

***Enterobacter sakazakii*: An Emerging Food Pathogen**

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Introduction

Prompted by recent *Enterobacter sakazakii* infections in neonates and the large percentage of neonates in intensive care units being fed dry infant formula, FDA has sent a warning letter to health professionals recommending that powdered infant formula not be used in neonatal intensive care settings. To address the concerns of manufacturers and users of powdered infant formula, this white paper summarizes relevant information on illnesses associated with *E. sakazakii* infections, the protocols for detection of this pathogen, distinguishing biochemical, molecular, and pathogenic characteristics of this pathogen, the ecology of *E. sakazakii*, and regulations regarding the pathogen in foods.

General Characteristics

E. sakazakii is a gram-negative straight rod belonging to the Enterobacteriaceae family and qualifies as a coliform bacterium. Having dimensions of 3 μm in length and 1 μm in width, the cells are motile by peritrichous flagella and do not form spores. Prior to 1980, *E. sakazakii* was referred to as a “yellow-pigmented” *Enterobacter cloacae* but it was reclassified based on differences in DNA-DNA hybridization, biochemical reactions, antibiotic susceptibility, and production of yellow-pigmented colonies. Two scanning electron microscopic images of this organism may be found on the SciMat web site (http://www.magma.ca/~scimat/E_sakaza.htm).

Illnesses Associated with *E. sakazakii*

Infections associated with *E. sakazakii* have been relatively rare. A review article published in *Medicine* in 2001 summarized the literature from 1960 to 1999 and found in the English literature only 31 cases of *E. sakazakii* infections in neonates, infants, and children, and 4 cases among adults. In the foreign-language literature for which English abstracts were available, 5 additional cases of *E. sakazakii* infections were located. Since that review, three additional case studies have been reported. One involved 12 neonates in Belgium associated with an outbreak in June-July 1998, the second involved 2 neonates and 3 infants in Israel (reported in 2001), and the third occurred in Tennessee in 2001 and involved one neonatal fatality and several other infants who were colonized with the pathogen. An additional case of *E. sakazakii* meningitis resulting in an infant's death in Belgium and an *E. sakazakii* infection in a hospitalized neonate in Chattanooga, TN, occurred in 2002. Despite the low frequency of reported infections, the high mortality rate (33%) and severe neurologic impairment in many survivors has generated a high level of concern for *E. sakazakii* infections.

Risk Factors of Infected Patients

Among the 31 cases involving neonates, infants, and children, ages ranged from 3 days to 4 years with 50% \leq 1 week of age and about 75% $<$ 1 month of age. Low birth weights of 2.5 kg or less have also been identified as a distinguishing feature in about 75% of infected patients. These conditions suggest that infected individuals may be immunocompromised or are lacking sufficient colonization of the gastrointestinal tract with normal bacterial flora to compete with the opportunistic pathogen, *E. sakazakii*. Similarly, in the adult cases, most had underlying

diseases (i.e. malignancies) that could have predisposed them to infection by *E. sakazakii*. In otherwise healthy individuals, *E. sakazakii* infections are unknown. There are cases, however, from which *E. sakazakii* has been isolated from specimens of patients and no signs of infection have been observed.

Clinical Symptoms of *E. sakazakii* Infections

Onset of *E. sakazakii* infections is characterized by signs and symptoms typical of infections caused by other gram-negative organisms. These symptoms include poor feeding response, irritability, jaundice, grunting respirations, and instability of body temperature. In a large number of the neonatal cases, infection progressed to meningitis (an acute inflammation of the membranes of the brain and spinal cord), with survivors suffering from severe neurological impairment. Ventriculitis (inflammation in the ventricles of the brain), brain cysts and abscesses, cerebral infarction, and late development of hydrocephalus (abnormal increase in the amount of cerebrospinal fluid within the cranial cavity) are characteristic of central nervous system infections. Other medical conditions have also been associated with *E. sakazakii* infections, including bacteremia (bacteria in the blood) as well as necrotizing enterocolitis (localized death of small and large intestine tissues). After the first signs of sepsis appear, death may occur within a few hours to several days. In survivors, continued colonization by *E. sakazakii* varies from 2 to 8 weeks.

Treatment and Mortality Rates of *E. sakazakii* Infections

E. sakazakii infections before 1985 were frequently treated with ampicillin, gentamicin, and/or chloramphenicol. After 1985, the third-generation cephalosporins were commonly used in conjunction with ampicillin and gentamycin.

Higher case-fatality rates have been reported for premature or low birth weight infants than full-term or infants with birth weights ≥ 2.5 kg (50% versus 30%). Improvements in treatment, however, have affected the case-fatality rate, with 62% of meningitis patients dying before the use of third-generation cephalosporins and only 14% dying after the introduction of third-generation cephalosporins. Only one death has been reported in the absence of meningitis, a neonate exhibiting bacteremia.

Source of *E. sakazakii* Infections

In early cases of *E. sakazakii* infections, an environmental source of the organism could not be identified. Vertical transmission from the mother's birth canal to newborns was ruled out as cases occurred after caesarean section, and colonization of newborns at birth was not demonstrated. In more recent cases, environmental sampling has yielded evidence of contaminated blenders used to prepare infant formula and recovery of *E. sakazakii* isolates from a dish brush and a stirring spoon. To date, no subtyping has been reported for the isolates recovered from the blenders, whereas the plasmid profiles of the brush and spoon isolates did not match those of the patients. Dried infant formula, on the other hand, has been identified epidemiologically as the source of *E. sakazakii* in three outbreaks of neonatal meningitis and linked to one outbreak of necrotizing enterocolitis. In these outbreaks, *E. sakazakii* isolates from implicated milk powders either had the same plasmid profile, multilocus enzyme profiles, or pulsed-field gel electrophoresis profiles as those isolates from the patients. Presently, approved technology is not available to render powdered milk formulas commercially sterile. Hence, *E. sakazakii* could be present in formulas at substantially lower levels than those considered acceptable by the Codex Alimentarius for levels of coliforms in milk-based powdered infant formula. Prolonged periods of

unrefrigerated storage after hydration of the powdered infant formula before feeding may subsequently enable substantial growth of the organism.

Detection of *E. sakazakii*

Recently, FDA released a protocol for inclusion in the FDA's Bacteriological Analytical Manual (FDA/BAM) for quantitative enumeration of *E. sakazakii* in dehydrated powdered infant formula (<http://www.cfsan.fda.gov/~comm/mmesakaz.html>). In this method, a total of 333 g of product is assayed per sample with three replicate enrichments of 100, 10 and 1 g. Nine portions of Enterobacteriaceae Enrichment (EE) broth are pre-warmed at 45°C before addition to each of the nine subsamples. Samples are gently, but well mixed to suspend solids. Each sample is incubated at 36°C overnight followed by addition of a 10-ml aliquot to 90 ml pre-sterilized EE broth that is also incubated at 36°C overnight. This second EE enrichment culture is streaked and directly plated onto two violet red bile glucose agar (VRBG) plates each. These plates are incubated at 36°C overnight, and up to five typical colonies are streaked onto Trypticase Soy Agar (TSA) and incubated at 25°C for 48-72 h. Only typical yellow-pigmented colonies on TSA are selected and confirmed by the API 20E biochemical identification system. The MPN per g of product is calculated using the table in the FDA/BAM, Appendix 2 (Most Probable Number from Serial Dilutions).

Media evaluated for the direct plating of food or clinical samples for isolating *E. sakazakii* include MacConkey agar, MacConkey-sorbitol agar, eosin methylene blue (EMB) agar, deoxycholate agar, tergitol 7 agar, xylose-lysine-desoxycholate (XLD) agar, and violet red bile (VRB) agar. Recovery was less on XLD agar than on tergitol 7 agar. On MacConkey, EMB, or deoxycholate agars, *E. sakazakii* strains formed light to dark pink colonies with no precipitated bile around them. Unfortunately, a high percentage of false-negative results occur with direct plating methods. Addition of a preenrichment step to the direct plating approach (lauryl sulfate tryptose broth incubated for 48 h at 44°C and plated onto violet red bile agar for incubation at 44°C for 48 h) reduced the false-negative rate to 8% when combined with direct plating of environmental samples. Recovery of presumptive *E. sakazakii* colonies may be accomplished after transfer to a non-selective medium (trypticase soy agar) and formation of bright buttery yellow colonies on incubation at 25°C for 48 h. Diminished pigment production and lighter yellow colonies will be observed if incubated at 36°C or in the dark, or if multiple transfers of the organism have occurred. Final confirmation of *E. sakazakii* colonies can be done via several systems such as API 20 E™, API ZYM™, or Vitek™ assays.

Biochemical Reactions of *E. sakazakii*

Two major differences between *E. sakazakii* and the other *Enterobacter* species were observed in a study of 226 *Enterobacter* strains (of which 129 were *E. sakazakii*). α -Glucosidase activity was demonstrated in all *E. sakazakii* strains but in none of the other *Enterobacter* strains. Absence of the enzyme phosphoamidase was also unique to *E. sakazakii* isolates. *E. sakazakii* is also distinguished by its ability to ferment sucrose, raffinose, and α -methyl-D-glucoside, but not D-sorbitol, dulcitol, adonitol, or D-arabinol. Seventy to 90% of *E. sakazakii* strains would be classified as coliforms based on their ability to produce gas from lactose at 35°C. In addition, some could be identified as fecal coliforms based on their ability to produce gas in EC broth at 44.5°C within two days. We found that 2 of 4 environmental isolates of *E. sakazakii* produced gas in EC broth held at 44.5°C for 24 h.

The API ZYM™ system may be used to confirm *E. sakazakii* isolates. This system consists of a plastic strip and 20 cupules, with 19 cupules containing substrates and buffers, and 1 cupule serving as a negative control. The strip assays for alkaline phosphatase, butyrate esterase, caprylate esterase-lipase, myristate lipase, leucine

arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, phosphoamidase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase, and α -fucosidase activities. After aerobic incubation in the dark at 36°C for 4 h, presence and degree of enzymatic activity is scored as color intensities 0 – 5 in accordance with a color comparison chart provided by the manufacturer. Distinguishing responses of *E. sakazakii* are the absence in practically all strains of myristate lipase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, β -glucuronidase, α -mannosidase, and α -fucosidase activities. *E. sakazakii* isolates also produce a strong caprylate esterase-lipase reaction and a moderate acid phosphatase reaction. Alteration in the enzyme profile occurs when cells are treated ultrasonically. In particular, positive responses previously observed by *E. sakazakii* for leucine arylamidase, β -galactosidase, β -glucosidase, α -glucosidase, acid phosphatase, and N-acetyl- β -glucosaminidase reactions become negative for ultrasound-treated cells.

Subtyping of *E. sakazakii*

To identify sources of infection, subtyping has been conducted on both environmental and clinical isolates. Phenotypic typing is based on secondary characteristics of bacteria including biochemical reactions, antibiograms, serotyping, and bacteriophage typing. However, the power of these phenotypic methods to differentiate *E. sakazakii* isolates has been poor. For example, fewer differential patterns have been discerned through antibiograms than have been found with molecular typing methods such as ribotyping. Variability in antibiotic resistance that occurs among different colonies (clones) selected from each isolate upon re-testing also severely hampers the usefulness of antibiograms.

The most common molecular methods used for epidemiologic subtyping of *E. sakazakii* include chromosomal DNA restriction analysis, plasmid typing, ribotyping, pulsed-field gel electrophoresis (PFGE) and random amplified polymorphic DNA (RAPD) typing. A study by Health Canada investigators characterizing *E. sakazakii* isolates from dried infant formulas and clinical isolates revealed that biotyping should be used in outbreak situations as a screening tool, whereas RAPD or PFGE should be used to more definitively establish relatedness among isolates. Distinguishable differences in isolates of *E. sakazakii* have been reported with these latter genomic subtyping methods, even in situations when no differences were observed by ribotyping. Overall, a high discriminatory index of 1.000 was reported in PFGE analysis of 18 *E. sakazakii* isolates that indicated a high level of genomic diversity in these strains. The disadvantage in using the electrophoretic typing techniques, however, is the inability to compare results between laboratories because such methods have not been standardized.

Antibiotic Susceptibility of *E. sakazakii*

Reports from 1960-1999 of antibiotic susceptibility of *E. sakazakii* indicate the organism is typically susceptible to ampicillin, tetracycline, chloramphenicol, gentamicin, and the third-generation cephalosporins. However, a study of *E. sakazakii* isolates from patients at the University of Massachusetts Medical School in 1995–1996 revealed these bacteria were uniformly resistant to ampicillin, cefazolin, and extended-spectrum penicillins, and were not uniformly susceptible to the third-generation cephalosporins or the quinolones. The occurrence of antibiotic resistance observed in the UMass isolates is consistent with the recent trend of increased acquisition of antibiotic resistance among all *Enterobacter* species. Although *E. sakazakii* isolates are typically susceptible to aminoglycosides, such antibiotics are not recommended for primary treatment because of poor penetration into the central nervous system.

Pathogenicity of *E. sakazakii*

To date, specific mechanisms of pathogenicity or associated virulence factors of *E. sakazakii* have not been identified. Hence, it is unknown whether the predilection of *E. sakazakii* for neonates reflects intrinsic virulence of the organism or the pathogen's opportunity to be an early colonizer. Given that there is an immunologic similarity between enterotoxins of *E. coli* and other coliforms, there is a strong possibility that such toxins, mediated by plasmid(s) transmissible to other organisms resident in the human intestine, are also virulence factors of *E. sakazakii*. In a review article, Nazarowec-White and Farber reported that *E. sakazakii* was pathogenic in suckling mice when inoculated orally and intraperitoneally at levels of 10^5 and 10^3 CFU, respectively. They reported the production of an enterotoxin-like compound by *E. sakazakii*, but provided no supporting data.

Ecology of *E. sakazakii*

Powdered milk substitute infant formulas are the principal sources from which *E. sakazakii* has been isolated. *E. sakazakii* was isolated from 20 of 141 dried infant formulas from 35 countries in a 1988 survey. A Canadian study of dried infant formulas determined that *E. sakazakii* was present in contaminated samples at cell numbers less than 1 cfu/100g. Detection of such low levels of contamination requires the use of enrichment culture methods. Lack of effective enrichment procedures may account for the inability of investigators prior to 1990 to detect the organism in environmental samples, including surface water, soil, mud, rotting wood, grain, bird dung, rodents, domestic animals, cattle and raw cow's milk. Since 1990, *E. sakazakii* has been isolated from ultra-high-temperature (UHT) milk in cartons, ground meat, fermented bread (khamir) and thermal mineral water springs.

In formula powders, the source of contamination is not known, but post-process contamination from the factory environment seems likely because these products receive thermal treatments prior to and during drying. Dupont Qualicon reports on their internet site (<http://www.qualicon.com/entsak.html>) that *E. sakazakii* contamination was detected in three factories used to produce dried infant formulas. Using the RiboPrinter® system to discriminate 30 *E. sakazakii* isolates from one factory, eight RiboGroup pattern sets were identified. Personnel movement and unhygienic practices were identified by Dupont Qualicon personnel as mechanisms for cross-contamination within the processing facilities. A study sponsored by Orkin Pest Control, Atlanta, GA, revealed the importance of proper sanitation practices in foodservice to prevent *E. sakazakii* contamination, as household flies collected at the backdoor areas and rear dumpsters of four restaurants in Gainesville, FL, were found to carry *E. sakazakii*. Similarly, *E. sakazakii* has been isolated from the gut flora of fruit flies, with no differences in distribution between males and females. Surprisingly, the organism was only isolated from a culture of fruit flies propagated in 1998 and not from a colony of flies over 30 years old. Since flies have been demonstrated to spread bacteria to equipment surfaces, it is important that they be prevented from entering food processing facilities.

Growth of *E. sakazakii*

Two different morphological colony types occur when fresh isolates of *E. sakazakii* are streaked for purity. One type is dry or mucoid with scalloped edges and also rubbery when touched with a loop, whereas the other is typically smooth and nondistinct. The latter type predominates when the isolate is subcultured. Differences in virulence or phenotypic traits between these two colony types is unknown.

E. sakazakii will grow on nonselective media [e.g. tryptic soy agar (TSA)] commonly used in enteric bacteriology. On TSA, colonies 2-3 mm in diameter form within 24 h at 36°C, and if grown at 25°, the colonies will typically be 1-1.5 mm in diameter. Growth is also rapid in tryptic soy broth (TSB) with an increase of 10^5 to 10^9 cfu/ml occurring overnight.

When grown in the presence of D-glucose and citrate either aerobically or anaerobically, there are no obvious requirements by *E. sakazakii* for vitamins, amino acids, or other organic growth factors. The pH ranges for growth can vary considerably, although good growth occurs between pH 5 and 9. Minimum growth temperatures of 5.5 – 8°C have been reported for both clinical and food isolates in reconstituted infant formulas, whereas *E. sakazakii* cell numbers at 4°C decreased or remained constant during storage. The maximum temperature at which visible growth of *E. sakazakii* in TSB occurred was at 47°C. While lag and generation times were generally less for food isolates compared to clinical strains, the differences were not statistically significant. Average generation times were 40 min at 23°C and 4.98 h at 10°C, which are less than for most other bacteria typically found in milk or milk products. Hence, *E. sakazakii* would be expected to predominate if reconstituted formula is stored at nonrefrigerated temperatures. Determination of the competitiveness of *E. sakazakii* has only been reported against *E. cloacae* and in that case, *E. sakazakii* grew to large numbers, whereas there was no growth of *E. cloacae*. The production of a colicin(s) by *E. sakazakii* may enable its predominance. Growth of *E. sakazakii* on surfaces and in biofilms has not been evaluated, although an extracellular polysaccharide which is produced by multiple strains of *E. sakazakii*, would likely serve to enable attachment of the organism to surfaces and provide protection from disinfectants.

Thermal Tolerance of *E. sakazakii*

Heat resistance of ten strains (5 clinical and 5 food isolates) of *E. sakazakii* has been determined at 52, 54, 56, 58, and 60°C in reconstituted dried infant formula. D-values of 54.8, 23.7, 10.3, 4.2 and 2.5 min were obtained for each of these temperatures, respectively. Compared to other *Enterobacteriaceae* isolates, *E. sakazakii* is one of the most thermotolerant organisms in this Family. However, extrapolating thermal inactivation data to HTST pasteurization (72°C, 15 sec) conditions reveals that *E. sakazakii* will not survive pasteurization. Although *E. sakazakii* has been isolated from UHT milk, it is unknown if post-pasteurization contamination occurred or if the organism survived UHT treatment. When reconstituted infant formula was boiled (85-100 sec depending on milk type) by exposure to microwaves (2450 MHz, 600W), survival of *E. sakazakii* was dependent on the milk formula. From an initial inoculum of 100 cfu/ml, no *E. sakazakii* was detected in four commercial brands; however, in another commercial brand, 20 cfu/ml were detected after microwave heat treatment.

Current Regulations Affecting Occurrence of *E. sakazakii* in Powdered Infant Formulas

Regulations governing the prevalence of *E. sakazakii* in powdered infant formulas falls under the hygienic requirements for allowable levels of coliforms. For the Codex Alimentarius, these requirements include 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. Based upon these test parameters, dry milk-based infant formula that contains *E. sakazakii* at levels of < 1 organism per 100 g of formula would not be reliably detected.

Future Research on *E. sakazakii*

More research is needed to determine virulence factors, genetic diversity, appropriate recovery and subspeciation methods, environmental niches, and methods for control of this emerging pathogen in foods and the environment.

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