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Review

Enterobacter sakazakii: A coliform of increased concern to infant health

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Abstract

The first cases of neonatal meningitis believed to have been caused by *Enterobacter sakazakii* were reported in 1961. Prompted by several subsequent outbreaks of *E. sakazakii* infections in neonates and an increasing number of neonates in intensive care units being fed rehydrated powdered infant formula, considered to be a source of the pathogen, public health authorities and researchers are exploring ways to eliminate the bacterium or control its growth in dry infant formula, processing environments and formula preparation areas in hospitals. Reviewed here are advances in taxonomy and classification of *E. sakazakii*, methods of detecting, isolating and typing the bacterium, antibiotic resistance, clinical etiology and pathogenicity. Outbreaks of *E. sakazakii* infections in neonates and adults are summarized. Reports on the presence of *E. sakazakii* in clinical settings, the environment and foods and food processing facilities are reviewed. Tolerance of the pathogen to environmental stresses, its behavior in powdered and rehydrated infant formulae and hazard analysis and risk management are discussed. Research needs are presented.

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Keywords: *Enterobacter sakazakii*; Infant formula; Neonate; Meningitis

Contents

1. Introduction	2
2. Taxonomy and biochemical characterization	3
3. Isolation, identification and typing	5
4. Clinical etiology and pathogenicity	7

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5.	Outbreaks and cases of infections	11
5.1.	1958, St. Albans, England	11
5.2.	1965, Denmark	12
5.3.	1979, Macon, GA, USA	12
5.4.	1981, Indianapolis, IN, USA	12
5.5.	1983, The Netherlands	12
5.6.	1985, Athens, Greece	12
5.7.	1986–1987, Reykjavik, Iceland	13
5.8.	1987, Boston, MA, and New Orleans, LA, USA	13
5.9.	1988, Memphis, TN, USA	13
5.10.	1990, Baltimore, MD, USA	13
5.11.	1990, Cincinnati, OH, USA	14
5.12.	1995/1996, Boston, MA, USA	14
5.13.	1998, Belgium	14
5.14.	2000, Winston Salem, NC, USA	14
5.15.	1993, 1995, 1997–2000, Israel	14
5.16.	2001, Knoxville, TN, USA	15
6.	Antibiotic resistance	15
7.	Sources of contamination	17
7.1.	Clinical sources	17
7.2.	Environmental sources	17
7.3.	Foods as sources of <i>E. sakazakii</i>	19
8.	Inactivation	20
8.1.	Thermal resistance	20
8.2.	Osmotic and desiccation resistance	21
8.3.	Antimicrobial activity of chitosans and its oligomers	22
9.	Presence and behavior in infant formula	22
10.	Hazard analysis and risk management	25
11.	Research needs	26
	References	27

1. Introduction

Pangalos (1929) reported that a yellow-pigmented coliform was the causative bacterium in a case of septicemia in an infant. The first reported account implicating a bacterium now known as *Enterobacter sakazakii*, taking its name from Riichi Sakazaki, a Japanese microbiologist, as the causative agent in meningitis, however, is that of Urmenyi and Franklin (1961). They described two cases of terminal neonatal meningitis that occurred in 1958 in St. Albans, England. The next definitive report of infection caused by *E. sakazakii* described a child in Denmark that survived meningitis but experienced severe mental and neurological impairment (Joker et al., 1965). Reports from both England and Denmark listed the causal agent as an atypical yellow-pigmented *Enterobacter cloacae*.

The first recorded use of the name *E. sakazakii* was by Farmer et al. (1977) and Brenner et al. (1977). *E. sakazakii* was later distinguished from *E. cloacae* based on differences in DNA relatedness, pigment production, biotyping, and antibiotic susceptibility patterns (Farmer et al., 1980; Izard et al., 1983). Five names previously used for the coliform, including the Urmenyi and Franklin bacillus, yellow coliform, yellow *Enterobacter*, pigmented *cloacae* A organism, and most notably, yellow-pigmented *E. cloacae* were listed.

Infections caused by *E. sakazakii* have been rarely reported. The bacterium has been implicated most frequently in causing illness in neonates and children from 3 days to 4 years of age, with at least 76 cases of *E. sakazakii* infections and 19 deaths in infants and children being reported (Iversen and Forsythe, 2003).

At least nine cases of *E. sakazakii* infections in adults have been documented. Infectious neonatal and childhood cases have been reported to occur in at least eight countries and nine states in the United States.

Sources of *E. sakazakii* associated with infant infections have, in most cases, not been confirmed. Studies corroborated by epidemiologic investigations, however, have implicated rehydrated powdered infant formula (a non-sterile product) as well as equipment and utensils used to prepare rehydrated formulae in hospital settings (Bar-Oz et al., 2001; Biering et al., 1989; Clark et al., 1990; Muytjens and Kollee, 1990; Muytjens et al., 1983; Noriega et al., 1990; Simmons et al., 1989; Van Acker et al., 1990). Voluntary recalls of infant formula containing *E. sakazakii* have occurred in the United States, which have led to decisions by manufacturers, hospital staff, and the U.S. Food and Drug Administration (FDA) to pursue higher microbiological standards and hygienic practices. This has resulted in an increased effort to seek solutions to eliminate infections caused by a bacterium about which sources of contamination, ecology, and virulence characteristics are not well understood.

2. Taxonomy and biochemical characterization

Taxonomy, classification and nomenclature of genera in the family Enterobacteriaceae have evolved over the years based on various distinctions in serology, morphology, biochemical traits and genetic characteristics. There are 14 species or biogroups in the genus *Enterobacter* (Farmer, 1995). *E. sakazakii*, a motile, peritrichous, Gram-negative, non-sporulating, straight rod, previously known as yellow-pigmented *E. cloacae*, is considered a biovar in the genus *Enterobacter* (Monroe and Tift, 1979; Muytjens et al., 1983; Nazarowec-White and Farber, 1997b). Major differences between *E. sakazakii* and other *Enterobacter* species have been traditionally thought to be its inability to ferment D-sorbitol and its ability to produce an extracellular deoxyribonuclease (Farmer et al., 1980). However, some strains of *E. sakazakii* more recently have been shown to ferment D-sorbitol (Heuvelink et al., 2001).

Based on DNA–DNA hybridization showing yellow-pigmented strains to have less than 50% homology with non-pigmented strains, Brenner (1974)

suggested that yellow-pigmented *E. cloacae* should comprise a new species. Phenotypic characterization and differentiation based on biochemical traits, serotyping, bacteriophage typing and antibiotic resistance are frequently among the first steps used to distinguish characteristics of isolates (Arbeit, 1995; Einstein, 1990; Nazarowec-White and Farber, 1999). Some have suggested using phenotype tests (e.g., biotyping, bacteriocin typing, serotyping and phage typing) to differentiate *Enterobacter* species; however, none of these tests has proven effective in distinguishing strains within the species, nor can they be used for all species of *Enterobacter* (Gaston, 1988; Grattard et al., 1994; Nazarowec-White and Farber, 1999; Poilane et al., 1993). Iversen et al. (2004f) investigated the phylogenetic relationships of *E. sakazakii* using 16S ribosomal DNA and *hsp60* sequencing. They found that strains were distributed among four clusters, indicating taxonomic heterogeneity. The type strain 16S rDNA sequence was 97.8% similar to that of *Citrobacter roseri* and 97.0% similar to that of *E. cloacae*. Studies have shown that the *Enterobacter* genus is polyphyletic (Loc-Corrillo et al., 2004). Strains currently classified as *E. sakazakii* fall into two distinct groups which can be further subdivided based on *hsp60* sequences. Both genotypes include clinical strains and do not correspond to biochemical profiles.

Farmer et al. (1980) extended the work of Brenner (1974) and Brenner et al. (1977) by further distinguishing 57 strains of yellow-pigmented *E. sakazakii* based on DNA hybridization, antibiotic susceptibility and biochemical reactions. Other distinguishing characteristics of the bacterium include greater pigment production at temperatures less than 36 °C, with optimum pigment production at 25 °C, survival of cells in stock cultures stored at 17–30 °C without transfer for up to 8 years, utilization of citrate as a sole carbon source, 31–49% DNA–DNA homology with *E. cloacae*, and 57% guanine+cytosine ratio (Farmer et al., 1980). Production of the diffusible yellow pigment is unstable with repeated subculturing.

Freshly isolated *E. sakazakii* may produce colonies with two distinct morphologies (Farmer et al., 1980). One colony type is described as being either dry or mucoid, crenated (notched or scalloped) and leathery or rubbery when touched with a wire loop, i.e., very little biomass adheres to the loop and the colony snaps back to the agar when touched. These characteristics

may be attributable to the production of a heteropolysaccharide (Harris and Oriol, 1989). A second colony morphology has been described as smooth and more amenable to removal of cells from colonies with a wire loop. Cells that produce leathery colonies when subcultured from stock cultures may revert to the production of typical smooth colonies, some of which exhibit very slight pigment production. This differentiation in phenotypicity has been more recently described by Iversen and Forsythe (2003) as being either “matt” or “glossy.” Farmer et al. (1980) observed that *E. sakazakii* grown in broth tends to clump and sediment, a characteristic that has been also noted by others (Nazarowec-White and Farber, 1997b). Approximately 58% (33 of 57) of the strains examined were considered to be fecal coliforms on the basis of gas production in *Escherichia coli* (EC) broth incubated at 44.5 °C for 48 h. It is recommended that screening be done to confirm that isolates of *E. cloacae* presumptive for *E. sakazakii* be tested for yellow pigment production, D-sorbitol fermentation and a delayed-reaction (7 days) DNase test.

In addition to phenotypic characterization of *E. sakazakii*, advances have been made in fingerprinting DNA and RNA by several techniques, e.g., PCR, randomly amplified polymorphic DNA (RAPD) PCR, pulsed-field gel electrophoresis (PFGE), chromosomal DNA restriction analysis, ribotyping and plasmid typing (Grant and Kroll, 1993; Farber, 1996; Nazarowec-White and Farber, 1999). Nazarowec-White and Farber (1999) ribotyped *E. sakazakii* with the *EcoR1* restriction endonuclease and found that 18 isolates were represented by 10 ribotypes. This analysis has been determined to be more discriminatory than that of restriction endonuclease analysis (REA) (Clark et al., 1990). In another study, 30 *E. sakazakii* isolates from an infant formula factory comprised only 8 ribotypes (Anonymous, 1996). Kornacki (1998) isolated 17 *EcoR1* ribotypes from a factory environment. Nazarowec-White and Farber (1999) analyzed 18 isolates by PFGE using the restriction endonuclease *Xba1* and found each to have a distinct pattern. Characterization was superior to ribogrouping in that two sets of three isolates, comprising only two ribogroups, were distinguishable as six distinct pulsovars.

Farmer et al. (1980) examined 57 isolates of *E. sakazakii* and observed that all grew at 25 °C and 45 °C. None of the isolates grew at 4 °C or 50 °C,

whereas at 47 °C, 7 (12%) of the isolates failed to grow. Breeuwer et al. (2003) examined 22 strains of *E. sakazakii*, all capable of growing in brain heart infusion broth at 47 °C. Nazarowec-White and Farber (1997c) reported that 12 strains of *E. sakazakii* grew at temperatures between 41 and 45 °C. *E. sakazakii* and *Klebsiella pneumoniae* have been reported to grow more rapidly than *Pseudomonas aeruginosa*, *E. coli*, *Staphylococcus aureus*, *Mycobacterium terrae* and *Candida albicans* in rehydrated infant formula (Kindle et al., 1996). It was surmised that rapid growth of *E. sakazakii* might account for nosocomial neonatal infections associated with the pathogen. Colonies formed on trypticase soy agar were reported to be 2–3 mm in diameter after 24 h at 36 °C and 1–1.5 and 2–3 mm after 24 h and 48 h of incubation, respectively, at 25 °C.

E. sakazakii has been shown to exhibit substantial resistance to acid pH. Edelson-Mammel and Buchanan (2004a) examined survival characteristics of 12 strains of *E. sakazakii* in tryptic soy broth adjusted to pH 3.0 and 3.5 with HCl. Ten of twelve strains showed less than a 1-log decline over a 5-h period at 37 °C; reductions in TSB at pH 3.0 were 4.9 to >6.3 log CFU/ml. There was no correlation in acid resistance based on 1-h/pH 3.0 results and previously determined heat resistance of test strains (Edelson-Mammel and Buchanan, 2004c). Skladal et al. (1993) examined the fermentation of milk inoculated with 10–15 CFU of *E. sakazakii* per 500 ml and incubated at 30 °C. Changes in pH and the production of L-lactate and D-lactate were monitored. *E. sakazakii* fermented milk rapidly, reducing the pH from 6.6 to 5.6 in less than 20 h. Concentrations of L-lactate and D-lactate reached 0.40 mM and 10.7 mM, respectively.

Conditions influencing the production of an exopolysaccharide (EPS) by *E. sakazakii* have been studied (Scheepe-Leberkuhne and Wagner, 1986). Distinguishing features of the complex were its high viscosity and gel formation. Attempts were made to optimize the production of EPS by increasing the carbon/nitrogen ratio in the growth medium. Production was highest in media supplemented with glucose and at an incubation temperature of 27 °C. Maximum amounts of EPS produced by *E. sakazakii* in nutrient broth were obtained with a carbon/nitrogen ratio of 20.2:1 when glucose (15 g/l) was the carbon source

(Scheepe-Leberkuhne and Wagner, 1986). Others have shown that glucose enhances EPS production by *Enterobacter aerogenes*, *Xanthomonas campestris* (Sutherland, 1983) and *Arthrobacter viscosus* (Gasdorf et al., 1965), and patents have been filed for the production of gums from *E. sakazakii* (Harris and Oriol, 1989; Yang, 2002).

3. Isolation, identification and typing

The FDA (2002d,f) developed a method to isolate and enumerate *E. sakazakii* in dehydrated powdered infant formula (Table 1). This method is the same as that proposed by Muytjens et al. (1988) and Nazarowec-White and Farber (1997c), with the exceptions that the powdered infant formula is rehydrated with distilled water in the FDA method rather than with buffered peptone water, and enriched samples (0.1 ml) are streaked on violet red bile glucose (VRBG) agar rather than pour-plating 1.0 ml in VRBG agar.

Table 1
Method for analyzing powdered infant formula for presumptive *E. sakazakii*^a

Step	Time	Temperature
• Sterilize can lid margins and sampling spoons	N/A	N/A
• Dilute 100 g, 10 g, and 1 g of powdered infant formula with pre-warmed sterile distilled water at 1:10 ratio, mix and incubate	Overnight	36 °C
• Add 10 ml of each suspension to 90 ml of Enterobacteriaceae enrichment broth and incubate	Overnight	36 °C
• Mix suspensions and surface plate 0.1 ml on VRBG agar, streak on VRBG agar with a 10 µl inoculating loop onto three quadrants for isolation and incubate	Overnight	36 °C
• Pick five presumptive-positive <i>E. sakazakii</i> colonies from both sets of VRBG plates and subculture by streaking onto TSA and incubate	48–72 h	25 °C
• Select yellow-pigmented colonies only and confirm per manufacturer's instructions for the API 20E [®] biochemical confirmation system	N/A	N/A
• Calculate the most probable number (MPN) after determining the number of positive tubes at each dilution	N/A	N/A

^a FDA (2002d).

Leclercq et al. (2002) examined the growth of *E. sakazakii* on fecal coliform agar, a medium developed by Hsing-Chen and Wu (1992), and found the medium to support slightly higher recovery than violet red bile lactose agar. It has been suggested that the distinguishing quality of Tween 80 esterase production by most strains of *E. sakazakii* could be used to help confirm presumptive isolates (Aldova et al., 1983; Postupa and Aldová, 1984).

E. sakazakii is α -glucosidase positive, which can be demonstrated in media containing 4-nitrophenyl- α -D-glucopyranoside (Muytjens et al., 1984). This assay has been recommended as a supplementary confirmation test to avoid false-positive test conclusions (Muytjens, 1985) and is a basis for the differential reaction on Druggan, Forsythe, and Iversen agar, a medium developed for isolating *E. sakazakii* (Iversen et al., 2004c). A 4-h α -glucosidase reaction was used to differentiate 29 presumptive *E. sakazakii* isolates from 152 environmental samples originating from three milk powder factories from other coliforms, two of which produced α -glucosidase, but within 24 h and not 4 h (Kandhai et al., 2004b).

A medium for presumptive detection of *E. sakazakii* in infant formula was developed by Leuscher et al. (2004). It is based on the production of α -glucosidase, which differentiates *E. sakazakii* from other species in the family Enterobacteriaceae. *E. sakazakii* forms yellow colonies that fluoresce under UV light on nutrient agar supplemented with 4-methyl-umbelliferyl α -D-glucoside (α -MUG). Other representatives of Enterobacteriaceae and non-Enterobacteriaceae, e.g., *Acinetobacter* sp., *Escherichia hermannii*, *Cedaceae lepagii*, *Leclercia acecarboxylata* and *Enterobacter agglomerans* isolated from infant formulae, also produced yellow pigment, but colonies did not fluoresce under UV light. The medium performed well in an interlaboratory study designed to detect *E. sakazakii* in powdered infant formula (Leuscher and Bew, 2004). In an independent study, another medium containing α -MUG was also shown to be reliable for differentiation and isolation of *E. sakazakii* (Oh and Kang, 2004; Oh et al., 2004).

Kandhai et al. (2004b) developed a 4-h colorimetric screening assay based on α -glucosidase production by cells in yellow-pigmented colonies formed on tryptic soy agar. The colony is dispersed in saline containing paranitrophenyl- α -D-glucopyranoside, incubated at

37 °C and examined spectrophotometrically for the formation of yellow-colored paranitrophenyl hydrolysate. Using this method, *E. sakazakii* was isolated from 18 of 152 environmental samples collected from three milk powder production plants.

A chromogenic medium (Druggan–Forsythe–Iversen agar, DFI) was formulated for selective detection of *E. sakazakii* (Druggan et al., 2004; Iversen et al., 2004c). The medium is based on the α -glucosidase reaction, which is detected using 5-bromo-4-chloro-3-indolyl- α ,D-glucopyranoside (X α Glc). Ninety-five clinical and food isolates of *E. sakazakii* were detected on DFI agar 2 days sooner than using the FDA (2002d) method. Characteristics of 148 strains representing 17 genera of Enterobacteraceae other than *E. sakazakii* were compared using DFI agar and VRBG agar. Only 19 strains representing three genera gave false-positive results on DFI agar. This compares to 31 strains giving false-positive results using the standard method.

There is a need for improvement in the current, largely phenotypically based, approach for detecting and confirming presumptive *E. sakazakii* isolates. The Enterotube II[®] as well as the API 20E[®] systems have been used for presumptive-positive confirmations of *E. sakazakii* via biochemical characteristics (Biering et al., 1989; Cottyn et al., 2001; Gassem, 1999; Kandhai et al., 2004b, Monroe and Tift, 1979; Mosso et al., 1994; Muytjens et al., 1983; Nazaro-wec-White and Farber, 1999; No et al., 2002; Seo et al., 2003; Simmons et al., 1989; Van Os et al., 1996; Willis and Robinson, 1988). Recent findings, however, indicate that API 20E biochemical test strips can give false-negative as well as false-positive results (Iversen et al., 2004b). It was concluded that further biochemical characterization is needed to determine the traits most strongly associated with strains of *E. sakazakii* falling into two distinct genotypes. Seo et al. (2003) reported confirming *E. sakazakii* via API ZYM[®] and Vitek[®] assays. Cottyn et al. (2001) used fatty acid methyl ester (FAME) analysis as well as API 20E[®] strips and Biolog[®] strips as confirmation assays. Lee and Kim (2003) recovered and identified *E. sakazakii* from a semi-pilot potable water system via membrane filtration, m-Endo agar, API 20E[®] kits, and the Microbial Identification (MIDI) system in which whole-cell fatty acid methyl ester profiles were analyzed. Muytjens et al. (1983) identified *E.*

sakazakii by DNase reactions after incubating cultures for 2 and 7 days, in addition to using the API 20E[®] assay.

Selection of an appropriate screening approach has an important bearing on the validity of the identification. Iversen and Forsythe (2004a) reported that three strains identified as *E. sakazakii* by the API 20E[®] assay were identified as *E. cloacae*, *Enterobacter amnigenus*, and *Enterobacter cloacae/gergoviae* by API 32E[®], whereas 10 strains identified as *E. sakazakii* by API 32E[®] gave patterns consistent with *Pantoea* species by API 20E[®]. The close phenotypic similarities between *E. sakazakii* and *E. cloacae* and the existence of some sorbitol-positive *E. sakazakii* as well as some non-pigmented *E. sakazakii* strains causes one to wonder about the true incidence of *E. sakazakii* infections, given that *E. cloacae* is commonly reported in neonatal, nosocomial infections and has been traced to contaminated enteral feeding (Levy et al., 1989).

Seo et al. (2003) developed a real-time PCR assay to detect *E. sakazakii* by designing primers and probes using the partial macromolecular synthesis (MMS) operon of *E. sakazakii*. The assay was specific for differentiating *E. sakazakii* and *E. cloacae*, and almost 50 other genera of Enterobacteriaceae, allowing detection of as few as 100 CFU of *E. sakazakii* per ml of infant formula without enrichment. In a limited study of *E. sakazakii*-contaminated product (5 of 22 samples), the population was about 4 cells in 1000 g (Zink, 2003). Consequently, enrichment procedures would be necessary to increase the population to about 100 CFU/ml to enable detection. Given that the protocol for analyzing dry infant formula requires a 1:10 dilution in enrichment broth, about 15 generations or approximately 4–5 h at an optimal growth temperature would be required to reach this population.

Further characterization of *E. sakazakii* isolated from food and environmental samples can be accomplished using pulsed PFGE, RFLP, multilocus enzyme electrophoresis tests, or ribotyping. Other potential methods of analyses include testing for antibiotic resistance patterns (antibiograms), toxin assays, hemagglutination, serotyping and phage typing. Nazaro-wec-White et al. (2003) and Farber (2004) recommended that laboratories type all *E. sakazakii* isolates for molecular characteristics to facilitate epidemiolog-

ic investigations and as a means to identify new infection vehicles. Williams et al. (2004) recently described a method to differentiate strains of *E. sakazakii* based on protein biomarkers. The biomarkers were sequenced to provide insight into why certain strains were more thermal tolerant than others.

4. Clinical etiology and pathogenicity

Enterobacter species can create community infections, are responsible for approximately half of all nosocomially acquired infections and are often implicated in co-infections (Borderon et al., 1996; Chang et

al., 2000; Hervas et al., 2001; Huang et al., 2001; Leclerc et al., 2001; Nazarowec-White and Farber, 1997b; Wenger et al., 1997). Symptoms of *E. sakazakii* infections in infants, adults and elderly patients are listed in Table 2. Studies have shown that patients with extended hospital visits, especially in intensive care units, are at heightened risk for contracting infections from *Enterobacter* species (Al Ansari et al., 1994; Burchard et al., 1986; Flynn et al., 1987; Gallagher, 1990; Georghiou et al., 1995; Kuhn et al., 1991; McConkey et al., 1989; Pitout et al., 1997; Sanders and Sanders, 1997). Others who may be at heightened risk include those previously treated with antibiotics, the immunocompromised, the elderly,

Table 2
Symptoms of *E. sakazakii* infection

Symptom	Reference
Infections reported in infants include	
Meningitis leading to ventriculitis, brain abscess, infarction and cyst formation	Gallagher and Ball, 1991.; Kleiman et al., 1981; Kline, 1988a; Nazarowec-White and Farber, 1997b; Ries et al., 1994; Willis and Robinson, 1988
Conjunctivitis	Bar-Oz et al., 2001; Block et al., 2002; Reina et al., 1989
Bulging fontanelles, destruction of the frontal lobes of the brain, seizures, spastic quadriplegia, hypothermia, fever, Cheyne–Stokes respirations, bradycardia, poor feeding, irritability, jaundice, grunting respirations, instability of body temperature, hemorrhagic cerebral necrosis, meningitis, necrotic softened brain, cyst formation, liquefaction of cerebral white matter and severe neurologic complications	Willis and Robinson, 1988
Infections associated with catheters	Arseni et al., 1985; Block et al., 2002
Meningitis patients displaying early signs of illness such as grunting, pallor, cyanosis and collapse, bulging fontanelle, convulsions, twitching and hypertonia	Muytjens et al., 1983
Necrotizing enterocolitis in neonates	Muytjens et al., 1983; Van Acker et al., 2001
Brain abscesses and meningitis in neonates	Burdette and Santos, 2000; Kleiman et al., 1981; Muytjens et al., 1983; Ries et al., 1994
Ventriculitis, cerebral infarction, brain cysts, late development of hydrocephalus and brain abscesses	Bar-Oz et al., 2001; Burdette and Santos, 2000; Gallagher and Ball, 1991
Bacteremia	Block et al., 2002; Burdette and Santos, 2000; Monroe and Tift, 1979; Nazarowec-White and Farber, 1997b; Noriega et al., 1990
Infections reported in adults include	
Coinfective agent (amongst three bacteria) isolated from an ulceration in the foot of a diabetic	Pribyl et al., 1985
Appendicitis	Reina et al., 1989
Biliary sepsis	Lai, 2001
Infections in elderly patients include	
Urosepsis	Hawkins et al., 1991; Nazarowec-White and Farber, 1997b
Bacteremia	Jimenez and Gimenez, 1982
Infection in a vascular graft and a thigh wound	Hawkins et al., 1991
Pneumonia	Dennison and Morris, 2002 Lai, 2001

patients with medical implants and those with acute, chronic, or serious illnesses (Pitout et al., 1997; Sanders and Sanders, 1997). In 1992, *Enterobacter* species were reported as being the fifth and third most common among those recovered from the urinary and respiratory tracts, respectively, of patients in intensive care units (Jarvis and Martone, 1992). At least one report suggests that human health can be negatively affected by the ingestion of pigmented bacteria, such as *E. sakazakii*, in drinking water (Rusin et al., 1997).

E. sakazakii infections are likely to involve newborns and infants and have been associated with sepsis, meningitis and necrotizing enterocolitis (Adamson and Rogers, 1981; Arseni et al., 1987; Biering et al., 1989; Bar-Oz et al., 2001; Borderon et al., 1996; Burdette and Santos, 2000; Clark et al., 1990; Chotpitayasonndh, 1994; Fotopoulos et al., 1997; Gallagher and Ball, 1991; Gebremariam, 1998; Greenberg et al., 1997; Hervas et al., 2001; Himelright et al., 2002; Huang et al., 2001; Iversen and Forsythe, 2003; Joker et al., 1965; Kleiman et al., 1981; Lai, 2001; LeCour et al., 1989; Monroe and Tift, 1979; Muytjens et al., 1983; Nazarowec-White and Farber, 1997b; Noriega et al., 1990; Sakata and Maruyama, 1997; Simmons et al., 1989; Tekkok et al., 1996; Urmenyi and Franklin, 1961; Willis and Robinson, 1988; Weir, 2002; Van Nierop et al., 1998; Van Acker et al., 2001; Wolf and Young, 1993). Only one case of *E. sakazakii* sepsis was identified among 10,660 very low-birth-weight infants, suggesting that outside of an epidemic situation, *E. sakazakii* infection is very rare in these infants (Stoll et al., 2004). At least nine cases have also been reported among adults, all immunocompromised with underlying medical conditions (Dennison and Morris, 2002; Emery and Weymouth, 1997; Hawkins et al., 1991; Jimenez and Gimenez, 1982; Pribyl et al., 1985; Lai, 2001).

The first reported cases of *E. sakazakii* were in 1958 in England (Urmenyi and Franklin, 1961). Additional cases of neonatal and infant *E. sakazakii* infections have been reported and further described in Canada, Belgium (Van Acker et al., 2001), Denmark, (Joker et al., 1965), Iceland (Biering et al., 1989), Germany, (Ries et al., 1994), Greece, (Arseni et al., 1985), Israel, (Bar-Oz et al., 2001; Block et al., 2002), The Netherlands, (Muytjens et al., 1983) Spain (Reina et al., 1989) and the United States (Burdette and Santos, 2000; Gallagher and Ball, 1991; Himel-

right et al., 2002; Kleiman et al., 1981; Lai, 2001; Monroe and Tift, 1979; Noriega et al., 1990; Simmons et al., 1989; Weir, 2002; Willis and Robinson, 1988). In the United States cases, neonatal *E. sakazakii* infections have been reported in at least nine states. Iversen and Forsythe (2003) reported that at least 76 cases of neonatal *E. sakazakii* infections were documented to occur worldwide between 1958 and 2003. Contributing factors in neonates that might increase the risk of infection include immunosuppression, premature birth and low birth weight (Health Canada, 2002; Himelright et al., 2002). One of the first two reported cases of neonatal *E. sakazakii* infection involved a full-term newborn, although premature birth often accompanies and appears to result in a predilection to infection (Urmenyi and Franklin, 1961). The International Commission for Microbiological Specifications for Foods (2002) classified *E. sakazakii* as a severe hazard for restricted populations, causing life-threatening or substantial chronic sequelae or illness of long duration.

Some common infective species of *Enterobacter*, particularly in newborns, include *meningitis*, *cloacae*, *aerogenes*, *agglomerans*, *hormechie*, *gergovial* and *sakazakii* (Nazarowec-White and Farber, 1997b; Weir, 2002; Willis and Robinson, 1988). Muytjens et al. (1983) estimated, however, that 50–75% of septicemia and meningitis cases in neonates are attributable to *Streptococcus agalactiae* and *E. coli* infections. *E. sakazakii* is an opportunistic pathogen most commonly affecting immunocompromised neonates (Naqvi et al., 1985; Willis and Robinson, 1988). The bacterium has been isolated from or implicated in numerous types of infections (Table 2). It is of interest that a review of 17 cases of neonatal meningitis revealed that patients with *E. sakazakii* infections fared worse than those with more frequently occurring meningitis caused by other Gram-negative bacteria, including *E. cloacae* (Willis and Robinson, 1988). Lai (2001) reviewed reported cases of *E. sakazakii* infections and outbreaks, eliminating those lacking relevant or clinical information. Observations in these reports as well as those in hospital records are summarized in Table 3.

Very few cases of *E. sakazakii* infections have been reported in adults, who are considered a low-risk group (Burdette and Santos, 2000; Gallagher and Ball, 1991; Kleiman et al., 1981; Muytjens et al.,

Table 3
Observations from case reports describing *E. sakazakii* infections^a

- In the University of Massachusetts Medical Center, 3.6% of patients with septicemia (from 1994 to 1996) were positive for *Enterobacter* species, 0.4% of patients with septicemia were positive for *E. sakazakii* in 1996, and five cases were diagnosed with nosocomially acquired *E. sakazakii* infections in 1995–1996.
- Thirty-one cases of infections in infants and children ranging from 3 days to 4 years linked to *E. sakazakii* were reported in the literature.
- 50% of children infected with *E. sakazakii* was <1 week old.
- 75% of children infected with *E. sakazakii* was <1 month old.
- 43% of those cases with meningitis experienced seizures.
- Case fatality rates of infants with non-meningeal *E. sakazakii* infections were 33%, whereas meningitis patients had a higher fatality rate of 45%.
- After 1985, patients were treated with the third-generation cephalosporins in addition to combinations of ampicillin, gentamicin and chloramphenicol, which reduced the case fatality rate in meningeal *E. sakazakii* patients from 62% to 14%.
- There were only four cases of *E. sakazakii* infections in adults reported in the literature and an additional four reported from the University of Massachusetts Medical Center.
- All adult *E. sakazakii* cases were in-patients ≥ 56 years of age, except for one 39-year-old male.
- Mean and median ages for the adult patients were 68 and 76 years, respectively.
- Case mortality rate of adult patients was 50%, with the University of Massachusetts Medical Center experiencing a 75% mortality rate.
- Most of the adult cases involved underlying complicating medical factors including 50% with malignancies.
- Sites of infection for the eight adult cases included bacteremia, osteomyelitis of the foot, pneumonia, and biliary sepsis.

^a Summarized from Lai (2001).

1983; Ries et al., 1994). Dennison and Morris (2002) described a case in which the patient required extended treatment with broad-spectrum antibiotics due to multi-antibiotic resistance of the infectious strain. Neonatal infections have been reported to arise via contact with *E. sakazakii* in the birth canal or through post-birth environmental sources (Monroe and Tift, 1979; Steere et al., 1975; Tift, 1977). Pathogenesis in neonates frequently involves bacteremia and/or sepsis, cerebrospinal fluid (CSF) infection and meningitis, brain abscess and infarction, ventricle compartmentalization due to necrosis of brain tissue and liquefaction of white cerebral matter, cranial cystic changes, fluid collection and dilated ventricles and hemorrhagic and non-hemorrhagic intercerebral infarctions leading to cystic encephalomalacia (softening

of the brain) and has been associated with necrotizing enterocolitis (Gallagher and Ball, 1991; Kleiman et al., 1981; Kline, 1988a; Ries et al., 1994; Weir, 2002; Nazarowec-White and Farber, 1997b). Bacterial meningitis infections generally occur following head trauma, neurosurgery, or the development of tumors, although some cases arise in the absence of other implicating factors (Chang et al., 2000; Fotopoulos et al., 1997; Huang et al., 2001). The onset of *E. sakazakii* septicemia and meningitis is sometimes without causal explanation in neonates and infants (Arseni et al., 1987; Biering et al., 1989; Burdette and Santos, 2000; Clark et al., 1990; Gallagher and Ball, 1991; Huang et al., 2001; Joker et al., 1965; Kleiman et al., 1981; Lai, 2001; LeCour et al., 1989; Monroe and Tift, 1979; Muytjens et al., 1983; Nazarowec-White and Farber, 1997b; Noriega et al., 1990; Ries et al., 1994; Sakata and Maruyama, 1997; Simmons et al., 1989; Tekkok et al., 1996; Urmenyi and Franklin, 1961; Willis and Robinson, 1988).

Meningitis, an acute inflammation of the meninges surrounding the brain and the spinal chord, frequently results in mortality. Infection is most commonly caused by *Haemophilus*, meningococci and pneumococci in infants, as well as *E. coli*, *Enterobacter*, *Citrobacter diversus* and *Listeria* (Iversen and Forsythe, 2003; Kline, 1988a,b; Nazarowec-White and Farber, 1997b). Meningitis is the most frequently reported condition in neonatal *E. sakazakii* infections, resulting in ca. 90% of the cases leading to brain abscesses (Burdette and Santos, 2000; Gallagher and Ball, 1991; Kline, 1988a). These infections often increase inner cranial pressure, requiring aspiration of fluid and drainage of cerebral infarction, sometimes including insertion of a ventriculoperitoneal shunt to prevent cerebral damage (Muytjens et al., 1983).

Low birth weight (i.e., under 2.5 kg) has been identified as a contributor to higher risk of contracting illness (Muytjens et al., 1983). Neonatal meningitis caused by *Enterobacter* species is predictably high, with up to 92% mortality reported in cases involving *E. cloacae* (Rance et al., 1962). Meningeal *E. sakazakii* infection has been reported as arising between the fourth and fifth day after birth and can be fatal within a few hours to several days following the first clinical signs (Muytjens et al., 1983). Neonatal brain abscesses from Gram-negative rods are most frequently caused by *C. diversus* and *E. coli* (Foreman et al., 1984;

Gallagher and Ball, 1991; Kline, 1988a,b). Infant mortality for *E. sakazakii* meningitis is 40–80%, with death often occurring within hours of infection (Adamson and Rogers, 1981; Arseni et al., 1987; Joker et al., 1965; Kleiman et al., 1981; Muytjens et al., 1983; Nazarowec-White and Farber, 1997b; Urmenyi and Franklin, 1961; Willis and Robinson, 1988). Seizure activity has been reported in about one-third of the cases of neonatal *E. sakazakii* meningitis, with physiological responses including grunting, bulging fontanelles, convulsions, twitching and an increase in cranial circumference (Muytjens et al., 1983; Weir, 2002). These infections can cause hemorrhagic and non-hemorrhagic intercerebral infarctions, leading to cystic encephalomalacia (Ries et al., 1994). Up to 20% of newborns develop serious neurological complications following infection (Gebremariam, 1998).

The pathogenesis of neonatal *E. sakazakii* meningitis has not been fully defined. The process is believed to be by translocation of the bacterium through the chordus plexus and subsequent cellular invasion by means of pathogenic secretory factors (e.g., elastases, glycopeptides, endotoxins, collagenases and proteases) used to increase blood–brain barrier permeability, thus gaining access to the nutrient-rich cerebral matter (Iversen and Forsythe, 2003). Others have reported a similarity between the tropism of *E. sakazakii* and *C. diversus* for invasion of and infection in the central nervous system (Willis and Robinson, 1988; Kline, 1988a,b; Burdette and Santos, 2000).

E. sakazakii infections historically have been thought to originate from maternal vaginal contamination during passage of the infant through the birth canal; however, studies suggest that this hypothesis is implausible (Muytjens et al., 1983). For suspect cases of neonatal meningitis, many have recommended early detection by means of cranial ultrasonography, computed tomography scan (CT scan), or magnetic resonance imaging (MRI) (Bar-Oz et al., 2001; Burdette and Santos, 2000; Gallagher and Ball, 1991; Iversen and Forsythe, 2003; Kline, 1988b). Monroe and Tift (1979) were the first to report a case of *E. sakazakii*-associated bacteremia in the absence of meningitis.

Many neonatal cases of *E. sakazakii* meningitis may have some relationship to necrotizing enterocolitis, which is associated with several bacterial pathogens and is the most common gastrointestinal disease in newborns (Muytjens et al., 1983; Van Acker et al.,

2001). The illness affects ca. 2–5% of premature neonates, leads to death in 10–55% (Peter et al., 1999) and is characterized by ischaemia, bacterial colonization of the intestinal tract and increased levels of protein in the gastrointestinal lumen, the latter often attributable to the consumption of infant formula (Iversen and Forsythe, 2003). A positive correlation between necrotizing enterocolitis and oral formula feeding has been suggested (Kosloske, 1984; Iversen and Forsythe, 2003; Van Acker et al., 2001). Babies fed only infant formula rather than breast milk are 10 times more likely to contract necrotizing enterocolitis (Lucas and Cole, 1990). Another study confirmed that in 125 infants, prior to the administration of antibiotics, *Enterobacter* species were the most prevalent bacteria, present in 29% of neonates (Chan et al., 1994).

The specific virulence factors of *E. sakazakii* remain elusive; however, pathogenicity of and production of exotoxins, aerobactin and hemagglutinin by *E. cloacae* have been documented (Keller et al., 1998). Until Pagotto et al. (2003) used the suckling mouse model, no animal model specifically addressing the minimum infectious dose, lethal dose, or virulence of *E. sakazakii* had been described. In these experiments, mice were challenged intraperitoneally and perorally to test for the production of enterotoxin by nine clinical isolates and eight food isolates of *E. sakazakii*, in addition to the type strain (ATCC 29544). The study also included negative and positive controls. Mice were challenged, after which they were euthenized and the intestines were examined for distension and fluid accumulation. Cell cultures (CHO, Vero, and Y-1 lines) were also tested for cytopathic effects caused by the bacterium. Four of eighteen strains (three clinical isolates and one food isolate) or 22% tested positive for production of enterotoxin. One strain of *E. sakazakii* was toxic to all three cell lines. Another strain was positive for enterotoxin production but negative for cytopathicity against cell cultures. Minimum lethal doses for the three *E. sakazakii* strains were the same, regardless of the method of challenge. All 18 strains were lethal to mice at oral doses of 10^8 CFU/mouse; however, the type strain, ATCC 29544, tested negative for enterotoxin production. One clinical isolate and one food isolate were lethal via the peroral route at 10^7 CFU, and one clinical isolate and one food isolate were lethal by intraperitoneal injection at populations as low as 10^5

CFU. The authors concluded, by extrapolating from the suckling mouse model, that a minimum lethal dose in neonates would most likely require an unusually high number of viable cells such as might occur over time in temperature-abused infant formula. A recent study showed that lipopolysaccharide from *E. sakazakii* is toxic to N2a cells via the MTT test (Iversen et al., 2004e). Protease, phosphatase and lipase activities may contribute to host cell death.

In the more than 76 documented cases of neonatal and infant *E. sakazakii* infections, the infectious dose was not determined. Iversen and Forsythe (2003) speculated that a good first estimate for infection should be close to that postulated for *E. coli* O157:H7, *Listeria monocytogenes* 4b, or *Neisseria meningitidis*, i.e., ca. 1000 CFU. They noted that *Enterobacter* would not encounter extremely harsh pH conditions in the upper gastrointestinal tract of neonates and would pass rapidly into the small intestines. The conclusion by Iversen and Forsythe (2003) that outbreaks of *E. sakazakii* infections are due to gross temperature abuse or poor hygienic practices was questioned by Havelaar and Zwietering (2004), who provided another approach to assessing the risk of infections. Iversen and Forsythe (2004b) subsequently questioned the model, noting that it is based on assumptions and therefore needs to be verified.

Based on a level of contamination of ca. 0.36–66 CFU/100 g reported by Muytjens et al. (1988) and Nazarowec-White and Farber (1997c), and 18 g of powder reconstituted in a single feeding, at least 14 generations would be needed to produce 6000 CFU/feeding. This would require ca. 7 h at 37 °C vs. 17.9 h at 21 °C, 1.7 days at 18 °C, 7.9 days at 10 °C, and nearly 9 days at 8 °C (Iversen and Forsythe, 2003). However, these calculations are theoretical, as a fraction of a bacterial cell per bottle will not occur. The formula in the bottle will be either contaminated or not contaminated.

Adegbola and Old (1983) described fimbrial haemagglutinin in the genus *Enterobacter*. Two of four strains *E. sakazakii* were fimbriate mannose sensitive, haemagglutinin positive, produced thick fimbriae 7–8 nm in diameter and tested positive for a strong antibody coating. The other two strains were non-fimbriate and haemagglutinin negative. They observed that with increasing haemagglutinating power, the proportion of fimbriate bacteria increased.

5. Outbreaks and cases of infections

The number of documented outbreaks and cases of neonatal *E. sakazakii* infections are few. Summaries of some of the reports describing these infections are presented here.

5.1. 1958, St. Albans, England

The first two documented cases of neonatal *E. sakazakii* meningitis occurred in 1958 (Urmenyi and Franklin, 1961). At that time, however, the bacterium was described as an unusual pigmented strain of the *cloacae* group. The two infants were born within a week of each other and died within 2 days of each other. The first infant was born to a mother experiencing mild toxemia. The child was readmitted to the hospital 12 h after discharge due to “left-sided fits” and jaundice. Additional complications included an increased pulse rate, enlarged liver and bulging anterior fontanelle from meningitis. The *E. sakazakii* isolate from the patient was sensitive to chloramphenicol and streptomycin; however, the infant died after 48 h treatment with oxytetracycline. The second infant was one of a pair of twins. Five days after birth, she abruptly collapsed with complications from jaundice, cerebral trauma and a hive-like rash and died shortly thereafter. Postmortem examinations revealed that white cranial matter in both infants had degenerated into a soft hemorrhagic mass. Brain, cerebrospinal fluid (CSF) and bronchial swabs in the first infant, and brain, bronchial, liver and marrow swabs in the second infant tested positive for *E. sakazakii*. Environmental samples taken several days after infection, including swabs of the suspect incubator, were not positive for *E. sakazakii*. Nevertheless, it was surmised that the reason only one of the twins succumbed to the infection might be that the child was placed in the same incubator that had been used for an infected infant. The clinical isolate produced only slightly yellow pigmentation when cultured on nutrient agar at 37 °C, yet produced a non-diffusible yellow-gold pigment when incubated at room temperature. This was the first confirmed case now recognized to have been caused by *E. sakazakii*. Septicemia due to a yellow-pigmented coliform, however, had been reported previously (Pangalos, 1929).

5.2. 1965, Denmark

Joker et al. (1965) reported a case of neonatal meningitis caused by *E. sakazakii*, described as an atypical *Enterobacter*, forming yellow colonies on ordinary media. The patient was a 3.2-kg (6.9-lb) female delivered as a primigravida (first-time pregnancy) after 27 h of labor. Two days after birth, the child was suspected of having meningitis. *Enterobacter* was isolated from three specimens of cerebrospinal fluid but blood, feces and throat cultures tested negative. The child appeared to have recovered at 27 days and was discharged. At 2 months of age, she was readmitted to the hospital with convulsions and an electroencephalogram showed severely abnormal brain waves. Following an aggressive antibiotic administration regimen of streptomycin, chloramphenicol, ampicillin, sulfadiazine, sulfadimidine, sulfamerazine and sulfacombin, the child recovered at about 4 months of age, yet experienced extreme mental impairment. Joker et al. (1965) did not state whether the child was fed breast milk or rehydrated powdered infant formula but attributed meningitis to the prolonged delivery period and cited a publication implicating extended labor as a causal factor in neonatal meningitis (Groover et al., 1961).

5.3. 1979, Macon, GA, USA

Monroe and Tift (1979) reported a male term infant (birth weight 2.6 kg) as having *E. sakazakii* bacteremia at 7 days of age. Ampicillin administration for 10 days ameliorated the situation. Environmental samples of the nursery and/or samples from other patients and personnel were not taken. The physicians surmised that the source of infection was post-birth as evidenced from the 6-day delay in symptoms. This was the first reported case of non-meningeal bacteremia caused by *E. sakazakii*.

5.4. 1981, Indianapolis, IN, USA

Kleiman et al. (1981) reported a full-term, 5-week-old infant developing meningoencephalitis and cerebral ventricular compartmentation resulting in bulging fontanelles and grand mal seizures. Ampicillin and gentamicin treatments were administered and the child was discharged. Two months later, the circum-

ference of the child's head increased and a ventriculo-peritoneal shunt was performed. Tan 2–3-mm-diameter colonies of *E. sakazakii* formed on chocolate agar incubated under an atmosphere containing 10% carbon dioxide and 90% air and on CDC-anaerobe blood agar under a 10/5/85% mixture of hydrogen, carbon dioxide and nitrogen, respectively. Yellow-pigmented colonies developed when the culture was incubated aerobically for 48 h at 25 °C. The child recovered from the acute illness after antibiotic therapy but was severely developmentally delayed.

5.5. 1983, The Netherlands

Over a 6-year period, eight cases of neonatal meningitis involving *E. sakazakii* were reported in The Netherlands, with two of the eight patients also experiencing necrotizing enterocolitis (Muytjens et al., 1983). The bacterium was isolated from the blood and CSF of all eight patients, two of whom were delivered by Caesarean section. It was also confirmed that at least four of the eight infants were not colonized at birth (Muytjens, 1985). Twenty-three *Enterobacter* isolates were recovered from CSF of six patients, with eight of these testing positive for *E. sakazakii*. The remaining 15 were *E. cloacae*, *E. agglomerans* and *E. aerogenes*. *Enterobacter* species but no *E. sakazakii* was isolated from the blood of patients. *E. sakazakii* isolates indistinguishable from those from CSF were recovered from prepared infant formula as well as from utensils used to prepare the formula. Muytjens and Kollee (1990) later reported that upon opting for sterile liquid formula in place of powdered formula, no further cases of *E. sakazakii* infections were reported in the following 8 years.

5.6. 1985, Athens, Greece

Arseni et al. (1985) reported a case of neonatal septicemia caused by *E. sakazakii* co-mingled with *K. pneumoniae* in the neonatal intensive care unit of a children's hospital. The patient was born premature and sepsis was discovered 3 days after birth. Only *E. sakazakii* was isolated from the umbilical catheter as a pure culture, although both bacteria were isolated from blood. The two isolates were resistant to ampicillin, netilmicin, cefotaxime and amikacin. While the patient was being treated, an unspecified number of

other infants was colonized, yet not infected by *E. sakazakii*, as revealed by rectal and throat swabs.

5.7. 1986–1987, Reykjavik, Iceland

Three cases of *E. sakazakii* meningitis in full-term male neonates, two of whom were more than 38 weeks old, in Iceland were reported by Biering et al. (1989). One infant was treated with antibiotics but was severely mentally retarded and quadriplegic after recovery from infection. The second child was diagnosed with Down syndrome and died of complications from the *E. sakazakii* infection 5 days after birth. The third child was treated with antibiotics, after which he developed a seizure disorder and was moderately delayed in all developmental areas. All infants had been fed a rehydrated powdered infant formula administered within 2 h of preparation. *E. sakazakii* was not found on formula preparation utensils, in the preparation kitchen, or in environmental samples. An isolate was recovered from a time-indefinite bottle of refrigerated infant formula but not from freshly prepared formula. *E. sakazakii* was recovered in rehydrated samples from five packages of rehydrated formula incubated for 4 h at 36 °C. Formula from all lots tested was positive for the coliform. In addition, *E. cloacae* and *E. agglomerans* were recovered from the powdered formula. *E. sakazakii* was also recovered from urine, as well as from groin and anal swabs of a 3-day-old asymptomatic male child. Twenty-two of the twenty-three isolates from rehydrated formula were identical in biotype, antibiotic profile and plasmid profile to the four neonatal strains (Clark et al., 1990). It was concluded that infections must have originated from the rehydrated infant formula stemming from unknown contributing factors in the infected infants, as many neonates, including a twin brother of one infected patient, had received the same contaminated infant formula with no detectable pathogenicity. One of the causal factors may have been that formula bottles were occasionally left in heaters at 35–37 °C for lengthy periods of time.

5.8. 1987, Boston, MA, and New Orleans, LA, USA

Willis and Robinson (1988) reported that two infants, 4 weeks and 8 days of age, developed *E. sakazakii*-induced meningitis, necessitating ventricu-

lar shunts. Infections led to cerebral destruction, developmental damage and severe neurologic complications in both babies but subsided following treatment with moxalactam. No information was provided concerning possible sources of contamination; however, it was stated that the patients were never geographically proximate.

5.9. 1988, Memphis, TN, USA

Simmons et al. (1989) reported an outbreak of neonatal *E. sakazakii*-induced septicemia and meningitis clearly linked to powdered infant formula which contained *E. sakazakii* and *E. cloacae* at populations of 8 CFU/100 g and 48 CFU/100 g, respectively. The outbreak involved four pre-term neonates that exhibited one or more symptoms of bacteremia, septicemia, urinary tract infection, abdominal distension and bloody diarrhea or stool. A blender was implicated as the possible source of contamination after testing positive for *E. sakazakii* and *E. cloacae*. Other bacterial contaminants included *Pseudomonas fluorescens* and *Pseudomonas maltophilia*. The latter of the pseudomonads was isolated from CSF and stools of two of the patients. The blender was routinely rinsed with potable water and hand-cleaned on occasion with hexachlorophene and chlorhexidine. *E. sakazakii* infections of infants did not occur after use of the contaminated blender was discontinued and only sterilized blenders were used. It was determined that all isolates from the infant formula and three isolates from infants had the same plasmid and multilocus enzyme profile (Clark et al., 1990).

5.10. 1990, Baltimore, MD, USA

Noriega et al. (1990) reported a case involving a 6-month-old female who had developed septicemia following small bowel complications, including an exploratory laparotomy and a gastrostomy tube. Blood cultures were positive for both *E. sakazakii* and *Leuconostoc mesenteroides*, while stool cultures were negative for both bacteria. The central venous catheter tip contained *E. sakazakii*. Following treatment with vancomycin and ampicillin, the infection resided. The authors reported that the blender used to rehydrate the powdered infant formula was heavily contaminated with *E. sakazakii* and *L. mesenteroides*. The hospital

implemented a quality control protocol in which the blender was washed and autoclaved daily following each use. Rehydrated formula was also terminally pasteurized after receipt at the hospital and before administration.

5.11. 1990, Cincinnati, OH, USA

Gallagher and Ball (1991) reported that an infant developed complications 2 days after birth. Ampicillin and cefotaxime were prescribed after a blood culture tested positive for the presence of *E. sakazakii*. Ultrasound and CT scans 4, 6, and 20 days after birth revealed a hemorrhage, abscess and brain infarction. After 28 days of antibiotic treatment, the problem was abated and the child was discharged. Twelve days later, the infant developed meningitis and antibiotic therapy was commenced. CT scans revealed a large cranial cyst purulent with *S. aureus*.

5.12. 1995/1996, Boston, MA, USA

Five cases of *E. sakazakii* infection were reported between January 1995 and December 1996 involving individuals 3, 39, 73, 76 and 82 years of age (Lai, 2001). All patients were experiencing complicative, potentially immunosuppressive illnesses and were being treated accordingly. Symptoms and treatments included carcinoma with radiation therapy, insertion of a gastrostomy tube and emergent tracheostomy, jaundice and hepatic complications, aortic surgery with accompanying abdominal distress and fever and cecal volvulus. Only the two youngest patients survived following antibiotic treatment with gentamicin, cefotaxime, ceftazidime, cefuroxime axetil and clindamycin.

5.13. 1998, Belgium

Van Acker et al. (2001) reported an outbreak of 12 cases of neonatal necrotizing enterocolitis in which four patients required operative treatment and male twins died within 3 weeks of each other in a Belgium neonatal intensive care unit (NICU). All 12 patients had been fed reconstituted infant formula prior to the illness, 10 of whom were fed with formula manufactured by a single manufacturer. Six of the twelve patients tested positive for *E. sakazakii* via blood

culture, anal swabs and stomach aspirates. Two *E. sakazakii* strains with two differing yet unspecified morphologies were isolated from one patient. Eleven strains of *E. sakazakii* were isolated from the six *E. sakazakii*-positive patients. A survey of unopened cans of powdered infant formula yielded 14 *E. sakazakii* isolates. Suspecting a link between the formula and the necrotizing enterocolitis, feeding of the formula was discontinued. The manufacturer's quality control records revealed that five samples were analyzed prior to distribution of the product. One sample contained 20 coliforms/g, with <1 coliform/g in the remaining four samples. The Codex Alimentarius standard requires a minimum of four of five control samples with <3 coliforms/g, and a maximum of one of five samples with >3 but ≤20 coliforms/g (FAO, 1994). Belgian law is more stringent, requiring <1 coliform/g in all samples.

5.14. 2000, Winston Salem, NC, USA

Burdette and Santos (2000) reported that a 3.3-kg (7.28-lb) female neonate born at week 35 of gestation developed symptoms typical of *E. sakazakii* infection, i.e., brain abscess 6 days after birth, including a high fever, irritability and seizure activity. A CT scan showed abnormal cerebritis-like indicators in the frontal lobe of the brain. Magnetic resonance imaging was used to identify infected cavities and lesions. The bacterium was isolated from both blood and CSF but not from urine. The acute condition cleared up after administration of ampicillin, cefotaxime and intravenous bactrim and draining purulent fluid via an emergency craniotomy. This fluid tested positive for *E. sakazakii*. The authors stated that this was the first reported case of *E. sakazakii* being isolated directly from a drained cranial abscess. Five weeks after admission, the child was discharged with no apparent neurological or developmental deficits.

5.15. 1993, 1995, 1997–2000, Israel

Bar-Oz et al. (2001) reported two cases of neonatal meningitis that occurred in the NICU of a hospital in Israel in December 1999–January 2000. Both infants were underweight at birth, weighing 2.1 kg (4.75 lb) and 0.6 kg (1.37 lb), respectively. The first child was a full-term healthy female that had been fed rehydrated

powdered infant formula and developed complications on the fourth day after birth. *E. sakazakii* was recovered from the CSF and blood. The child developed seizures and was confirmed by magnetic resonance imaging (MRI) and CT scans as having infarction, liquefaction and cavitation of the brain. The child required cefotaxime and a ventriculoperitoneal shunt to reduce cranial pressure. The second child, born 9 weeks premature, was fed rehydrated infant formula by an enteral feeding tube. Nine days following birth, the child was diagnosed with an upper gastrointestinal hemorrhage and *E. sakazakii* was recovered from blood but not the CSF. The child recovered after a 10-day treatment with cefotaxime. As both children were born via Caesarean section, contamination attributable to passage through the birth canal was excluded. In addition to the two infected infants, three additional infants had *E. sakazakii*-positive stools for 3, 4 and 8 weeks, respectively, demonstrating colonization without infection. Antibiotic treatment did not eliminate *E. sakazakii* from these patients. Samples from the blender and rehydrated formula from the preparation kitchen were positive for *E. sakazakii*, yet samples of all other rehydrated formula, infant formula powder and preparation personnel were negative. The blender, noted to have a small crack in the base (Block et al., 2002), was no longer used for formula preparation but continued to test positive for *E. sakazakii* for 5 months.

Block et al. (2002) described four additional cases of *E. sakazakii* bacteremia, three of which occurred at hospitals in Israel. The first case involved in a full-term formula-fed neonate in 1993. The second case occurred in 1995 in which *E. sakazakii* conjunctivitis developed in a female delivered by Caesarean section. The third case involved a 6-year-old boy in 1997 who had received a bone marrow transplant at the age of 3 days. *E. sakazakii* was isolated from a broviac catheter. A fourth case in 1998 involved a female 6 days after birth who was delivered vaginally and developed *E. sakazakii* meningitis.

5.16. 2001, Knoxville, TN, USA

Himelright et al. (2002) and Weir (2002) described a 2001 outbreak of *E. sakazakii* infections in Tennessee in which 49 infants in a NICU were screened, with 10 infants testing positive for the bacterium and 1

dying of meningitis following 9 days of intravenous treatment with antibiotics. Seven of the ten *E. sakazakii*-positive neonates were colonized but did not show symptoms of infection. The source of the bacterium was traced to a powdered infant formula specific for individuals with nutritional and malabsorption problems. Following this outbreak, the hang time, i.e., the length of time a given rehydrated infant formula is administered to an infant, was reduced from 8 h to 4 h (Himelright et al., 2002).

6. Antibiotic resistance

The frequency of clinical treatments of patients with broad spectrum antibiotics is thought to be a contributing cause of increased numbers of multi-antibiotic-resistant microorganisms, including *Enterobacter* species (Borderon et al., 1996; Burwen et al., 1994; Delphine et al., 2001; Ehrhardt and Sanders, 1993; Hervas et al., 2001; Huang et al., 2001; Landry et al., 1991; Pitout et al., 1997; Sakata and Maruyama, 1997; Sanders and Sanders, 1997; Snyderman, 1991; Wust et al., 1994). Pitout et al. (1997) cited numerous studies showing that as the number of patients in hospitals increases, so increases the number of isolates of *Enterobacter* resistant to quinolones, β -lactams and trimethoprim-sulfamethoxazole (Burwen et al., 1994; Landry et al., 1991; Pitout et al., 1997; Sanders and Sanders, 1997; Wust et al., 1994). The amount of antibiotics utilized also increases as the size of the hospital increases, thus potentially exposing microorganisms to more as well as a wider array of antimicrobials. This theory is supported by Sakata and Maruyama (1997), who reported a steady emergence of multiple multi-antibiotic-resistant strains of *E. cloacae* from 1982 to 1995 corresponded with the type and volume of antibiotics administered. Effective means to treat antibiotic-resistant *Enterobacter* have been limited. Carbapenems or fluoroquinolones are commonly used to treat infected patients, although resistance to both antibiotics has been noted (Sanders and Sanders, 1997).

Because bacterial meningitis necessitates effective treatment, antibiotic susceptibility is of increased concern with neonatal *E. sakazakii* infections (Eng et al., 1987). *E. sakazakii* has been reported as being more sensitive than other *Enterobacter* species to some

antibiotics, including typical susceptibility to aminoglycosides, ureidopenicillins, ampicillin and carboxypenicillins (Adamson and Rogers, 1981; Farmer et al., 1980; Hawkins et al., 1991; Jimenez and Gimenez, 1982; Muytjens and van der Ros-van De Repe, 1986; Monroe and Tift, 1979; Nazarowec-White and Farber, 1999; Willis and Robinson, 1988). Farmer et al. (1980) tested 10 strains of *E. sakazakii* for antibiotic susceptibility and determined that the minimum inhibitory concentrations (MICs) to chloramphenicol and ampicillin were moderate, at 4–8 µg/ml and 2–4 µg/ml, respectively. Examination of 24 strains of *E. sakazakii* using the Kirby–Bauer disk method revealed that 96% were sensitive to nalidixic acid (30 µg), 100% to gentamicin (10 µg), 92% to streptomycin (10 µg), 100% to kanamycin (30 µg), 87% to tetracycline (30 µg), 100% to chloramphenicol (30 µg), 100% to ampicillin (10 µg) and 87% to carbenicillin (100 µg). The same strains were less susceptible to penicillin (0% at 10 U), cephalothin (13% at 30 µg), sulfadiazine (67% at 250 µg), and colistin (71% at 10 µg).

E. sakazakii infections have been traditionally treated with ampicillin in combination with gentamicin or chloramphenicol (Lai, 2001). The ampicillin–gentamicin treatment has been dubbed as the gold standard (Willis and Robinson, 1988). Unfortunately, *E. sakazakii* has developed resistance to these antibiotics by means of transposable elements and to β-lactams by the production of β-lactamase (Delphine et al., 2001; Lai, 2001; Muytjens et al., 1983; Pitout et al., 1997). *Enterobacter* species are known to be prolific in their ability to inactivate broad spectrum penicillins and cephalosporins due to β-lactamase production, which appears to be increasing among strains of *E. sakazakii* (Chow et al., 1991; Emery and Weymouth, 1997; Lai, 2001). For this reason, a shift to carbapenems or the newer third-generation cephalosporins in combination with an aminoglycoside or trimethoprim–sulfamethoxazole has been suggested. Trimethoprim has also been suggested as a viable alternative (Lai, 2001; Weir, 2002). Lai (2001) described cases of five *E. sakazakii* infections in which one or more of the isolates were resistant to cefazolin, ampicillin, cefotaxime, ceftazidime, piperacillin–tazobactam, gentamicin, ofloxacin and cefuroxime. One or more isolates were sensitive to cefotaxime, ceftazidime, piperacillin–tazobactam, gentamicin, ofloxacin, tri-

methoprim–sulfamethoxazole and imipenem–cilastatin. One strain from a 76-year-old patient was susceptible only to trimethoprim–sulfamethoxazole and aminoglycosides. A second strain from the same patient was susceptible to these antibiotics in addition to quinolones. Pitout et al. (1997) tested eight strains of *E. sakazakii* for the presence of β-lactamases. Antibiotic susceptibility testing was against ampicillin, ampicillin–sulbactam, amoxicillin–clavulanic acid, ticarcillin, ticarcillin–clavulanic acid, piperacillin, piperacillin–tazobactam, aztreonam, cephalothin, cefazolin, cefoxitin, cefotaxime, ceftriaxone, ceftazidime, cefepime and imipenem. Some of the eight strains were sensitive to the three β-lactams, ampicillin, cephalothin, and cefoxitin, while all wild-type *E. sakazakii* strains were susceptible to ampicillin, cefoxitin and cephalosporins. All eight strains tested for β-lactamases were positive for Bush group 1 β-lactamase (cephalosporinase). Block et al. (2002) examined *E. sakazakii* isolates from six neonatal and childhood infections and reported all as being β-lactamase positive, most likely representing Bush group 1 β-lactamase.

Nazarowec-White and Farber (1999) tested the antibiotic resistance of 17 strains of *E. sakazakii* and found four antibiotic susceptibility patterns (antibiograms). Five food isolates and all but one clinical isolate had the same antibiogram pattern as the type culture, viz., resistance to sulphisoxazole and cephalothin, yet susceptible to ampicillin, cefotaxime, chloramphenicol, gentamicin, kanamycin, polymyxin-B, trimethoprim–sulphanmethoxazole, tetracycline and streptomycin. The authors concluded that antibiogram patterns were the least discriminatory of five analyses used to distinguish bacterial strains, i.e., biotyping, ribotyping, PFGE, RAPD and antibiotic susceptibility testing. Muytjens et al. (1983) reported that, although the *E. sakazakii* isolates in cases they investigated were susceptible in vitro to ampicillin, gentamicin, chloramphenicol and kanamycin, six of eight patients responded poorly and died.

Muytjens and van der Ros-van De Repe (1986) tested the antibiotic susceptibility of 195 *E. sakazakii* isolates, 157 of which were from confirmed sources, against 29 antibiotics. Some of the sources (numbers of isolates in parenthesis) included the respiratory tract (35), digestive tract (31), utensils (21), CSF (17), superficial wounds (12), urine (9), upper respi-

ratory tract (9) and blood (5). They reported using an agar dilution method with a base of Mueller–Hinton agar to determine MICs of antibiotics. *E. sakazakii* was the most susceptible of eight *Enterobacter* species evaluated for sensitivity to 29 antimicrobials. Concentrations of 24 of the 29 antibiotics necessary to inhibit at least 90% of the strains were ≤ 8 $\mu\text{g/ml}$. Antibiotics having MICs greater than 8 $\mu\text{g/ml}$ were chloramphenicol (16 $\mu\text{g/ml}$), cefaloridin (16 $\mu\text{g/ml}$), cefsulodin (32 $\mu\text{g/ml}$), cephalothin (>128 $\mu\text{g/ml}$) and sulfamethoxazole (>128 $\mu\text{g/ml}$). Concentrations necessary to inhibit 90% of the *E. sakazakii* strains were at least twofold lower than those required for *E. cloacae*. Kleiman et al. (1981) reported less, yet only moderate, resistance of an *E. sakazakii* strain isolated from a 5-week-old female with meningoen- cephalitis to cephalothin (MIC=16 $\mu\text{g/ml}$).

Willis and Robinson (1988) detailed two cases of *E. sakazakii*-induced neonatal meningitis that, after being unresponsive to ampicillin–gentamicin therapy, resulted in abatement via treatment with moxalactam. Naqvi et al. (1985) eliminated *E. sakazakii* infection in one patient by using cefotaxime. Block et al. (2002) concluded that general assumptions concerning antimicrobial therapy for *E. sakazakii* cannot be made, and treatment should be guided by clinical judgment and in vitro susceptibility testing.

7. Sources of contamination

7.1. Clinical sources

Farmer et al. (1980) reported that most *E. sakazakii* isolates from infected patients originate from CSF, blood, sputum, throat, nose, stool, gut, skin, wounds, bone marrow, eye, ear and breast abscess. Most isolates are rare if not sporadic. However, in a 7-month period, the bacterium was isolated from the respiratory tract of 29 patients in one hospital. Although *E. sakazakii* infections in newborns have been suspected as arising via passage through the mother's birth canal, this hypothesis seems untenable based on *E. sakazakii* infections in neonates born by Caesarean section (Bar-Oz et al., 2001; Muytjens and Kollee, 1990; Muytjens et al., 1983; Urmenyi and Franklin, 1961). Analysis of plasmid DNA profiles has revealed that three or four of the five isolates from the same

hospital were presumptive-positive for the same strain (Muytjens et al., 1983). Fecal, vaginal and cervical swabs of the mothers were negative for *E. sakazakii*. Samples from the anus, skin, nose, umbilical chord and outer ear of two infants on their day of birth were negative for the *Enterobacter*. Another infant who was tested for the presence of *E. sakazakii* in the outer ear, nose, umbilicus and gastric aspirate proved negative for the organism. These observations, coupled with infection of three infants delivered by Caesarean section, seem to also preclude contamination upon passage through the birth canal. The organism may persist in clinical settings over extended periods of time. For instance, three isolates in the same hospital over 11 years appeared to be the same strain based on ribotyping (Nazarowec-White and Farber, 1999).

7.2. Environmental sources

Enterobacter species have been reported as being frequently isolated from soil, water, animals, sewage and human fecal samples (Sakazaki, 1974). Hallmann et al. (1997), in a review of endophytic bacteria in agricultural crops, noted that the genus *Enterobacter* is associated with the phytic flora. *Enterobacter* species have been isolated from corn roots and stems, cucumber roots, rough lemon roots and grapevine stems (Bell et al., 1995; Fisher et al., 1992; Gardner et al., 1982; Mahaffee and Kloepper, 1997; McNroy and Kloepper, 1995).

It has been hypothesized that just as the primary reservoir for the coliform *E. coli* is feces, the reservoir for *E. sakazakii*, in addition to other coliforms, e.g., *Klebsiella oxytoca*, *K. pneumoniae*, *E. cloacae*, and *Citrobacter* species, may be primarily environmental and from plant materials (Mossel and Struijk, 1995). Iversen and Forsythe (2003) speculated that the principal environmental sources of *E. sakazakii* are water, soil and vegetables, and a secondary means of contamination may be vectors such as flies and rodents. However, Muytjens and Kollee (1990) did not recover *E. sakazakii* from bovine milk, cattle, domesticated animals, rodents, bird dung, grain, rotting wood, mud, soil, or surface water in The Netherlands. This is in contrast to our studies, including one industry study in the United States, in which *E. sakazakii* was recovered from 20 of 49 factory environmental sampling

sites (data not published). [Kandhai et al. \(2004a\)](#) reported isolating *E. sakazakii* from 35 of 147 samples from a survey of 9 food factories and 16 households in The Netherlands. Factory samples were taken by sampling vacuum cleaner bags or by scraping or sweeping surfaces while household samples were taken exclusively from vacuum cleaner bags. Fourteen of the sixty-eight samples (21%) from a powdered milk factory were positive for *E. sakazakii*. In a chocolate factory, cereal factory, potato flour factory, pasta factory and spice factory, 2 of 8 (25%), 4 of 9 (44%), 4 of 15 (27%), 6 of 26 (23%) and 0 of 5 samples, respectively, were positive for the bacterium. Five of sixteen household samples (31%) were positive for *E. sakazakii*. It was recommended that the widespread nature of *E. sakazakii* needs to be taken into consideration when designing preventive control measures ([Kandhai et al., 2004a](#)). [Arts \(2004\)](#) made two additional recommendations, viz., enhancement of the promotion of and support for breast feeding and inclusion of a warning on infant formulae and other breast-milk substitutes that the product might be contaminated with *E. sakazakii* and other microorganisms.

[Kandhai et al. \(2004b\)](#) evaluated a colorimetric screening assay to detect *E. sakazakii* in 152 dry samples of scrapings from dust, vacuum cleaner bags and spilled products from three milk powder plants. The bacterium was isolated from 18 of 152 (11.8%) samples. [Cruz et al. \(2004\)](#) isolated nine strains of *E. sakazakii* from 20 samples of dust; two strains were isolated from 48 samples of water in Mexico.

[Kuzina et al. \(2001\)](#) first reported isolating *E. sakazakii* from the Mexican fruit fly, *Anastrepha ludens*, and [Hamilton et al. \(2003\)](#) were the first to isolate *E. sakazakii* from the midgut of the stable fly larvae, *Stomoxys calcitrans*, suggesting that it may be a reservoir for the bacterium. Stable flies feed on blood of livestock and other domesticated animals, as well as humans and feral hosts, but are most commonly found in the vicinity of cattle. *S. calcitrans* can be found in all countries reporting *E. sakazakii* infections. Strains of *E. sakazakii*-producing mucoid or matt colonies have been isolated from the stable fly ([Hamilton et al., 2003](#)). *E. sakazakii*, *Erwinia carotovora*, *Micrococcus luteus*, *Providencia stuartii* and *Serratia marcescens* were isolated from farm-reared larvae.

E. sakazakii has been isolated from a physician's stethoscope and an uninoculated bottle of bacterial culture medium ([Farmer et al., 1980](#)). [Iversen and Forsythe \(2003\)](#) suggested that the capsule produced by *E. sakazakii* might increase its ability to attach to surfaces and form biofilms. [Iversen et al. \(2004d\)](#) studied biofilm formation by *E. sakazakii* grown in an infant milk formula. The bacterium adhered to silicon, latex and polycarbonate in greater numbers than to stainless steel. A capsulated strain formed denser biofilms compared to a noncapsulated type strain. They recommended that bottles and utensils used to prepare infant formulae should be cleaned thoroughly as soon as possible after use to eliminate or minimize the formation of biofilms, which could be sources of infection. [Zogaj et al. \(2003\)](#) observed that an extracellular matrix, cell clumping, pellicle formation and biofilm formation by *E. sakazakii* was associated with the expression of cellulose and curli fimbriae.

[Lee and Kim \(2003\)](#) constructed a semi-pilot galvanized iron pipe model to assess the microbial quality of a public drinking water system. Municipally treated potable water was circulated in the iron pipe for 12 weeks and examined for the presence of bacteria. *E. sakazakii* was isolated from one influent sample and one effluent sample. [Mosso et al. \(1994\)](#) surveyed the bacterial quality of 26 thermal mineral water springs in Spain. Three of the springs were classified as hypothermal (<30 °C), ten were mesothermal (30–40 °C), and thirteen were hyperthermal (>40 °C). Forty isolates of *Enterobacter* species, including *E. sakazakii*, *E. agglomerans* and *E. amnigenus*, and six unidentified isolates were recovered from 13 of the 26 springs surveyed. Ten of thirty-one *Enterobacter* isolates from hyperthermal springs were identified as *E. sakazakii*. [Van Os et al. \(1996\)](#) isolated *E. sakazakii* from grass silage in The Netherlands.

E. sakazakii has been isolated from floor drains, air, a vacuum canister, broom bristles, a room heater and electrical control box, transition socks, a clean-in-place (CIP) valve, a floor dryer, floor and condensate in a dry product processing environment in the United States (unpublished data). [Iversen and Forsythe \(2003\)](#), in reviewing relevant literature, reported a number of other environmental sources from which *E. sakazakii* has been isolated, including air in a hospital ([Masaki et al., 2001](#)), clinical materials ([Janicka et al., 1999](#); [Tuncer and Ozsan, 1988](#)), rats

(Gakuya et al., 2001), soil (Neelam et al., 1987), rhizosphere (Emilani et al., 2001), sediment and wetlands (Espeland and Wetzell, 2001), crude oil (Assadi and Mathur, 1991) and cutting fluids (Suliman et al., 1988).

7.3. Foods as sources of *E. sakazakii*

Krieg and Holt (1984) stated that *E. sakazakii* was more prevalent in foods and the environment than in clinical settings. The bacterium has been isolated from a can of previously unopened non-fat dried milk (Farmer et al., 1980). Muytjens et al. (1988) cultured members of the family Enterobacteriaceae from 52.5% of 141 milk-substitute infant formulae obtained from 35 countries. Populations did not exceed 1 CFU/g of any product. *E. sakazakii* was detected in 20 of 141 (14.2%) samples from 13 of the 35 countries. In a survey of infant formula products from 11 countries, Leuscher et al. (2004) isolated *E. sakazakii* from 8 of 58 (13.8%) samples. Iversen et al. (2004a) surveyed 82 samples of powdered infant formula milk and 404 other food products for the presence of *E. sakazakii*, *Salmonella* and other Enterobacteriaceae. The bacterium was isolated from 2 of 82 (2.4%) formulae, 5 of 49 (10.2%) dried infant foods, 3 of 72 (4.1%) milk powders, 2 of 62 (3.2%) cheese products and various dry food ingredients, including 40 of 122 (37.8%) herbs and spices. *Salmonella* was not isolated from the dry infant formula milk, dried infant foods, or milk samples. It was concluded that hygienic production of formula and milk powder as monitored by control of *Salmonella* and enumeration of Enterobacteriaceae did not control *E. sakazakii*.

Several reports have implicated rehydrated powdered infant formula as a source of *E. sakazakii* in neonatal infections (Biering et al., 1989; Block et al., 2002; Clark et al., 1990; Himelright et al., 2002; Muytjens et al., 1983, 1988; Noriega et al., 1990; Simmons et al., 1989; Smeets et al., 1998; Van Acker et al., 2001; Weir, 2002). The first outbreak of *E. sakazakii* linked to powdered infant formula from a previously unopened can was in 2001 (CDC, 2002; Himelright et al., 2002; Weir, 2002). In another outbreak, powdered formula tested negative for *E. sakazakii*, yet the blender used to prepare the rehydrated formula was positive (Noriega et al., 1990). It

was suggested that contamination could have arisen from a previous batch of powdered infant formula that was contaminated. The blender was washed in a dish-washing machine daily; however, it was suspected that the cleaning procedure was not sufficient to eliminate bacterial contamination. Caric (1993) indicated that the drying and filling areas of a food factory are potential sources of contamination. Nazarowec-White and Farber (1997a) stated that microbial pathogens can gain access to the powder from the environment or from the addition of ingredients at the powder stage. At least one strain of *E. sakazakii* (NCTC 8155) originated from dried milk (Farmer et al., 1980; Iversen and Forsythe, 2003; Thornley, 1960).

In an outbreak of five cases of neonatal meningitis, *E. sakazakii* was isolated from a stirring spoon and a dish brush used to prepare infant formula and from prepared formula (Muytjens et al., 1983). Isolates were indistinguishable from clinical isolates by means of antibiograms, biogroups, pigment production and general morphology, with the exception of biochemical differences in one clinical isolate. Samples of water and powdered formula were negative for the coliform.

Gassem (1999) analyzed khamir, a fermented bread made from the Baydah and Hamra varieties of sorghum in southwest Saudi Arabia, for the presence of coliforms. They prepared the bread by combining the sorghum with water, onion, garlic, lemon juice and fenugreek and incubating the mixture for 24 h at 30 °C. Products prepared from the two varieties had pH values of 3.92 and 3.85, respectively. Six *E. sakazakii* isolates were among the six coliforms detected in these breads. In another study (Gassem, 2002), 14 samples of sobia, a traditional fermented beverage made in western and central provinces of Saudi Arabia, were examined for microbiological quality, titratable acidity and pH. Titratable acidity ranged from 0.04% to 0.30%, based on percent lactic acid, with the pH ranging from 3.37 to 5.53. *E. sakazakii* was most frequently isolated (1/3 of all samples) among 12 different bacteria, including lactics and coliforms recovered from the beverage.

Soriano et al. (2001) examined 370 food samples from restaurants in Spain to determine the incidence of pathogenic bacteria as well as the presence of spoilage microorganisms that might serve as food safety indicators. Lettuce, pork, beef, chicken and

Spanish potato omelettes were analyzed. *E. sakazakii* was recovered from 1 of 40 samples of raw lettuce but not from ready-to-eat lettuce or the other food products. Cottyn et al. (2001) analyzed rice harvested from various sites in the Philippines for bacterial flora; of the 428 bacterial isolates examined, 184 were Gram-positive and 244 were Gram-negative. The most prevalent (25%) of the Gram-negative isolates were from the family Enterobacteriaceae, with the genus *Pantoea* and *Enterobacter* predominating. Four seed lots yielded 20 *E. sakazakii* isolates and five lots yielded 9 isolates of *E. cloacae*.

Other sources reported to harbor *E. sakazakii* include water, pipes and biofilm (Al-Hadithi and Al-Edani, 1995; Bartolucci et al., 1996; Oliver, 1997), beer mugs (Schindler and Metz, 1990), sour tea (Tamura et al., 1995), cheese, minced beef, sausage meat and vegetables (Leclercq et al., 2002). Nazarowec-White and Farber (1997b) reported a personal communication from R. Foster who isolated the coliform from ground meat. In a Norwegian study, *E. sakazakii* was isolated from mung bean sprouts (Robertson et al., 2002). Nine strains of *E. sakazakii* were isolated from 50 samples of alfalfa sprouts in Mexico (Cruz et al., 2004). We have isolated the organism from rice starch, rice flour and eggs (unpublished data).

8. Inactivation

8.1. Thermal resistance

The atypically high number of *Enterobacter* species in powdered infant formulae has been explained by some as resulting from the relatively high thermal resistance of some species in the genus (Buchanan, 2003; Nazarowec-White and Farber, 1997a; Van Acker et al., 2001). Other studies suggest that persistence of *E. sakazakii* following pasteurization treatment is doubtful (Breeuwer et al., 2003; Buchanan and Edelson, 1999; Edelson-Mammel and Buchanan, 2004c; Nazarowec-White and Farber, 1997a; Nazarowec-White et al., 1999). One study revealed that the milk processing plant environment as well as ultra-high-temperature pasteurized milk cartons to be sources of *E. sakazakii* contamination (Skladal et al., 1993). This finding, however, does not conclu-

sively prove that the organism is capable of surviving pasteurization.

Nazarowec-White and Farber (1997a) were the first to describe thermal inactivation and resistance characteristics of *E. sakazakii* in rehydrated powdered infant formula. The heating medium was an infant formula with the highest fat content (3.8 g/100 ml) of all formulae sold in Canada. Ten strains of *E. sakazakii* (five food isolates and five clinical isolates) at populations of $7 \log_{10}$ CFU/ml were heated at 52, 54, 56, 58, and 60 °C. The resulting *D* values were $D_{52\text{ °C}}=54.8$ min, $D_{54\text{ °C}}=23.7$ min, $D_{56\text{ °C}}=10.3$ min, $D_{58\text{ °C}}=4.20$ min, $D_{60\text{ °C}}=2.50$ min, with a pooled *z* value of 5.82 °C. The authors calculated that a 6–7 \log_{10} kill would require heating at 60 °C for 15–17.5 min. Iversen et al. (2004d) determined *D* values for *E. sakazakii* in a rehydrated powdered milk formula. *D* values of 16.4, 5.1, 2.6, 1.1 and 0.3 min at 54, 56, 58, 60, and 62 °C, respectively, were reported for the type strain. *D* values for a capsulated strain were generally less but *z* values for type and capsulated strains were 5.8 and 5.7 °C, respectively. High-temperature short-time (HTST) pasteurization (71.2 °C for 15 s) used to process dried infant formula would theoretically result in ca. a 21-*D* kill (Iversen et al., 2004d), which indicates that *E. sakazakii* cannot survive a commercial pasteurization process.

Nazarowec-White et al. (1999) developed a linear model for heat inactivation of *E. sakazakii* during HTST pasteurization of bovine milk by taking into account physical stresses such as shear force. Inoculated formulae were heated at 58–68 °C in 1 °C increments for 3, 10, 16, 30, and 60 s. *D* values were compared with those of *L. monocytogenes* calculated by Piyasena et al. (1998). The authors concluded that at 68 °C, *E. sakazakii* was more thermotolerant than *L. monocytogenes*.

Edelson-Mammel and Buchanan (2004c) examined the thermal resistance of 12 strains of *E. sakazakii* using a submerged vessel method reported in a previous study from the same laboratory (Buchanan and Edelson, 1999). Rehydrated infant formula was inoculated with *E. sakazakii* at ca. $8 \log_{10}$ CFU/ml and heated at 56, 58, 60, 65 and 70 °C. Isolates exhibited an almost 20-fold divergence in thermal resistance, with strain ATCC 51329 being the least thermally resistant. The most thermally resistant strain was a clinical isolate. The respective mean $D_{58\text{ °C}}$

values for the two strains were 0.51 and 9.87 min. Approximately half of the strains had $D_{58\text{ }^{\circ}\text{C}}$ values of <0.83 min, whereas half had D -values of >5 min. It was concluded that the two divergent thermally resistant groups could be divided into two distinct phenotypes. The z value of the most thermally tolerant strain was calculated to be $5.6\text{ }^{\circ}\text{C}$, very close to that reported by Nazarowec-White and Farber (1997a). This strain was further examined by inoculating powdered infant formula with a cell suspension, then rehydrating the powder in baby bottles with deionized water at 50, 60, 70, 80, 90 and $100\text{ }^{\circ}\text{C}$. Populations were reduced by 0.3 and 1.3 log_{10} CFU/ml at 50 and $60\text{ }^{\circ}\text{C}$, respectively, while treatment at all higher temperatures reduced populations by $>4\text{ log}_{10}$ CFU/ml, which was below the lowest detection limit of 100 CFU/ml. It should be recognized that the behavior of cells after desiccation resulting from exposure to dry powder may be different than that of cells not exposed to a dry environment.

Breeuwer et al. (2003) performed thermal inactivation studies using five strains of *E. sakazakii* in the stationary phase. $D_{58\text{ }^{\circ}\text{C}}$ values ranged from 0.39 to 0.60 min, with a mean of 0.48 min. The z values of two strains were 3.1 and $3.6\text{ }^{\circ}\text{C}$, respectively. These values are lower than those reported in other studies (Edelson-Mammel and Buchanan, 2004c; Nazarowec-White and Farber, 1997c). However, while the latter studies tested the thermal stability of *E. sakazakii* in rehydrated infant formula, Breeuwer et al. (2003) used disodium hydrogen phosphate/potassium dehydrogenate phosphate buffer with a neutral pH as a heating medium. Differences in composition of heating media serve as a plausible explanation for the divergence in observed D values. The increased amount of fat, protein and carbohydrate in the infant formula may protect *E. sakazakii* against thermal inactivation, thus resulting in higher D values. Spray or roller-dried milk, the primary ingredient in infant formula, casein, and whey proteins (β -lactoglobulins, α -lactalbumins, serum albumin and immunoglobulins) may also affect thermal inactivation rates.

Several studies have evaluated the effects of microwave heating on the destruction of microorganisms in milk. The mechanism by which microwaves cause the death of microbial cells is thought to involve thermal as well as non-thermal effects associated with electromagnetic radiation (Goldblith and Wang,

1967; Kindle et al., 1996; Lechowich et al., 1969; Najdovski et al., 1991; Rosenberg and Sinell, 1989; Vela and Wu, 1979). Kindle et al. (1996) examined the effects of electromagnetic radiation on (2450 MHz) *E. sakazakii* strain ATCC 29544 (type culture) and two other strains. Cells were inoculated at a population of 5 log_{10} CFU/ml into five rehydrated powdered infant formulae. Formulae were heated until the first signs of boiling, then cooled and analyzed for populations of surviving cells. Four of the five samples were negative for *E. sakazakii* following microwave treatment and one formula contained 20 CFU/ml. Differences in formula composition could account for different rates of inactivation of *E. sakazakii*. Thermal inactivation may also have been influenced by the concentration of solutes in various formulae. The bactericidal efficacy of microwave treatment of milk is the basis for recommending its use over more traditional methods to rewarm rehydrated powdered infant formula (Kindle et al., 1996). They reported that microwaving infant formula in baby bottles for 85–100 s to a temperature of $82\text{--}93\text{ }^{\circ}\text{C}$ can result in a $>4\text{ log}_{10}$ CFU/ml destruction of *E. sakazakii*. However, Edelson-Mammel and Buchanan (2004a,b,c) suggested that due to the scalding hazard, a rehydration temperature of $70\text{ }^{\circ}\text{C}$ would be more appropriate and added that rehydration at this temperature would virtually assure that a serving would not contain *E. sakazakii*.

8.2. Osmotic and desiccation resistance

E. sakazakii appears to have an unusual ability to survive when exposed to dry conditions. Survival of nine clinical and food strains of *E. sakazakii* in dry infant formula milk was studied by Caubilla-Barron et al. (2004). Initial reductions were 2–4 logs and reductions after 6 months were 4–7 logs. Rehydration with water at $60\text{ }^{\circ}\text{C}$ resulted in a 3-log decrease in viable cell number compared to rehydration at ambient temperature or $45\text{ }^{\circ}\text{C}$. Iversen and Forsythe (2003) speculated that its survival under such conditions for periods of up to 2 years may be attributable to capsule formation. Breeuwer et al. (2003) described the resistance of *E. sakazakii* to high osmolarity and discussed mechanisms bacteria use to achieve this condition. Bacteria are known to prevent intercellular dehydration via accumulation of ions (e.g., K^{+}) and compat-

ible solutes (e.g., trehalose, proline, glycine betaine) that can increase the intracellular osmolarity and maintain a shell of water around macromolecules (Kempf and Bremer, 1998; Potts, 1994; Leslie et al., 1995). An increase in the trehalose concentration in growth media has been shown to increase the resistance of stationary phase cells of *E. coli* to desiccation (Welsh and Herbert, 1999). Increasing the compatible solute concentrations in media used to culture *E. coli* and *Salmonella* has also proven effective in increasing osmotic resistance (Kempf and Bremer, 1998).

Breeuwer et al. (2003) tested the osmotic resistance of *E. sakazakii* at a_w 0.934 (in brain heart infusion broth supplemented with sorbitol) at 25 °C and observed it to be more resistant than *E. agglomerans*, *E. coli*, *Salmonella senftenberg*, *Salmonella typhimurium* and *Salmonella enteritidis* to inactivation. The population of one strain of *E. sakazakii* decreased by ca. 90% cycle during storage for 2 months. Populations of *Salmonella*, *E. coli*, *K. pneumoniae*, *Serratia rubidea* and *Citrobacter freundii* suspended in 75% sorbitol (a_w 0.811) decreased by 6 log₁₀ CFU/ml within 14 days. Populations of two strains of the *E. sakazakii* exposed to the same conditions decreased only 3–4 log₁₀ CFU in the same period of time, with an additional two strains being detectable after 4 weeks. The phase of growth played an important role in survival. For stationary phase cells suspended in a 75% sorbitol solution, the population of one strain of *E. sakazakii* decreased ca. 90% within 3 weeks, while cells in exponential growth phase decreased by 5 log₁₀ within 1 week. Additional studies involved desiccating cells in phosphate buffer for 1 h at 20.7% relative humidity and holding pellets for 46 days before analyzing for survivors (Breeuwer et al., 2003). Exponential phase *E. sakazakii* decreased by ca. 7 log₁₀ in 10 days, while stationary phase cells decreased by only 1–1.5 log₁₀ during a 46-day trial. This is in contrast to stationary phase *E. coli* cells, which declined by >4 log₁₀ in 46 days. When trehalose was added to the *E. sakazakii* cell suspension prior to desiccation, viable exponential phase cells were reduced by ca. 2.5 log₁₀ vs. ca. 7 log₁₀ in the absence of the polyhydroxyl solute. Trehalose or glycine betaine, however, did not provide greater osmotic protection against sorbitol. The trehalose content in *E. sakazakii* was 500% higher in stationary phase cells than in exponential phase cells.

The genetic basis for survival of *E. sakazakii* when exposed to dry conditions a_w 0.23 was studied by Breeuwer et al. (2004). Desiccation results in an induction of seven genes from the heat shock regulon, four genes from the cyclic AMP receptor protein regulon, six genes involved in the stringent response and a number of genes involved in trehalose synthesis and cell wall functions such as lipid A and lipopolysaccharide biosynthesis. It was concluded that the response of *E. sakazakii* to dry stress involves a genome-wide expression of functionally different groups of genes.

8.3. Antimicrobial activity of chitosan and its oligomers

Chitosan, present in the shells of crustaceans such as shrimp, lobster and crab, is a non-toxic biopolymer (No et al., 2002). The ability of chitosan and chitosan oligomers to inhibit tumors and lower LDL cholesterol levels, as well as their antimicrobial activities, has been described (Kendra and Hadwiger, 1984; Knorr, 1984; Muzzarelli, 1977; No et al., 2002; Sekiguchi et al., 1994; Sudarshan et al., 1992; Sugano et al., 1992; Tokoro et al., 1988). It has been theorized that antimicrobial activity might be attributable to an interaction between negatively charged bacterial surface residues and positively charged chitosan molecules (Hadwiger et al., 1981; No et al., 2002; Sudarshan et al., 1992; Young et al., 1982). Studies supporting the antimicrobial activity of chitosan are inconclusive, although it has been demonstrated that water-soluble chitosan preserves tofu against microbial spoilage (Chun et al., 1999). No et al. (2002) isolated seven different bacteria from 10 commercially produced South Korean brands of tofu, one of which was identified as *E. sakazakii*. They studied the antibacterial effects of six chitosans and six chitosan oligomers in tofu immersion solutions to determine MICs. Five of six chitosans at a concentration of 0.1% exhibited strong inhibition and one exhibited weak inhibition, with an MIC of 400 µg/ml for all but the lowest molecular-weight chitosan.

9. Presence and behavior in infant formula

Dried cow (bovine) milk and milk products are potential sources of bacteria pathogenic to humans.

In one study, coliforms were detected in 3 of 124 samples of spray dried milk, 6 of 54 samples of roller dried milk and 13 of 38 samples of infant formula from 10 factories at populations of >1 CFU/g (Ghodesker et al., 1980). Populations higher than 90 CFU/g were found in 25, 10 and 5 samples, respectively, and >1 CFU/g was detected in 3, 6 and 13 samples, respectively. Multiple bacterial pathogens known to occasionally be found in powdered infant formulae include species of *Klebsiella*, *Citrobacter*, *Enterobacter*, *Yersinia*, *Staphylococcus* and *Streptococcus* (Anderson et al., 1984; Baldwin et al., 1984; Casewell et al., 1981; Fagerman, 1986; Gill and Gill, 1981; Muytjens et al., 1988; Schroeder et al., 1983; Simmons et al., 1989). The FDA has published bulletins (FDA, 1988, 2002b) highlighting the dangers of bacterial contamination of enteral formula products, most of which contain powdered milk as the major ingredient. In one study, 28% of 208 enteral formulae for nosocomial patients was reported to contain bacteria (Navajas et al., 1992).

Powdered infant formula has an a_w of ca. 0.2 and is formulated so as to mimic the nutritional profile of human milk rather than cow milk (Breeuwer et al., 2003). Nazarowec-White and Farber (1997a) noted ways that cow milk is modified so as to achieve this goal, e.g., reducing protein and mineral content, increasing the amount of whey protein, increasing the carbohydrate content, increasing the Ca/P ratio, modifying the fat and adding vitamins. Production of powdered infant formula is achieved by either “wet” or “dry” processing (Caric, 1993). The wet process involves combining all essential ingredients with liquid skimmed milk and fat components and heating the mixture at ca. 81 °C for 20 s. All components are then added to the mixture and heated to 107–110 °C for 60 s, followed by concentrating in a falling film evaporator. The mixture is finally heated at 80 °C prior to spray drying (Caric, 1993; Nazarowec-White and Farber, 1997a). In the dry process, pasteurized evaporated skim milk is dry blended with the balance of essential ingredients (essential fatty acids, vitamins, whey, stabilizers and emulsifiers), pasteurized for 60 s at 110 °C and spray dried. It has been noted that problems associated with this method include a higher probability of post-processing contamination, ingredient mixing difficulties and ingredient separation, including lactose segregation (Iversen and Forsythe, 2003;

Lambert-Legace, 1982; Nazarowec-White and Farber, 1997a,c). It is often the case that dry and wet processing procedures are combined by adding the more soluble ingredients during the liquid phase and the less soluble ones into the spray-dried powder matrix (Caric, 1993; Nazarowec-White and Farber, 1997a,b). A definitive statement concerning which process is inherently more or less likely to result in contamination of products with *E. sakazakii* cannot be made. This is because in-factory contamination is most likely to occur at some point between the spray drying and packaging steps. Critical to preventing product contamination is how the enteric population in the drying and post-drying pre-packaging factory environments is controlled. Factories will differ in many ways (e.g., age, building materials, design and cleanability) that will influence the level of efficiency in controlling the microbial ecology therein. Hence, risk of formula contamination can be expected to be a function of the particular factory environment rather than solely manufacturing processes.

The 1980 Infant Formula Act, revised in 1986, regulates the production and distribution of infant formula in a unique fashion by setting minimum standards for 29 nutrients (Baker, 2002; FDA, 1985). The act mandates adherence to good manufacturing practices (GMPs) and clear labeling, although there is no requirement for sterility. Baker (2002) reported that the FDA limit for bacterial aerobic plate counts in infant formula powder is 4 log₁₀ CFU/g. Zink (2003) noted that additional restrictions imposed on powdered infant formula by the FDA are ≤3.05 MPN/g for coliforms and *S. aureus*, ≤100 CFU/g for *Bacillus cereus* and zero tolerance for both listeriae and salmonellae.

E. sakazakii has been isolated at varying frequencies from infant formulae examined in several studies and surveys (Block et al., 2002; Muytjens et al., 1988; Nazarowec-White and Farber, 1997b; Postupa and Aldová, 1984; Van Acker et al., 2001). One of the most notable surveys was conducted by Muytjens et al. (1988) in which the organism was detected in 14.9% of 141 samples of powdered infant formulae originating from 35 countries. Positive samples originate from 13 countries. *E. sakazakii* was the third most commonly isolated bacterium, just behind *E. agglomerans* and *E. cloacae*; however, none of the Enterobacteriaceae exceeded 1 CFU/g. Simmons et al.

(1989) reported that *E. sakazakii* could out-compete *E. cloacae*, the second most common *Enterobacter*, despite *E. sakazakii* being present in products at lower populations than other bacteria. Muytjens et al. (1988) suggested that contamination of powdered infant formulae with Enterobacteriaceae must occur post-processing and following the final thermal treatment.

In a survey in the former Czechoslovakia, four strains of *E. sakazakii* were recovered from powdered milk and two strains were recovered from powdered infant formula (Postupa and Aldová, 1984). Other reports have confirmed or implicated powdered infant formula as a source of bacteria responsible for meningitis and associated with neonatal necrotizing enterocolitis (Bar-Oz et al., 2001; Biering et al., 1989; Clark et al., 1990; Muytjens and Kollee, 1990; Noriega et al., 1990; Simmons et al., 1989; Van Acker et al., 2001). Some in the pediatric community, however, question the ability of *E. sakazakii* to induce necrotizing enterocolitis. The CDC (2002) reported an outbreak of neonatal *E. sakazakii* infections linked to infant formula. Studies have also confirmed the link between powdered infant formula and neonatal infection (Muytjens et al., 1983; Postupa and Aldová, 1984; Muytjens et al., 1988; Biering et al., 1989; Noriega et al., 1990). The CDC has reported a definitive link between the presence of *E. sakazakii* in powdered infant formula in an unopened can and an outbreak of *E. sakazakii* infection (CDC, 2002; Baker, 2002).

Nazarowec-White and Farber (1997c) examined powdered infant formula manufactured by five companies (48 cans/company) and isolated *E. sakazakii* at an average population of 0.36 CFU/100 g from eight cans, i.e., 3.3% of the cans analyzed. They also determined the minimum growth temperature of 10 food and clinical isolates by inoculating rehydrated infant formula with 3.0 log₁₀ CFU of *E. sakazakii*/ml. Minimum temperatures were between 5.5 and 8.0 °C (7 °C for the ATCC 29544 type strain). Iversen et al. (2004d) reported that six clinical and food strains grew between 6 and 45 °C, with an optimum of 37–43 °C, and Kandhai et al. (2004c) reported that the bacterium grew in reconstituted infant formula at 8–47 °C. Studies have indicated that up to 20% of household refrigerator temperatures are kept at ≥ 10 °C (Daniels, 1991; Harris, 1989; Rhodehamel, 1992;

Van Garde and Woodburn, 1987), thus providing temperatures at which *E. sakazakii* will grow. Nazarowec-White and Farber (1997c) reported the lag times and generation times for the 10 *E. sakazakii* strains in infant formula held at 4, 10 and 23 °C. No differences in behavior of these strains were noted among test formulae and growth did not occur at 4 °C. The lag times at 23 and 10 °C were 2–3 h and 19–47 h, respectively, and mean generation times were 0.67 and 4.64 h, respectively. *E. sakazakii* grew more rapidly than *Salmonella* or *E. coli*. Nazarowec-White and Farber (1997c) calculated that it would take at least 10 h for *E. sakazakii* initially at 1 CFU/ml to reach 7 log₁₀ CFU/ml in rehydrated infant formula stored at room temperature, and much less time if held at 35–37 °C. Iversen et al. (2004d) reported that 70 strains grew to ca. 10⁹ CFU/ml overnight in TSB at 37 °C and 44 °C but none grew within 24 h at 47 °C. The growth phase of *E. sakazakii* inoculated into powdered infant formula had no significant effect on lag time or growth rate in reconstituted formula (Kandhai et al., 2004c).

Lihono et al. (2004) examined the use of probiotic cultures to control the growth of *E. sakazakii* in rehydrated infant formula at 30 and 35 °C. *Enterococcus faecium* was more inhibitory than *Lactobacillus acidophilus* or *Pediococcus acidilacticii*, largely as a result of decreased pH caused by the production of acids.

The rate of inactivation of *E. sakazakii* in powdered infant formula held at room temperature has been studied (Edelson-Mammel and Buchanan, 2004b). The population decreased by approximately 2.5 logs (6.0 log CFU/ml to 3.5 log CFU/ml of rehydrated formula) during the initial 5 months. Over the course of the subsequent year, the population decreased an additional 0.5 log, indicating that *E. sakazakii* can survive for an extended period of time in powdered infant formula.

An outbreak of *E. sakazakii* infections in Tennessee in 2001 was the first reported in the United States that resulted in a voluntary recall of product by a manufacturer (Himelright et al., 2002; FDA, 2002e; Weir, 2002). An epidemiologic investigation of microbial cultures from water and hospital environments failed to isolate *E. sakazakii*, while PFGE patterns of isolates from opened and unopened cans of infant formula were identical to the neonatal patient isolates.

In a survey of 16 NICUs, 25% reported using powdered infant formula as the sole food source while 31% reported using powdered formula in addition to other formulae (Himelright et al., 2002). Guidelines for the preparation of powdered infant formula in NICUs are outlined. A voluntary recall of ca. 1.5 million cans of another brand of dehydrated infant formula containing *E. sakazakii* occurred in 2002 (FDA, 2002a).

10. Hazard analysis and risk management

It has been demonstrated that powdered infant formula is not commercially sterile and may harbor *E. sakazakii*. However, studies have not established the minimum number of cells needed to cause clinical symptoms, barring poor preparation, temperature abuse, refrigeration and hygienic practices that have been frequently implicated as contributing factors to infections (Block et al., 2002; Clark et al., 1990; Iversen and Forsythe, 2003; Smeets et al., 1998). In an attempt to lower the risk of infant formula causing neonatal *E. sakazakii* infections, interim guidelines were issued in the U.S. (Himelright et al., 2002), drawing from recommendations issued by the American Dietetic Association (1991) for proper procedures for preparing, feeding and storing powdered infant formula in health care facilities (Table 4). It should be noted that these guidelines are for health care facilities and do not apply to home settings (Baker, 2002). In a letter to health professionals, the FDA (2002c) delineated further recommendations that were later revised to eliminate the recommendation of rehydrating formula with boiling water. Justification for the revision to remove the boiling water step was based on problems associated with the practice, including a potential for loss of heat-sensitive nutrients, changes in physical characteristics of some formulae, the inability to ensure destruction of *E. sakazakii* and possible injury to formula preparation personnel and the infant as a result of scalding. The FDA (2002b) noted the possibility of *E. sakazakii* infections in hospitalized neonates, indicating that the most likely contributing factor is the use of milk-based powdered formula. It was stressed that the likelihood of contracting infections is greater in premature or other immunocompromised infants; however, the warning did not

Table 4

Guidelines for preparation and handling infant formula^a

- Formula products should be selected based on nutritional needs; alternatives to powdered forms should be chosen when possible.
- Trained personnel should prepare powdered formula using aseptic techniques in a designated preparation room.
- Manufacturer's instructions should be followed; product should be refrigerated if not fed immediately and discarded if not used within 24 h after preparation.
- The administration or "hang" time for continuous enteral feeding should not exceed 4 h.
- Written hospital guidelines should be available in the event of a product recall, including notification of health-care providers, a system for reporting, follow-up of specific formula products used, and retention of recall records.

^a Summarized from Himelright et al. (2002).

apply to liquid infant formula, which is sold as a commercially sterile product, or to healthy full-term infants at home. Guidelines for Preparation of Formula and Breastmilk in Health Care Facilities, published by the American Dietetic Association (1991), provides 132 guidelines in eight chapters focused on physical facilities, equipment, utensils and supplies, personnel, formula preparation and handling, expressed human milk, delivery and bedside management of infant feedings, microbiology and infection control and quality assurance.

The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) jointly convened a workshop on *E. sakazakii* in early 2004 (FAO/WHO, 2004) in response to a request for scientific advice from the Codex Committee on Food Hygiene to provide input for the revision of the Recommended International Code of Hygienic Practice for Goods and Infants and Children. An extensive list of recommendations to FAO, WHO, Codex, their member countries, and Non-Governmental Organizations (NGOs) was issued (FAO/WHO, 2004) (Table 5). The extent to which these recommendations have been accepted and implemented by the infant formula industry and in settings where infant formula is reconstituted and fed to infants is unknown.

It has been suggested that thermal treatment of rehydrated infant formula may be a practical way of minimizing neonatal risk to *E. sakazakii* infection (Edelson-Mammel and Buchanan, 2004c; Jaspas et al., 1990; Kindle et al., 1996; Muyltjens and Kollee, 1990; Nazarowec-White et al., 2003). Simmons et al.

Table 5

Joint FAO/WHO recommendations to the powdered infant formula industry and infant caregivers concerning processing, preparing and handling powdered and reconstituted products^a

- In situations where infants are not breast-fed, caregivers, particularly of infants at high risk, should be regularly alerted that powdered infant formula is not a sterile product and can be contaminated with pathogens that can cause serious illness and provided with information that can reduce the risk.
- In situations where infants are not breast-fed, caregivers of high-risk infants should be encouraged to use, whenever possible and feasible, commercially sterile liquid formula or formula which has undergone an effective point of use decontamination procedure (e.g., use of boiling water to reconstitute or by heating reconstituted formula).
- Guidelines should be developed for the preparation, use and handling of infant formula to minimize risk.
- The infant food industry should be encouraged to develop a greater range of commercially sterile alternative formula products for high-risk groups.
- The infant food industry should be encouraged to reduce the concentration of prevalence of *E. sakazakii* in both the manufacturing environment and powdered infant formula. To this end, the infant food industry should consider implementing an effective environmental monitoring program and the use of Enterobacteriaceae rather than coliform testing as an indicator of hygienic control in factory production lines.
- In revising its Code of Practice, Codex should better address the microbiological risks of powdered infants formula and, if deemed necessary, include the establishment of appropriate microbiological specifications for *E. sakazakii* in powdered infant formula.
- FAO/WHO should address the particular needs of some developing countries in establishing effective measures to minimize risk in situations where breast-milk substitutes may be used in exceptionally difficult circumstances, e.g., feeding infants of HIV-positive mothers or low-birth-weight infants.
- The use of internationally validated detected and molecular typing methods for *E. sakazakii* and other relevant microorganisms should be promoted.
- Investigation and reporting of sources and vehicles, including powdered infant formulae, of infection by *E. sakazakii* and other Enterobacteriaceae should be encouraged. This could include the establishment of a laboratory-based network.
- Research should be promoted to gain a better understanding of the ecology, taxonomy, virulence and other characteristics of *E. sakazakii* and on ways to reduce its levels in reconstituted powdered infant formula.

^a Summarized from FAO/WHO (2004).

(1989) recommended using refrigeration and limiting hang time to prevent or retard the growth of *E. sakazakii*. Jaspar et al. (1990) recommended powdered infant formula preparation interventions as a means of preventing infections caused by *E. sakazakii*. Suggestions included disinfecting blenders as well

as boiling spoons, bottles and nipples prior to formula preparation. They also recommended storing rehydrated formula at refrigeration temperatures as well as heating the formula in a microwave oven just prior to feeding. Others have echoed the last two suggestions and have cautioned that bottle warmers may pose a risk of prolonged exposure to temperatures at which *E. sakazakii* can rapidly grow (Muytjens and Kollee, 1990).

Iversen and Forsythe (2003) made recommendations focused on reducing the probability of neonatal and infant infections caused by infant formulae. These include controlling the initial populations of *E. sakazakii* in raw materials on receipt, reducing populations during heat treatment of raw milk and related ingredients, preventing an increase in population of *E. sakazakii* by avoiding post-processing contamination, applying microbiological criteria and providing appropriate information and preparation instructions, e.g., labeling and consumer education.

11. Research needs

Studies involving *E. sakazakii* have focused on methods to eliminate the coliform from powdered infant formula, thermal resistance, environmental reservoirs, pathogenicity, antibiotic resistance, exopolysaccharide production, development of rapid methods detection, enumeration and identification, subtyping and predictive modeling, but additional research in these and other areas is needed. The urgency for more information in some areas is greater than in others. One study using a suckling mouse model to determine virulence mechanisms and minimum infectious dose has suggested the possibility of enterotoxin production by *E. sakazakii* (Pagotto et al., 2003) but other virulence factors associated with the bacterium remain unknown. Potential correlations between pathogenicity and pigmentation, shape and texture of colonies DNase production and the use of other animal models and cell cultures as enterotoxin assay systems need to be investigated (Pagotto et al., 2003).

Lai (2001), recognizing the propensity of *E. sakazakii* to infect certain individuals or groups of individuals in particular ways, commented that tropism for

the central nervous system in neonates and infants remains a mystery. Pathogenicity studies are underway and virulence factors of the bacterium are slowly being characterized; however, the ability of *E. sakazakii* to affect some full-term healthy neonates but not others in the same setting is enigmatic. The 1986 Reykjavik, Iceland, outbreak involving one of two twin boys is a case in point (Biering et al., 1989). Research has not fully explored traditional or technologically advanced treatments for their efficacy in eliminating the pathogen from the powdered milk or powdered infant formula. Baker (2002) recommended researching irradiation of powdered infant formula as an approach to control *E. sakazakii*. It was also suggested that research into protecting neonates from pathogens by using pro- or pre-biotics should be conducted.

Studies to determine conditions that influence survival and growth or cause death of *E. sakazakii* in dry and reconstituted infant formulae are needed, given the likelihood that post-process contamination is the principle route of contamination. Other areas in need of research attention include studies of conditions affecting biofilm formation by *E. sakazakii* in processing plants and hospital settings (e.g., in tubes used for enteral feeding), competitive exclusion to control or prevent growth, efficacy of sanitizers, methods to recover and resuscitate injured cells and evaluation of practices associated with preparing and feeding infant formulae in hospitals and in the home. Surveys of neonatal wards, NICUs and food processing environments for the presence of *E. sakazakii* and an evaluation of hygienic practices in hospitals and the home that may contribute to neonatal infections would also provide information of value when developing intervention strategies to eliminate *E. sakazakii* infections.

Iversen and Forsythe (2003) recommended further work to define the role of capsule production as it relates to desiccation resistance and thermal destruction, as well as characterization of virulence factors. Further investigation should also be done in the areas of phage typing, serotyping, virulence factors, tolerance to desiccation, heat and pH, lag times across a range of temperatures and in an array of food matrices, biofilm formation and the use of bacteriocins, organic acids, disinfectants and other chemicals to control the growth of the *E. sakazakii*.

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