

Isolation of *Enterobacter sakazakii* and other *Enterobacter* sp. from food and food production environments

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Received 15 May 2006; received in revised form 26 July 2006; accepted 31 July 2006

Abstract

Enterobacter sakazakii and other *Enterobacter* species have caused foodborne illnesses through consumption of a variety of foods, including infant foods. The prevalence of *E. sakazakii* and other *Enterobacter* sp. in infant food and milk formula, milk powder, cereal products, spices, sugar and food production environments were studied. A total of 106 samples were tested for the presence of *E. sakazakii* and other *Enterobacter* sp. The presence of *E. sakazakii* and *Enterobacter* sp. was detected using the FDA enrichment procedure and a chromogenic medium. *E. sakazakii* was isolated from 2/15 infant food formula, 2/8 infant milk formula, 1/18 cereal products. However none of the powder milk, spices, sugar and environmental samples were positive for *E. sakazakii*. *E. agglomerans* was isolated from infant food formula, infant milk formula, milk powder, cereal products, spices and environmental samples. *E. cloacae* was isolated from infant milk formula.

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Keywords: *Enterobacter sakazakii*; Infant formula; Production environments

1. Introduction

Enterobacter sakazakii is a Gram negative, facultative, rod-shaped bacterium. It belongs to the family *Enterobacteriaceae* and genus *Enterobacter* that contains a number of species including *E. agglomerans*, *E. cloacae*, *E. aerogenes* and *E. gergoviae*. The differentiation among these species is based on biochemical reactions, and serological and molecular techniques (Farmer, Asbury, Hickman, & Brenner, 1980; Farmer & Kelly, 1992; Hoffmann & Roggenkamp, 2003; Iversen, Waddington, On, & Forsythe, 2004d). *E. sakazakii*, *E. agglomerans*, and *E. cloacae* are considered the main species of this genus that are frequently isolated from clinical samples and food products (Farmer et al., 1980).

E. sakazakii is considered an opportunistic pathogen that has been implicated in severe forms of necrotizing colitis (Van Acker et al., 2001) and meningitis (Bar-Oz, Preminger, Peleg, Block, & Arad, 2001) especially in neonates with a mortality rate varying from 40% to 80% (Muytjens, Roelfos, & Jaspar, 1988). The International Commission for Microbiological Specification for Foods (ICMSF, 2002) has ranked *E. sakazakii* as “Severe hazard for restricted populations, life threatening or substantial chronic sequelae or long duration”.

E. sakazakii and *Enterobacter* species have been reported as frequently isolated from different environments including soil, rats, flies, milk powder factories, chocolate factories and households (Kandhai, Reij, Gorris, Guillaume-Gentil, & Van Schothorst, 2004a; Neelam, Nawaz, & Riazuddin, 1987). *E. sakazakii* has been also isolated from a wide range of foods including ultra high-temperature treated milk (UHT milk), cheese, meat, vegetables, grains,

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sorghum seeds, rice seeds, herbs, spices, fermented bread, fermented beverage, tofu, and sour tea (Gassem, 1999, 2002; Iversen & Forsythe, 2003, 2004a; Leclercq, Wanequ, & Baylac, 2002; Muytjens et al., 1988; Skaldal, Mascini, Sal Vadori, & Zannoni, 1993). Despite this, studies have confirmed the connection between neonatal *E. sakazakii* infection and infant milk formulas (Biering, Karlsson, Clark, Jonsdottir, & Ludvigsson, 1989; Muytjens et al., 1988; Nazarowec-White & Farber, 1997b; Simmons, Gelfand, Haas, Metts, & Feruson, 1989; Van Acker et al., 2001).

The US Food and Drug Administration (FDA, 2002) has issued an alert to health care professionals about the risk associated with *E. sakazakii* infections among neonates fed with milk-based infant formula. The alert stated that a major contribution to the avoidance of *E. sakazakii* infections in premature babies and neonates is the prevention of contamination of infant milk formula during production and bottle preparation. However, knowledge of the etiological and ecological characteristics of *E. sakazakii* is sparse and its occurrence in factories that produce infant formulas and in hospital kitchens has not been studied in depth.

The objective of this study was to investigate the prevalence of *E. sakazakii* and *Enterobacter* sp. in commercial food products including infant food formula, infant milk formula, milk powder, cereal products, spices, and sugar and in two food factories producing infant formula and cereals products.

2. Materials and methods

All media materials used in the study were obtained from Oxoid, UK.

2.1. Food samples

A total of 59 different commercial food samples from different manufacturers were purchased from retail stores across Jordan. The samples composed of 15 infant food formula (recommended for above 6 months old infants), 8 infant milk formula (Recommended for from birth to 1 year old infants), 10 full cream milk powder, 18 cereal products, spices 5 and sugar 3. Food samples were manufactured or packaged in 13 different countries.

2.2. Samples from food production environments

A total of 47 dry swab samples were obtained from infant food formula and cereal factories using dry cotton swabs. These samples were collected from production areas, including floors, walls, equipment and spilled dry products.

2.3. Detection, isolation and identification of *Enterobacter* sp.

The procedure of FDA (2002) for detection, isolation, and identification of *E. sakazakii* and other *Enterobacter* sp. in food and environmental samples was followed.

An aliquot (10 ml) of homogenate sample (10 g of powder/90 ml sterile Peptone water) or swab sample was added to 90 ml Enrichment Broth (EE broth) which contains bile salt and brilliant green to suppress the growth of non-*Enterobacteriaceae*. The bottles were then incubated for 14–16 h at 36 °C.

Two plates of Violet Red Bile Glucose Agar (VRBGA) were inoculated (0.1 ml) by streak method from the EE broth culture. Another loopful of the suspension was streaked on VRBGA. The plates were incubated for 14–16 h at 36 °C. Five colonies of the red or purple colonies surrounded by purple halo were examined morphologically. *E. sakazakii* and other *Enterobacter* sp. grown on VRBGA appeared under microscope as short-rods in shape and was Gram negative. Typical colonies were streaked on Tryptic Soya Agar (TSA), plates were incubated for 24–72 h at 25 °C.

The isolated colonies that produce yellow pigment were identified using API 24E test. To avoid the lack of specificity of the FDA procedure for the isolation of *E. sakazakii* (Iversen et al., 2004), *E. sakazakii* isolates were confirmed by growing them on Druggan-Forsythe-Iversen (DFI) medium.

E. sakazakii ATCC 51329 was used as the positive control organism. The non *E. sakazakii* isolates were also identified.

3. Results and discussion

3.1. Isolation of *Enterobacter* sp. from commercial food samples

3.1.1. Infant formula

Table 1 summarizes the confirmed identification of *E. sakazakii* and other *Enterobacter* sp. in infant food and milk formula samples. 17.4%, 20% and 17.4% of the samples were found to be positive for *E. sakazakii*, *E. cloacae* and *E. agglomerans*, respectively. The positive strains of *E. sakazakii* formed yellow colonies on TSA after 24–72 h of incubation at 25 °C and blue-green colonies after 24 h of incubation at 37 °C on DFI medium. These results were expected and consistent with the others who have found a direct relationship between infant formula and *E. sakazakii* (Biering et al., 1989; Iversen & Forsythe, 2003; Muytjens et al., 1983; Nazarowec-White & Farber, 1997b; Noriega, Kotloft, Martin, & Schwalb, 1990; Simmons et al., 1989). Muytjens et al. (1988) tested 141 samples of powdered infant milk formula manufactured in different countries. They found that the *E. sakazakii* and other *Enterobacteriaceae* were isolated from 14.1% and 52.2% of the total samples, respectively. Nazarowec-White and Farber (1997b) surveyed the presence of *E. sakazakii* in 120 dried infant milk formula samples (five manufacturers) obtained from Canadian retail market and reported that the prevalence of this bacterium ranged between 0% and 12% of the samples/manufacturer. Iversen and Forsythe (2004a) isolated *E. sakazakii* from 2.4% of 82 powdered infant milk formulas.

Table 1
Enterobacter sakazakii and other *Enterobacter* sp. isolated from powdered infant food and milk formula and related foods

Food samples	Number of samples	<i>Enterobacter</i> sp.		
		<i>E. sakazakii</i>	<i>E. cloacae</i>	<i>E. agglomerans</i>
Infant food formula	15	+(2/15)	+(3/15)	+(2/15)
Infant milk formula	8	+(2/8)	–	+(2/8)
Milk powder (full cream)	10	–	–	+(2/10)
<i>Cereal products</i>				
Starch	3	–	–	+(1/3)
Ground rice	3	–	–	–
Semolina	3	+(1/3)	–	+(1/3)
Bread crumbs	3	–	–	–
Oat	3	–	–	–
Flour	3	–	–	+(1/3)
Mixed spices	5	–	–	+(1/3)
Fine sugar	3	–	–	–

Many studies have focused on the infant formula as the main source of this serious pathogen (Block et al., 2002; Muijtens et al., 1988; Nazarowec-White & Farber, 1997b; Postupa & Aldova, 1984; Van Acker et al., 2001). Despite the fact that formulas are exposed to heat treatment during processing *E. sakazakii* was still isolated from these products. Post-processing contamination of the infant formula from food production environments maybe responsible for the presence of this pathogen in infant formula.

Nazarowec-White and Farber (1997a) stated that *E. sakazakii* can gain access to the powder from the environment or from the addition of the ingredients at the powder stage. Iversen and Forsythe (2003) reported that the presence of *E. sakazakii* in powdered infant milk formula depends on the process conditions and nature of the product.

E. sakazakii was not the only species isolated from infant formula, but also, other species of the same genus were detected in some of the samples, namely, *E. cloacae* and *E. agglomerans*. *E. cloacae* was isolated from three samples (infant food formula), and *E. agglomerans* was isolated from four samples (2 from infant food formula and 2 from infant milk formula). These organisms, category B, have been associated with neonatal infections which include necrotizing enterocolitis, which is the most common important gastrointestinal illness in the new born (FAO/WHO, 2004; Iversen & Forsythe, 2004b). FAO/WHO (2004) has stated “Other *Enterobacteriaceae* are in category “B” because they are well-established causes of illness in infants (e.g. systemic infection, NEC and severe diarrhea) and have been found in powdered infant formula, but contaminated powdered infant formula has not been convincingly shown, either epidemiologically or microbiologically, to be the vehicle and source of infection in infants. These organisms include, for example: *Enterobacter agglomerans*, *Hafnia alvei*, *Klebsiella pneumoniae*, *Citrobacter koseri*, *C. freundii*, *Klebsiella oxytoca* and *Enterobacter cloacae*. The variety of the organisms present in the product could reflect on the hygienic condition of the production environments (Lehner & Stephan, 2004).

3.1.2. Milk powders

E. sakazakii was not isolated from any samples of milk powder (Table 1). However, *E. agglomerans* was found in 20% of the samples. The presence of *E. sakazakii* in these products is unlikely since the liquid milk is normally pasteurized before manufacturing the milk powders. It has been reported that pasteurization treatment is effective in the elimination of this pathogen (Iversen et al., 2004c; Nazarowec-White, McKellar, & Piyasena, 1999). Nazarowec-White and Farber (1997c) found that *E. sakazakii* was more heat-sensitive than other pathogenic organisms like *Listeria monocytogenes*.

Although it is not detected in milk powder samples, *E. sakazakii* is not ruled out from being a possible pathogen present in this product, putting in consideration that each sample represents itself only. *E. sakazakii* has been isolated from milk powder (Farmer et al., 1980; Iversen & Forsythe, 2004a). The potential for milk powder to be cross contaminated must be recognized; either directly or via surface and equipment that may become contaminated with *E. sakazakii* after being in contact with raw milk (Iversen & Forsythe, 2004a). The EU Microbiological criteria of milk powders for *Enterobacteriaceae* testing (Anonymous, 2005) requires <10 cfu/g of powder ($n = 5, c = 0 m < 10$ cfu/g).

3.1.3. Cereal products

Detection and isolation of *E. sakazakii* and other *Enterobacter* sp. from different samples of cereal products are given in Table 1. *E. sakazakii* was isolated from 33.3% of semolina samples (crushed wheat) and *E. agglomerans* was isolated from 33.3% of starch, semolina, flour and mixed spices samples.

These results are not unusual because *E. sakazakii* has been isolated from a wide range of foods including cheese, fermented bread, tofu, sour tea, cured meats, rice seeds, sesame seeds, lentils, ground maize, ground rice, and almond (Cottynne et al., 2001; Iversen & Forsythe, 2004a), it has also been found in khamir bread due to the organism being part of the sorghum seed surface flora (Gassem, 1999). It is

Table 2
Enterobacter sakazakii and other *Enterobacter* sp. isolated from environmental samples of infant formula and cereal products factories

Environmental samples	Number of samples	<i>Enterobacter</i> sp.		
		<i>E. sakazakii</i>	<i>E. cloacae</i>	<i>E. agglomerans</i>
<i>Infant formula factory</i>				
Floors	7	–	–	+(3/7)
Walls	4	–	–	+(1/4)
Spilled products	3	–	–	–
<i>Equipment</i>				
Hopper	2	–	–	+(1/2)
Sieve	2	–	–	–
Vibrator	2	–	–	–
Twin head device	2	–	–	–
Conveyer device	4	–	–	+(1/4)
<i>Cereal products factory</i>				
Floors	7	–	–	+(2/7)
Walls	4	–	–	–
<i>Equipment</i>				
Roller	2	–	–	+(1/2)
Shaker	2	–	–	–
Mixer	2	–	–	+(1/2)
Conveyer	4	–	–	+(1/2)

believed that *E. sakazakii* may occur in a wider range of food ingredients; however, these have not been investigated in detail.

3.2. Isolation of *Enterobacter* sp. from factory environment

3.2.1. Infant formula factory

Swab samples were analyzed for the presence of *E. sakazakii* and other *Enterobacter* sp. (Table 2). *E. sakazakii* was not isolated from any of the samples collected from the factory. *E. agglomerans* was the only species that was isolated from 35.3% of the samples. Nazarowec-White and Farber (1997a) stated that processing environments and ingredients play a major role in the contamination of infant formula with pathogenic microorganisms. *E. sakazakii* and other *Enterobacter* sp. have been reported as being frequently isolated from food processing milk plant environments (Bar-Oz et al., 2001; Noriega et al., 1990; Skaldal et al., 1993). The factory environment of milk powder and infant formula has been studied extensively (Bar-Oz et al., 2001; Kandhai et al., 2004a, 2004b). *E. sakazakii* and *Enterobacter* sp. were isolated from different locations of the plant including floors, scraping, cleaning equipment (Kandhai et al., 2004a), drying and filling areas (Caric, 1993) and blender used to prepare the rehydrated formula (Bar-Oz et al., 2001). Although the swabs in this study were taken from places similar to those reported to be contaminated in similar factories, none of these swabs contained *E. sakazakii*.

3.2.2. Cereal products factory

E. sakazakii was not isolated from any samples collected from the factory (Table 2). *E. cloacae* and *E. agglomerans* were the main isolates from 38.5% of the collected samples. Kandhai et al. (2004a) isolated *E. sakazakii* from samples

obtained from different locations in food factories. They found that 21%, 25%, 44%, and 27% of the samples were positive for the bacterium in milk powder, chocolate, cereal products and potato flour factories, respectively. Generally, the presence of *E. sakazakii* in the environment of factories reflects the poor hygienic conditions and cross contamination (Gurtler, Kornacki, & Beuchat, 2005).

4. Conclusion

The results of this study show that *E. sakazakii* was found in infant formula and semolina samples. *Enterobacter* species like *E. cloacae* and *E. agglomerans* were isolated from infant formula and cereal product samples. *E. agglomerans* was isolated from milk powder and environmental samples.

More research is needed to determine conditions that influence survival and growth of *E. sakazakii* in dry and reconstituted infant formula. Also, studies of conditions affecting biofilm formation by *E. sakazakii* in food factories and hospital settings are required.

Acknowledgements

The authors thank the Deanship of Scientific Research at Jordan University of Science and Technology for funding the project. Also the authors thank Dr. Elizabeth Martin at University of Arkansas, USA for reviewing the manuscript.

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