Microbial Detection of *Enterobacter sakazakii*: Food and Clinical

A White Paper

Contaminants And Natural Toxicants Subcommittee Meeting
***Enterobacter Sakazakii* Contamination**
In Powdered Infant Formula
March 18-19, 2003

by

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E. sakazakii was first associated with cases of neonatal meningitis in 1958 (11). In 1980 while proposing *E. sakazakii* as a new species of *Enterobacteriaceae* the source of one isolate was reported to be from a can of dried milk (4). No detail of the isolation method was described. In 1982, during the investigation of eight neonatal meningitis cases caused by *E. sakazakii* in the Netherlands, Muytjens, et al., 1983 (6) recovered the organism from prepared formula but not from the formula powder itself. In a later study, Muytjens, et al. 1988 (7) suggested that the inability to isolate *E. sakazakii* from powdered formula may have been due to its unequal distribution within the powdered formula or "its presence at such a low concentration that it escaped detection by conventional methods." Conventional methods, as described by the Food and Agriculture Organization (FAO) can detect a lower limit of 3.0 colony forming units (CFU)/g.(5). In 1984, six strains of *E. sakazakii* were isolated from samples of powdered formula using deoxycholate-citrate agar incubated at 37°C for 48 hours; again, no quantitative methods were used (9).

Muytjens et al., 1988 (7) described the first quantitative method for the isolation of *E. sakazakii* from powdered infant formula. In this method, triplicate 100, 10 and 1 gram samples of powdered formula were mixed with 900, 90 and 9 ml, respectively, of buffered peptone water (1:10 dilutions) at 45°C until completely dissolved. Following overnight incubation at 36°C, 10 ml of each culture was inoculated into 90 ml of *Enterobacteriaceae* enrichment broth (EE broth) and incubated at 36°C. After overnight incubation, 1 ml from each enrichment culture was mixed in duplicate with 20 ml of fluid

violet red-bile-glucose agar and poured into petri dishes. The agar was allowed to solidify, then incubated overnight at 36°C. Suspected colonies of *Enterobacteriaceae* were subcultured to sheep blood agar and eosin-methylene blue agar. The API 20E system was used to identify strains. Colonies were confirmed as *E. sakazakii* by testing for the production of yellow colonies on nutrient agar after 48 hr at 25°C, extracellular DNase production on toluidine blue agar and a positive alpha-glucosidase reaction. The levels of *Enterobacteriaceae* present in the formula sample were determined by the most probable number procedure. From 35 countries, 141 powdered formula samples were analyzed. *E. sakazakii* was isolated from 20 samples collected from 13 of the countries. The levels of *E. sakazakii* recovered ranged from 0.36 to 66 CFU/100 g. The lowest level of detection in this method was 0.36 CFU/100 g.

In 1989, Simmons et al. (10) used a similar method to isolate *E. sakazakii* from powdered formula associated with an outbreak in a neonatal intensive care unit located in Memphis, Tennessee. In this study, 50, 10, and 1 gram samples of the implicated infant formula were dissolved in 450, 90, and 9 ml of buffered peptone water, respectively. No additional sample was available. After overnight incubation, 10 ml of each culture was inoculated into 90 ml of EE enrichment broth, incubated overnight, and plated onto violet red-bile-glucose agar. Suspected colonies of E. *sakazakii* were subcultured onto MacConkey agar and confirmed with the API-20E system. No additional biochemical tests were described. The level of *E. sakazakii* present in the powdered formula sample was estimated be 8 CFU/100g.

Following an outbreak of neonatal meningitis in Iceland in 1986 and 1987, *E. sakazakii* was isolated from five different lot numbers of unopened packages of

powdered infant formula; in all, five of seven packages were positive (3). Using an amount of sterile water recommended by the manufacturer, 500 and 1000 gram packages of powder were dissolved and divided into 100 ml samples. Following incubation at 36°C for 4 hours, the samples were subcultured to blood agar and MacConkey agar plates. No quantitative data were reported. Isolates were confirmed by the API 20E system followed by biotyping, plasmid DNA determinations and antibiograms.

During the investigation of a 1998 outbreak of necrotizing enterocolitis that occurred in a neonatal intensive care unit in Brussels, Belgium (12), van Acker et al. isolated *E. sakazakii* from liquid formula prepared from powder (Nestle Alfare). The liquid formula was cultured by direct plating on trypticase soy agar supplemented with 5% sheep blood, on MacConkey agar and by culturing 1.0 ml in fastidious anaerobe broth. This report did not indicate which of these methods produced the positive results. The report stated that the manufacturer's quality control data for the implicated production lot of powder indicated that one of 5 sub-samples contained 20 coliforms per gram of powder and that the remaining 4 sub-samples contained less than 1 coliform per gram. These data support the hypothesis that the distribution of microbial contaminants in a lot of powder may be heterogeneous. If the distribution of coliforms (specifically, *E. sakazakii*) in lots of powdered formula is typically heterogeneous, or non-random, then it may be important to test sub-samples from each lot in order to improve the chances of correctly determining the acceptability of the lot (5).

In the same study (12), van Acker reported that unopened cans of the implicated Alfare powdered formula were tested for *E. sakazakii* by inoculating 3 grams of powder directly into fastidious broth. After 48 hr incubation at 37°C, the enrichments were plated

on tryptic soy agar supplemented with 5% horse blood, hemin T, and NAD; on tryptic soy agar with 5% horse blood and nalidixic acid; on MacConkey agar and on mannitol salt agar. The organism was isolated from several unopened cans of one of two production lots of formula in the hospital's inventory. No attempt was made to quantify the level of *E. sakazakii* present in the formula.

During a Canadian survey of the incidence of *E. sakazakii* in dried infant formula (8), the method of Muytjens et al. (7) was used with only minor modifications. The powder was suspended in sterile water and suspect colonies from the VRBG agar plates were subcultured to TSB-YE agar prior to confirmation using the API 20E system. No additional biochemical tests were performed and levels of *E. sakazakii* were determined by the most probable number technique. The sensitivity of this method was equal to that of the original method (7). *E. sakazakii* was isolated from 8 of 120 cans, representing 5 different manufacturers, at a level of 0.36 CFU/100 g.

E. sakazakii was isolated from commercial powdered formula during the investigation of a fatal infection in a hospitalized neonate (MMWR Weekly, April 12, 2002/51(14):298-300). The method used by the Centers for Disease Control and Prevention was based on the method of Muytjens, et al. 1988 (7). One hundred grams of powdered formula was mixed with 400 ml of phosphate buffered peptone water at 45°C. Following overnight incubation at 36°C, 1 ml of the culture was inoculated into 9 ml of brilliant green bile broth. After overnight incubation at 36°C, the enrichment culture was inoculated onto LesEndo agar and incubated overnight at 36°C. Colonies having a green metallic sheen were subcultured to TSA-5% sheep blood agar plates, incubated at 36°C overnight then held at room temperature for 24-48 hr. Yellow pigmented colonies were

re-cultured on TSA-5% sheep blood agar to confirm yellow pigment production. Presumptive *E. sakazakii* colonies were confirmed with the API –20E system.

The current recommended FDA procedure was derived from the Canadian method (8), which was based on the method of Muytjens, et al., 1988 (7). The FDA method calls for the direct spreading or streaking of an overnight EE broth rather than VRBG pour plates. Five presumptive colonies are subcultured to Trypticase Soy Agar and incubated at 25°C for 48-72 hours. Only yellow pigmented colonies from the TSA plates are confirmed using the API 20E biochemical identification system. No additional biochemical testing is recommended. The levels of *E. sakazakii* are determined as previously described (8); the lower limit of detection is 0.36/100g. Thus, the FDA method can detect levels of *E. sakazakii* much lower than the recommended FAO level of 3.0 CFU/g. of powdered infant formula (5).

E. sakazakii is isolated from clinical samples using standard methods for the isolation of *Enterobacteriaceae* (1). No special media has been developed for *E. sakazakii*, and it grows well on blood agar, MacConkey, eosin methylene blue, deoxycholate agar and tergitol 7 agars. Reduced plating efficiency was reported for XLD and HE agars (4). During surveillance culturing, using conventional enteric media, in a neonatal intensive care unit in Athens, Greece, *E. sakazakii* was isolated from throat and rectal swabs and from tracheal aspirates (2). Isolates of *E. sakazakii* from samples of blood and cerebrospinal fluid from cases of meningitis have been confirmed with either the Api-20 or the Enterotube II system (13). During an outbreak of necrotizing enterocolitis associated with *E. sakazakii*, anal and stomach aspirates were inoculated onto tryptic soy agar supplemented with horse blood, hemin T, and NAD; tryptic soy agar supplemented

with horse blood and nalidixic acid; MacConkey agar and mannitol salt agar. Blood cultures were performed using the BBL Septi-Chek system with brain heart infusion broth bottles (12). Investigators were able to recover *E. sakazakii* from blood, anal, and stomach sites.

In conclusion, the procedures for isolating *E. sakazakii* from powdered formula and clinical samples follow the standard microbiological methods for the isolation of other members of the family *Enterobacteriaceae*. Normally, sterile clinical samples pose no major problems for isolating *E. sakazakii*. However, because of the very low levels of *E. sakazakii* in powdered formula samples and its non-random distribution in the powder, larger quantities and sub-samples should be cultured for isolation. In both clinical and food microbiology laboratories, appropriate incubation times and temperatures should be applied in diagnostic tests for precise identification of *E. sakazakii*.

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