three maternity wards. The hospital A milk kitchen (school and hospital) has two clean areas (one exclusively for formula preparation) and one washing area. Four staff and two nutritionists work on each shift. This milk kitchen is responsible for preparing and distributing formula to daycare centers in its region. The average volume of formula prepared ranged from 25 to 30 liters per day. Formulas were rehydrated with fresh boiled mineral water and distributed in sterile plastic milk bottles. The temperature of the water was measured during each visit and was approximately 60°C. Twice the number of filled bottles needed for in-house use were separated, and half of them were immediately transported to the NICU and the nurseries. The other half were stored under refrigeration to be used 5 h later. The average temperatures for the refrigerator ranged from 17.5 (maximum) to 10.5°C (minimum), indicating storage temperature abuse during all four visits (data not shown). Although there were clear instructions from the nutritionists to administer the formula immediately, the nurses would often keep the formula in a water bath at the nursery or NICU for up to 1 h. The temperatures of this water bath were 60, 32.8, 46.8, and 50°C at the time of the four visits, respectively. Clearly, a consistent approach for storing formula was not used.

The milk kitchen at hospital B (public) was smaller than that at hospital A, with only one worker per shift and only 3 to 5 liters of formula prepared each day. This milk kitchen comprised one clean room and one washing area; the washing was done sometimes in the clean area. Formula was rehydrated and treated in a manner similar to that in hospital A, with some of the filled bottles kept under refrigeration to be used later. The average refrigerator storage temperatures ranged from 11.5 (maximum) to 9.3°C (minimum) (data not shown).

In both hospitals A and B, the temperature of the refrigerator used to store prepared formula was well above that considered a good manufacturing practice (GMP) and that given in the World Health Organization (WHO) guidelines (44). Rehydrated formula, as any other preprepared food, should be kept at temperatures below 5°C to avoid the growth of microorganisms.

Hospital C (private) has a medium-size milk kitchen, with two rooms (clean and washing) and two workers per shift. On average, 10 liters of formula were prepared each day. The formula was rehydrated with filtered tap water at room temperature in a blender. The product was sieved into a plastic jar and distributed into sterile milk bottles that were immediately sent to the nursery and NICU. Even though hospital C appeared to be operating in an organized and clean environment, the highest number of EB-positive samples (17 of 51) was found in this hospital. The samples most frequently contaminated were cleaning aids (sponges and brushes) and the plastic jar used to transfer the formula to the milk bottles. The work bench in the formula preparation area was also positive on two occasions, with EB populations of 3 × 10 CFU and 10² CFU per bench. Other samples that harbored EB included a rubber nipple, the internal surface of a blender, and the hands of one of the
workers. The only EB-contaminated rehydrated formula sample was from this hospital. However, E. sakazakii was not found in any samples from this hospital. After the first visit to hospital C, suggestions were made to the nutritionist to improve the general hygienic practices. Some of the suggestions were eventually implemented, e.g., sanitizing the blenders with sodium hypochlorite solution after washing. Boiled water cooled down to 60°C, as used in the other hospitals to rehydrate the PIF, was not being used during our first three visits. However, during the fourth visit, samples were prepared using both their normal procedure and using boiled water cooled down to 60°C. The total EB counts obtained were <5 CFU/ml for formula prepared with boiled water and 10² to 10³ CFU/ml for formula prepared with tap water. The protocol was repeated twice, with similar results. The results helped staff to understand why it was necessary to make this change in infant formula preparation. Recent WHO guidelines (44) suggest the use of water at 70°C to rehydrate PIF. However, the results of our study indicate that water at 60°C also can be effective for reducing the numbers of microorganisms. According to the nutritionists, the lower temperature is safer to work with and causes less nutrient loss in the product. In another study (36), the researchers found that PIF can be rehydrated with refrigerated water without increasing the risk of E. sakazakii growth if the rehydrated formula is kept for a short time at room temperature and efficient cold storage (≤4°C) is used.

In hospitals A and B, milk-based dehydrated formula from the two major manufacturers in Brazil (different commercial brands) was in use during each visit. Hospital C used formula from only the major producer.

Few studies have been reported regarding the occurrence of E. sakazakii in milk kitchens, and most of those studies were conducted to investigate the cause of outbreaks. Biering et al. (4), trying to identify the source of a meningitis outbreak in neonates caused by E. sakazakii in Iceland, evaluated the environmental conditions of a nursery and milk kitchen. They also examined the steps used to prepare infant formula in the hospital. The pathogen was isolated in only one sample of rehydrated PIF, which had been stored for an unknown period of time in a refrigerator in one of the nurseries. In addition, E. sakazakii was isolated from cans of dehydrated infant formula from five different lots found in the milk kitchen. The genetic profiles of the isolated strains from the dehydrated infant formula and from the affected neonates were identical, which led to the conclusion that the cause of this specific outbreak was the PIF.

To determine the cause of two cases of E. sakazakii infection in a hospital in Jerusalem, Block et al. (5) analyzed clinical and environmental samples and found the microorganism in samples from the blender and rehydrated infant formula.

The overall appearance of the milk kitchens evaluated in this study was deceiving. Although the milk kitchens in hospital A looked untidy and that in hospital C appeared to be very clean, EB populations were always low in the former and high in the latter. In contrast, the milk kitchen in hospital B looked clean and well maintained but was the only one where we found E. sakazakii. In this hospital, problems with GMPs were observed, which could have led to situations where cross-contamination of the prepared formula occurred from the environment.

Behavior of E. sakazakii. The growth of the three E. sakazakii strains isolated in this study and the control strain (CDC 7006) in both TSB and rehydrated infant formula incubated at 25°C is shown in Figure 1. All the strains behaved similarly compared with the control strain (P > 0.05). Growth rates in PIF were around 0.25 h⁻¹, and mean generation time was 0.36 h (from 0.35 to 0.37 h). After 1 and 4 h of incubation, there was a 0.5- and 2-log increase in numbers, respectively, for the strains grown in rehydrat-
ed infant formula that had no lag time (data not shown). These results differ from those of other studies in which there was a longer lag time for E. sakazakii spiked into rehydrated PIF (16, 17, 22, 24, 27, 32, 39). One explanation for these differences involves the intrinsic characteristics of the strains. Another is the incubation temperature used in this study (25°C). Lenatti et al. (27) reported different growth rates of E. sakazakii strains at different temperatures. However, Khandai et al. (24) found that growth curves obtained at 10, 21, and 29°C were comparable. They also verified that the physiological state of the cells had no apparent effect on lag time. In contrast, Telang et al. (39) reported no significant E. sakazakii population increase during 6 h at 22°C.

In TSB, the initial E. sakazakii population increased about 0.5 log by 3 h, with a multiplication rate slower than that in infant formula and a lag time ranging from 2.63 h for the control strain to 4.35 h for strain 2 (isolated from a cleaning sponge). A significant population increase (P < 0.05) was observed every 4 h of incubation up to 10 h and between 10 and 24 h, independent of the medium. E. sakazakii levels in rehydrated infant formula were higher (P < 0.05) than those in TSB from 4 to 10 h of incubation. However, between 10 and 24 h of incubation at 25°C, E. sakazakii grew at a faster rate in TSB than in infant formula. Other reports of the behavior of E. sakazakii at 25°C were not found.

Different strains of E. sakazakii studied by various investigators have been variable in terms of their growth characteristics, thermal resistances, and osmotic tolerances (10, 13, 22, 27, 32–34). The behavior of the strains isolated in this study, where we simulated the preparation conditions in milk kitchens and the formula feeding conditions in nurseries, was very similar, and rapid growth was observed. Strains of different origins demonstrating similar behavior, as seen in this study, also have been reported (24). The dose-response of E. sakazakii for humans has not been established. However, holding the prepared formula at NICU room temperature (abusive temperature), as in this study, is likely to increase the risk of infection.

According to the nutritionists who work in the milk kitchens visited in this study and according to the WHO guidelines (44), infant formula bottles should be given to infants as soon as the bottles arrive in the nursery. However, sometimes this does not happen. In the hospitals evaluated, the bottles remained in the water bath or at room temperature (25°C) for up to 1 h before being given to the infants. Some infants were fed by nasogastric probe or tubing, and the feeding time ranged from 30 min to 4 h, according to the physician’s recommendations. This hang time is in accordance with some U.S. recommendations (1, 41) but violates the WHO guidelines (44), which suggest that hang time should be no longer than 2 h at room temperature.

In this limited study, we found a low incidence of E. sakazakii in PIFs manufactured and sold in Brazil. Despite the low occurrence of the pathogen in the samples analyzed and the generally low numbers of EB found in milk kitchens, the hygienic procedures and infant formula preparation practices being used in neonatal wards in Brazil need improvement. Our small study uncovered some practices that could be unsafe. For example, maintaining formula for up to 4 h at room temperature, as observed in this study, is a high-risk practice that should be avoided because it can allow the multiplication of E. sakazakii to potentially hazardous levels. In a country such as Brazil, easily understandable guidelines for the preparation, use, and handling of PIF in health care facilities or in homes are needed. It is also necessary to continuously alert and educate caregivers of infants concerning this issue.

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ERRATA

In the article “Enterobacter sakazakii in Dried Infant Formulas and Milk Kitchens of Maternity Wards in São Paulo, Brazil” by Palcich et al., Journal of Food Protection 72(1):37–42, the correct MPN value is <0.3, not <0.03 as published in the Abstract and the Results and Discussion.

A corrected Figure 2 for the article “Temporal Patterns and Risk Factors for Escherichia coli O157 and Campylobacter spp. in Young Cattle” by Ellis-Iversen et al., Journal of Food Protection 72(3):490–496, appears here.