

DETECTION OF *ENTEROBACTER SAKAZAKII* IN SOUTH AFRICAN FOOD PRODUCTS

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that it has not previously, in its entirety or in part, been submitted at any university for a degree.

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ABSTRACT

It is estimated by the World Health Organisation (WHO) that thousands of millions of cases of foodborne diseases occur world-wide every year. *Enterobacter sakazakii* is a member of the family *Enterobacteriaceae* and has been identified as an occasional contaminant of powdered infant formula milk (IFM). *Enterobacter sakazakii* is an opportunistic emerging pathogen and has the ability to cause a severe form of neonatal meningitis. This organism was referred to as “yellow pigmented *Enterobacter cloacae*” until 1980 after which it was renamed as *E. sakazakii*.

The current method for the detection of *E. sakazakii* is very time consuming and includes pre-enrichment, enrichment in *Enterobacteriaceae* enrichment broth, subsequent plating on violet red bile glucose agar and subculturing on tryptone soy agar. In this study a polymerase chain reaction (PCR) method was developed for the identification of the presence of *E. sakazakii* in infant food products. A part of the 16S ribosomal RNA (rRNA) gene from *E. sakazakii* was amplified using the primer pair Esak2 and Esak3.

An internal amplification control (IAC) was constructed as part of the PCR detection method. The 850 base pair (bp) *E. sakazakii* PCR product was digested with *AluI* and the two fragments containing the primer binding sites were ligated, resulting in a 240 bp IAC. During this study a positive band for both the target DNA (850 bp) and the IAC (240 bp) was simultaneously observed when the IAC was added to the PCR mixture at a concentration of 0.72 pg.ml⁻¹.

Four of 22 South African food products tested positive for the presence of *E. sakazakii*, using both the PCR and recommended culturing methods. The PCR method was used successfully for the detection of *E. sakazakii* within three days and thus provides a possible alternative and improvement on the recommended current culturing methods. Other microorganisms present in the products tested included *Escherichia coli*, *Klebsiella pneumoniae*, *Raoultella terrigena* (“*Klebsiella terrigena*”) and *Chryseomonas luteola*.

Since *E. sakazakii* is usually present in low numbers in food products, it is possible that these few cells are unevenly distributed in the products, making it

important to take multiple samples when evaluating IFM and thereby ensuring that even low numbers of this pathogen are detected.

UITTREKSEL

Die Wêreld Gesondheidsorganisasie (WGO) beraam dat daar jaarliks duisende miljoene gevalle van voedselverwante siektes voorkom. *Enterobacter sakazakii* is 'n lid van die *Enterobacteriaceae* familie en word geïdentifiseer as 'n toevalligheidskontaminant van baba formule melk. *Enterobacter sakazakii* is 'n opportunistiese patoog en het die vermoë om 'n erge graad van neonatale meningitis te veroorsaak. *Enterobacter sakazakii* het tot 1980 bekend gestaan as die "geel gepigmenteerde *Enterobacter cloacae*", waarna dit hernoem is na *E. sakazakii*.

Huidige metodes vir die deteksie van *E. sakazakii* is baie tydrowend en sluit 'n voor verrykings stap asook verryking in *Enterobacteriaceae* verrykingsmedium in, waarna dit uitgeplaat word op *violet red bile glucose agar* (VRBGA) en verder uitgestreep word op *tryptone soy agar* (TSA). Tydens hierdie studie is 'n polimerase kettingreaksie (PKR) metode ontwikkel vir die identifikasie van *E. sakazakii* in baba voedselprodukte en in die besonder baba formule melk. 'n Gedeelte van die 16S ribosomale RNA (rRNA) geen van *E. sakazakii* is geamplifiseer deur die peilers Esak2 en Esak3 te gebruik.

'n Interne amplifikasie kontrole (IAK) is vervaardig as deel van die PKR deteksie metode. Die 850 basis paar (bp) *E. sakazakii* PKR produk is met die beperkingsensiem *AluI* verteer en die twee fragmente wat die peiler bindings punte bevat is geligeer waarna 'n IAK van 240 bp verkry is. Tydens hierdie studie is 'n positiewe band vir beide die teiken DNA (850 bp) en die IAK gelyktydig verkry deur die IAK teen 'n konsentrasie van 0.72 pg.ml^{-1} by die PKR reaksiemengsel te voeg.

Vier van die 22 Suid-Afrikaanse produkte het positief getoets vir die teenwoordigheid van *E. sakazakii*, deur beide die PKR asook kultuur tegnieke. Deur gebruik te maak van die PKR metode is *E. sakazakii* binne drie dae opgespoor en verskaf dus 'n moontlike alternatief en verbetering op die huidige aanbevole metode. Ander organismes wat teenwoordig was in die getoetste produkte sluit in *Escherichia coli*, *Klebsiella pneumoniae*, *Raoultella terrigena* ("*Klebsiella terrigena*") en *Chryseomonas luteola*.

Aangesien *E. sakazakii* gewoonlik in lae getalle voorkom in voedselprodukte, is dit moontlik dat daar 'n oneweredige verspreiding van hierdie enkele selle in die produkte is. Dis is dus noodsaaklik om veelvuldige monsters te neem wanneer formule melk getoets word om te verseker dat selfs lae getalle van die patogeen opgespoor word.

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Nothing is a waste of time if you use the experience wisely.

Auguste Rodin

Dedicated to my parents

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Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

CHAPTER 1

INTRODUCTION

Microbial foodborne illness has become an important and growing public health concern (WHO, 2004). Most countries with systems for reporting cases of foodborne illnesses have documented significant increases over the past few years in the incidence of foodborne diseases, including the presence of pathogens such as *Salmonella*, *Campylobacter jejuni*, enterohaemorrhagic *Escherichia coli* and parasites such as cryptosporidium, cryptospora and trematodes. The emergence of new pathogens, and pathogens not previously associated with food, is also becoming a major health concern.

One such an opportunistic emerging pathogen, *Enterobacter sakazakii*, is a motile, non-sporeforming, Gram-negative rod belonging to the family *Enterobacteriaceae*. The organism was until 1980 referred to as yellow pigmented *Enterobacter cloacae*, where after it was renamed *E. sakazakii* (Farmer *et al.*, 1980). Although researchers have failed to find an environmental source for this organism, dried infant formula milk (IFM) has been implicated as one of the modes of transmission in both outbreaks and sporadic cases of *E. sakazakii* meningitis (Muytjens *et al.*, 1983, 1988; Postupa & Aldova, 1984; Biering *et al.*, 1989; Noriega *et al.*, 1990). This organism can be the cause of rare, but life-threatening forms of neonatal meningitis, bacteremia, necrotizing enterocolitis (NEC) and necrotizing meningoencephalitis after ingestion (Muytjens & Kolleé, 1990).

Present microbial culturing methods for the detection of *E. sakazakii* involve a series of steps, including pre-enrichment, enrichment, plating unto violet red bile glucose agar (VRBGA) and subculturing unto tryptone soy agar (TSA) (Nazarowec-White & Farber, 1997; Anon., 2002). Since this process can take up to seven days, several researchers (Iversen *et al.*, 2004; Kandhai *et al.*, 2004; Leuschner *et al.*, 2004; Oh & Kang, 2004) have recently developed new media for the detection of this microorganism. These include a chromogenic medium using the indolyl substrate 5-bromo-4-chloro-3-indolyl- α -D-glucopyranoside (Iversen *et al.*, 2004), as well as

media based on two characteristic features of *E. sakazakii*, namely the production of yellow colonies when grown on TSA and its constitutive α -glucosidase, which is detected in a 4 h colorimetric assay (Kandhai *et al.*, 2004). Furthermore, a differential selective medium based on the presence of the enzyme α -glucosidase that metabolize the substrate 4-methyl-umbelliferyl- α -D-glucoside (α -MUG) to produce fluorescent colonies was developed by Leuschner *et al.* (2004). Similarly, Oh & Kang (2004) also used α -MUG as a selective marker to develop a differential medium for *E. sakazakii*. However, no standardised/validated or official method exists for the direct isolation of *E. sakazakii* from foods (Nazarowec-White *et al.*, 2003).

Since *E. sakazakii* is becoming an important foodborne pathogen test methods for its detection must result in prompt and accurate results. Polymerase chain reaction (PCR) analyses, as part of microbial diagnostics has been established in research laboratories (Malorny *et al.*, 2003) as a valuable alternative to traditional detection methods and is accurate and highly specific. Since current test methods for the detection of *E. sakazakii* are not selective enough for detecting only *E. sakazakii* and are also time consuming. PCR analysis could be used as a rapid and accurate detection method for determining the presence of *E. sakazakii* in IFM. The aim of this study was to detect *E. sakazakii* by a PCR method that including an internal amplification control and to compare this method with traditional culturing techniques, for the reliable and accurate detection of *E. sakazakii* from South African food products, specifically powdered IFM.

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CHAPTER 2

LITERATURE REVIEW

A. BACKGROUND

The World Health Organisation (WHO) estimates that world-wide thousands of millions of cases of foodborne diseases occur every year (Mogensen & Holm, 2003). One third of the population in developed countries is affected by foodborne illness each year. Food pathogens may be of bacterial, fungal or amoebal character and may be the cause of diseases either by direct infection or by producing toxins in the food. Most foods are perishable and liable to sustain pathogenic growth, with contamination occurring at all the stages of production and processing to consumption. A large number of preventive measures are necessary to reduce this contamination in developed, as well as in developing countries.

Ensuring the highest level of product quality and safety is the primary concern of infant formula milk (IFM) manufacturers (Anon., 2004a). Infants who are not breastfed require a suitable breast milk substitute and no other breast milk substitute is as safe as commercial IFM when produced according to international standards. However, breastfeeding is most beneficial to infants (Anon., 2004b) and it is recommended by the WHO that infants be exclusively breastfed for the first six months of their lives, continued with appropriate complementary feeding until the age of two years. Evidence shows that infants who are partially or not breastfed are at a significantly increased risk of morbidity and mortality due to diarrhoeal diseases. Despite the fact that there are mothers who cannot breastfeed due to physiological reasons, concerns about HIV-infected women breastfeeding infants are increasing (Anon., 2004b).

The microbiological standards of food have increased due to recent developments in food technology and the increased focus on food safety (Mogensen & Holm, 2003). However, a strict microbiological standard does not always ensure low risk foods. In actual fact eventual contaminants of food pathogens will face no

microbial competition and often new challenges arise such as contamination with new and highly virulent pathogens.

Enterobacter sakazakii is an occasional contaminant of powdered IFM (Iversen & Forsythe, 2003). *Enterobacter sakazakii* is an opportunistic emerging pathogen (Anon., 2004b) and has the ability to cause a severe form of neonatal meningitis. Little is known about its ecology, taxonomy, virulence and other characteristics. Even low levels of contamination of *E. sakazakii* in powdered IFM is considered to be a risk given the potential for multiplication during the preparation and holding time prior to the consumption of reconstituted formula. The presence of *E. sakazakii* in IFM, and its potential effect on infants could well be a significant public health problem in most countries world-wide.

B. ENTEROBACTER SPECIES

In recent years several members of the genus *Enterobacter* have been recognised as important pathogens (Sanders & Sanders, 1997) and cause nosocomial infections (infections that are acquired while a patient is in a hospital) (Gallagher, 1990). The genus *Enterobacter* is comprised of fourteen species (Manual of Clinical Microbiology, 2003), namely: *E. aerogenes*, *E. amnigenus* (biogroup 1 and 2), *E. asburiae*, *E. cancerogenus* (*E. tylosae*), *E. cloacae*, *E. cowanii* (*P. agglomerans*/Japanese NIH group 42), *E. dissolvens* (*Erwinia*), *E. gergoviae*, *E. hormaechei*, *E. intermedium*, *E. kobei*, *E. nimipressuralis* (*Erwinia*), *E. pyrinus* (*Erwinia*) and *E. sakazakii*. Established *Enterobacter* species, in particular *E. cloacae* and to a lesser degree *E. aerogenes*, have been frequently isolated from clinical specimens (Farmer & Kelly, 1992) and are the most common nosocomial pathogens responsible for a variety of infections (Sinave, 2003). These infections include bacteremia, lower respiratory tract infections, skin and soft tissue infections, urinary tract infections, intra-abdominal infections, septic arthritis, osteomyelitis and ophthalmic infections. *Enterobacter agglomerans* is also occasionally isolated from clinical specimens. *Enterobacter gergoviae* and *E. tylosae* are rarely isolated from the environment and their differentiation can be difficult (Farmer & Kelly, 1992).

Enterobacter species are in general responsible for 50% of nosocomial infections, mostly in immunocompromised patients (Leclerc *et al.*, 2001). Most of the *Enterobacter* species are innately resistant to antimicrobial agents that have been in use for a long time (Sanders & Sanders, 1997) and have the ability to rapidly develop resistance to recently developed agents.

Enterobacter species are opportunistic pathogens (Sanders & Sanders, 1997) and rarely cause disease in an otherwise healthy individual (Sinave, 2003). More epidemiological information is, however, required to allow reliable assessment of their potential as agents of foodborne illness (Gallagher, 1990) as little is known about the specific factors impacting their pathogenicity and virulence (Nazarowec–White *et al.*, 2003; Sanders & Sanders, 1997). Species of the genus *Enterobacter*, similar to other members of the family *Enterobacteriaceae*, produce an endotoxin that is known to play a major role in the pathophysiology of sepsis and other complications (Sinave, 2003).

Enterobacter sakazakii is a less common *Enterobacter* species, but a malicious cause of neonatal sepsis, and meningitis. Table 1 shows cases of *E. sakazakii* infections amongst neonates and infants including the age, number of deaths, symptoms and source of contamination. *Enterobacter* infections are observed most frequently in neonates, elderly individuals (Sinave, 2003) and HIV–positive individuals.

Enterobacter sakazakii

Enterobacter sakazakii is a motile peritrichous, non–sporeforming, Gram–negative rod and a facultative anaerobe belonging to the *Enterobacteriaceae* family (Nazarowec–White & Farber, 1997a). The organism was referred to as “yellow pigmented *Enterobacter cloacae*” until 1980 after which it was renamed as *E. sakazakii* (Farmer *et al.*, 1980). *Enterobacter sakazakii* was separated from *E. cloacae*, based on differences in DNA–DNA hybridization, biochemical reactions,

Table 1. Cases of *E. sakazakii* infection in neonates and infants (adapted from Iversen & Forsythe, 2003).

Year of outbreak	No. of neonates and infants	Age	Number of deaths	Symptoms	Source	References
1958	2	5 and 10 d	2	Meningitis	Unknown	Urmenyi & White–Franklin (1961)
1958	1	4 d	Unknown	Meningitis	Unknown	Jöker <i>et al.</i> (1965)
1958	1	7 d	0	Bacteremia	Unknown	Monroe & Tift (1979)
1958	1	5 weeks	Unknown	Meningitis and sepsis	Unknown	Adamson & Rogers (1981)
1958	1	5 weeks	0	Meningitis	Unknown	Kleiman <i>et al.</i> (1981)
1977–1981	8	Unknown	6	Meningitis	IFM	Muytjens <i>et al.</i> (1983)
1977–1981	NS	NS	NS	NS	NS	Postupa & Aldová (1984)
1984	11	varied from 2 d to 2 months	5	Colonisation (Respiratory distress syndrome/sepsis/anoxia/meningitis/ congenital defects)	Unknown	Arseni <i>et al.</i> (1987)
1984	1	21 d	0	Meningitis	Unknown	Naqvi <i>et al.</i> (1985)
1984	2	8 d and 4 weeks	0	Meningitis	Unknown	Willis & Robinson (1988)
1986–1987	3	5 d	1	Meningitis, septicemia	IFM	Biering <i>et al.</i> (1989); Clark <i>et al.</i> (1990)
1986–1987	4	NS	NS	Wound exudates, appendicitis, conjunctivitis	NS	Reina <i>et al.</i> (1989)
1981–1988	2	NS	2	Meningitis	Unknown	Lecour <i>et al.</i> , (1989)

Table 1. Continued.

Year of outbreak	No. of neonates and infants	Age	Number of deaths	Symptoms	Source	References
1988	4	28–34½ weeks	0	Sepsis/bloody diarrhoea	IFM, blender	Simmons <i>et al.</i> (1989); Clark <i>et al.</i> (1990)
1988	1	6 months	0	Bacteremia	IFM, blender	Noriega <i>et al.</i> (1990)
1988	1	2 d	0	Meningitis	NS	Gallagher & Ball (1991)
5 year period	NS	NS	NS	Necrotising enterocolitis	Unknown	Chan <i>et al.</i> (1994)
5 year period	1	NS	NS	Meningitis	Unknown	Reis <i>et al.</i> (1994)
5 year period	1	20 months	0	Wound infection	Unknown	Tekkok <i>et al.</i> (1996)
5 year period	1	6 d	0	Meningitis	NS	Burdette & Santos (2000)
1997	1	7 d	0	Meningitis	Unknown	Weekly Report (1997)
1998	12	varied from 4 d to 2 months	0	Enterocolitis	IFM	Van Acker <i>et al.</i> (2001)
1999–2000	NS	NS	NS	NS	IFM, blender	Block <i>et al.</i> (2002)
1999–2000	2	3 and 4 d	0	Bacteremia, meningitis	IFM & Blender	Bar–Oz <i>et al.</i> (2001)
1999–2000	1	3 years	0	Bacteremia	NS	Lai (2001)
2001	11	11 d	1	Meningitis, enterocolitis	IFM	Himelright <i>et al.</i> (2002)

NS: not specified in paper

IFM: infant formula milk

- : not stated

antibiotic susceptibility and the production of yellow pigmented colonies (Farmer *et al.*, 1980). DNA–DNA hybridization studies showed no clear generic assignment for *E. sakazakii* as it was 53–54% related to *Enterobacter* and *Citrobacter* species. When comparing the type strains of these two genera, it was found that *E. sakazakii* was 41% related to *C. freundii* and 51% related to *E. cloacae*. Since it was phenotypically closer to *E. cloacae*, it was assigned to the genus *Enterobacter* (Farmer *et al.*, 1980).

Enterobacter sakazakii is an occasional contaminant of powdered IFM and may cause a rare, but life–threatening form of neonatal meningitis, bacteremia, necrotising enterocolitis (NEC) and necrotising meningoencephalitis after ingestion (Iversen & Forsythe, 2003). This results in some form of developmental delay, neurologic sequela and hydrocephalus (Lai, 2001). Although little is known about the specific virulence mechanisms of the organism, it appears to infect the central nervous system.

Only a few developed countries have reported cases of *E. sakazakii* infections in contaminated powdered IFM (WHO, 2004). This absence of world–wide reports is probably due to a lack of awareness of the problem, rather than an absence of the illness itself. Limitations of current surveillance systems in most countries could also explain the lack of reported cases. The first reported case for *E. sakazakii* was by Urmenyi and White–Franklin (1961) implicating two infants (Table 1) who both died of generalised infection, including meningitis. The infection was considered to be caused by an unusual pigmented strain of the cloaca group.

A review of cases in infants reported in literature from 1961 to 2003 reveal only 48 cases of *E. sakazakii* induced illness among infants (Table 1). The US FoodNet 2002 survey found that the rate of invasive *E. sakazakii* infection among infants under one year old was 1 per 100 000 (based on the isolation of the organism from sterile sites only), whereas the rate among low–birth weight neonates was 8.7 per 100 000. In developing countries, there is a complete lack of information on the contamination of commercially available powdered IFM (Anon., 2004b). There has also been no surveillance of the disease burden resulting from the consumption of contaminated powdered IFM. In developing countries the proportion of particular subpopulations

consisting of low birth weight infants and infants with HIV infected mothers are higher than in developed countries. The use of IFM in these countries may, therefore, be increasing. This may result in more cases of infection when contaminated IFM is used.

C. ENVIRONMENTAL AND FOOD INCIDENCE

Enterobacter sakazakii is associated with powdered IFM and milk powder (Iversen & Forsythe, 2003), but has also been isolated from a variety of environments and food products including cheese, fermented bread, tofu, sour tea, cured meats, minced beef and sausage meat. The organism is one of the sorghum seed surface microorganisms and *E. sakazakii* has also been isolated from Khamir bread (Gassem, 1999). It is probable that soil, water and vegetables are the principle sources of contaminated food, since it is not part of the normal animal and human gut microorganisms. Rats and flies may be additional sources of contamination. However, Muytjens & Kollee (1990) could not isolate the organism from surface water, soil, mud, rotting wood, grains, bird dung, rodents, domestic animals, cattle and raw cow's milk.

Enterobacter sakazakii is an environmental organism, and is likely to be present in both manufacturing facilities and at home (CAC, 2004). Iversen *et al.* (2004a) found that the organism was able to adhere to and grow on latex, polycarbonate, silicon and to a lesser extent stainless steel. *Enterobacter sakazakii* can consequently attach to infant-feeding processing and preparation equipment.

Molecular epidemiology has clearly demonstrated that *E. sakazakii* present in powdered formula has caused serious human illness. During a study done by Muytjens *et al.* (1988) it was found that 52.2% of 141 (products from 28 of the 35 countries) powdered IFM tested contained *Enterobacteriaceae*. Of these, 25% contained *E. agglomerans* (35 isolates), 21% contained *E. cloacae* (30 isolates) and 14% contained *E. sakazakii* (20 isolates). *Klebsiella pneumonia* (13 isolates) was also one of the most frequently isolated organisms. *Enterobacter sakazakii* was cultured from unused IFM products obtained from 13 countries, although the formulas

met the requirements of less than 3 cfu.g⁻¹ as recommended by the Food and Agricultural Organization of the United Nations. It is not known whether the presence of members of the *Enterobacteriaceae* in prepared formula at the low concentrations determined during the specific study is associated with occasional cases of neonatal meningitis.

The presence of *Enterobacter sakazakii* and other *Enterobacteriaceae* in a variety of food products was investigated during a study done by Iversen & Forsythe (2004). A total of 82 powdered IFM and 404 other food products, including dried infant foods, milk powders, lactose powders, cheese products, fresh foods, herbs and spices and other dried foods were tested by using both the current Food and Drug Administration (FDA) detection method as well as using the newly developed chromogenic Druggan–Forsythe–Iversen (DFI) medium. By using this new DFI method *E. sakazakii* was isolated from 67 samples while only 19 samples tested positive using the conventional method. It is, therefore, clear that *E. sakazakii* is present in a wide variety of products, including high risk products such as powdered IFM, dried infant food and milk powder.

The incidence of *E. sakazakii* in dried IFM, the temperature range for growth, as well as the growth characteristics of *E. sakazakii* in reconstituted dried IFM was determined by Nazarowec–White & Farber (1997c). Strains of *E. sakazakii* were isolated from products available on the Canadian retail market and a total of 120 IFM samples from five different companies were evaluated for the presence of *E. sakazakii*. Positive samples (eight product samples) had *E. sakazakii* at levels of less than 1 cfu.100 g⁻¹ which is similar to the results reported by Muytjens *et al.* (1988). The minimum growth temperatures for both clinical and food isolates ranged from 5.5° – 8.0°C in brain heart infusion (BHI) broth. Results similar to those reported by Farmer *et al.* (1980) further showed that *E. sakazakii* could not grow at refrigeration temperatures of 4°C. The lowest reported temperatures that allowed growth of *E. sakazakii* were 5.7°C (Iversen *et al.*, 2004a), 5.5°C (Nazarowec–White & Farber, 1997c) and 3.4°C (Breeuwer *et al.*, 2003) suggesting that the organism is able to grow at refrigeration temperatures (Lehner & Stephan, 2004). Iversen *et al.* (2004a) found that the organism grew as low as 6°C in powdered IFM and optimally

at 37° – 43°C. It should, however, be noted that many home refrigerators have a temperature range of 7° – 10°C (Rhodehamel, 1992).

D. CHARACTERISTICS

Enterobacter sakazakii grows on bacteriological media used to isolate enteric bacteria, such as MacConkey, eosin methylene blue and deoxycholate agar (Iversen & Forsythe, 2003). It may form two types of colonies depending upon the media used and the specific strain studied. The one type of colony is usually dry or mucoid, with scallop edges and rubbery when touched with a loop (Farmer *et al.*, 1980). The second colony type is typically smooth and easily removed when touched with a wire loop. All the strains of *E. sakazakii* produce a large amount of cell mass after 24 h incubation in trypticase soy broth (Farmer *et al.*, 1980). This sediment appears to have a large amount of clumped cells and amorphous masses. With respect to water activity and pH, growth limits are unknown. A yellow, non-diffusible pigment is produced when grown on tryptone soy agar (TSA). The yellow pigment is more pronounced when incubated at 25°C than at 36°C. The colonies are usually 1 – 1.5 mm in diameter after 24 h and 2 – 3 mm after 48 h. At 36°C incubation colonies of 2 – 3 mm are formed after 24 h. Yellow colonies are also produced on nutrient agar (NA) (Muytjens *et al.*, 1988), which is not a unique trait since it is commonly found in the closely related genus *Pantoea* (formerly known as *Enterobacter agglomerans*), also associated with powdered IFM (Muytjens *et al.*, 1988; Iversen & Forsythe, 2004). It was found by Block *et al.* (2002) that one of the *E. sakazakii* isolates used during their study did not produce pigment.

Enterobacter sakazakii strains can grow over a wide temperature range of 6° – 47°C and have a doubling time of about 75 min at room temperature (21°C) in reconstituted IFM (Iversen & Forsythe, 2003). A study by Iversen *et al.* (2004a) showed that *E. sakazakii* grew in IFM at refrigeration temperatures, with a doubling time of *ca* 13 h. It is thus unlikely that sufficient multiplication will occur under refrigeration conditions to cause an infection.

Enterobacter sakazakii has a biochemical profile similar to that of *E. cloacae* (Table 2). Unlike *E. cloacae*, it is D-sorbitol negative and positive for extracellular deoxyribonuclease. On toluidine blue agar (36°C for 7 d), a delayed extracellular DNase reaction is produced. The organism is α -glucosidase positive (Muytjens *et al.*, 1984) which can be detected using 4-nitrophenyl- α -D-glucopyranoside after 4 h at 36°C. It also produces D-lactic acid, is mucate negative and the enzyme phosphoamidase is absent in *E. sakazakii* isolates. It was found by Postupa & Aldova (1984) that all of the *Enterobacter* species isolated from powdered milk and dried IFM produced Tween 80 esterase after 7 days incubation at 25°C and 37°C.

Nazarowec-White & Farber (1997b; 1999) determined decimal reduction times (D-value) and z-values for this organism in IFM and found that the D_{52} value was 54.8 min, while the D_{60} value was 2.5 min. When extrapolating the data to 72°C, it was found that the organism is very thermotolerant (z value 5.82°C). Nazarowec-White & Farber (1997b) also showed that *E. sakazakii* was one of the most thermotolerant amongst the *Enterobacteriaceae* in IFM. This thermal resistance was, however, not enough to withstand a standard pasteurisation process and suggests that the contamination of products occur during drying or filling. In contrast to this, Breeuwer *et al.* (2003) showed the *E. sakazakii* is not particularly thermotolerant, but that it can adapt to osmotic and dry stress. At 58°C the D-value for *E. sakazakii* ranged from 0.39 to 0.60 min (23.4 to 36 s), which is comparable to that of other *Enterobacteriaceae*. Edelson-Mammel & Buchanan (2004) observed D_{58} -values for *E. sakazakii* that ranged from 30.5 to 591.9 s, representing an almost 20-fold difference in the most and least heat resistant strains. The strains also appeared to fall in two distinct heat resistant phenotypes. It was also found that the most heat-resistant strain during their study had a z-value of 5.6°C, corresponding with the values reported by Nazarowec-White & Farber (1997b).

Dried and stationary phase *E. sakazakii* cells can survive at elevated temperatures (45°C) and the capacity to grow at temperatures as high as 47°C suggests that in warm and dry environments such as in the vicinity of drying equipment in factories, the organism has a competitive advantage over other members of the *Enterobacteriaceae* (Breeuwer *et al.*, 2003). Therefore, there is a

Table 2. Biochemical characteristics of opportunistic *Enterobacter* species (Iversen & Forsythe, 2003).

TEST	Results of reaction ^a				
	<i>E. sakazakii</i>	<i>E. cloacae</i>	<i>E. aerogenes</i>	<i>E. agglomerans</i>	<i>E. gergoviae</i>
Lysine decarboxylase	-	-	+	-	+
Arginine dihydrolase	+	+	-	-	-
Ornithine decarboxylase	+	+	+	-	+
KCN, growth in	+	+	+	V	-
Fermentation of:					
Sucrose	+	+	+	(+)	+
Dulcitol	-	(-)	-	(-)	-
Adonitol	-	(-)	+	-	-
D-sorbitol	-	+	+	V	-
Raffinose	+	+	+	V	+
α -methyl-D-glucoside	+	(+)	+	-	-
D-arabitol	-	(-)	+	-	+
Yellow pigment	+	-	-	(+)	-
DNase (7 d)	+	-	-	-	-

^a + represents 90 – 100% positive; (+) represents 75 – 89% positive; V represents 25 – 74% positive; (-) represents 10 – 24% positive; and – represents 0 – 9% positive.

risk for post–pasteurisation contamination of powdered products during processing or packaging with *E. sakazakii*. An important step to eliminate this bacterium from critical food production environments would be an understanding of its physiology and survival strategies.

It was found that the generation times for *E. sakazakii* at 10°C varied from 4.18 to 5.52 h, while it was only 40 min at 23°C (Nazarowec–White & Farber, 1997c), which differs from the doubling time (75 min at 21°C) found by Iversen & Forsythe (2003). Although the results in this study showed only low numbers and low incidence of *E. sakazakii* in IFM, the short lag and generation time may be a cause of concern. Improper storage of reconstituted dried IFM at ambient temperatures, for example on a bedside table for night feedings or during shopping, may permit growth of *E. sakazakii*.

According to Nair *et al.* (2004), the incorporation of an effective antimicrobial barrier could reduce the likelihood of outbreaks of *E. sakazakii* infection in infants after consumption of contaminated IFM. They studied the efficacy of monocaprylin, the monoglyceride of caprylic acid, which is naturally present in human breast milk, in inactivating *E. sakazakii* in reconstituted IFM. It was found that the presence of monocaprylin significantly reduced the population of *E. sakazakii*, especially at higher temperatures. It was concluded that monocaprylin could be used as an antimicrobial ingredient in IFM, but its effect on the sensory qualities of the product should be evaluated.

E. DETECTION METHODS USING CULTURE TECHNIQUES

Microbial detection methods for *E. sakazakii* have primarily involved pre–enrichment in buffered peptone water (BPW), enrichment in *Enterobacteriaceae* enrichment (EE) broth that inhibits the growth of non–*Enterobacteriaceae* such as lactic acid bacteria and subsequent plating on violet red bile glucose agar (VRBGA) (Nazarowec–White & Farber; 1997a; Anon., 2002). After this step, five *Enterobacteriaceae* colonies are picked and plated on TSA. These plates are then incubated at 25°C for 48 – 72 h to observe the typical yellow pigment produced by *E. sakazakii* (Nazarowec–White &

Farber, 1997a; Anon., 2002). Other *Enterobacteriaceae* species can outgrow *E. sakazakii* during the pre-enrichment and enrichment stages, leading to relatively few *E. sakazakii* colonies on VRBGA and subsequently a reduced chance of selecting and growing the organism on TSA. Consequently false negative results would be obtained and *E. sakazakii* contaminated IFM may be distributed.

Recently Leuschner *et al.* (2004) developed a differential selective medium for the isolation of *E. sakazakii*. This medium is based on the presence of an enzyme, α -glucosidase that can be used to differentiate between members of the family *Enterobacteriaceae*. The medium used consists of NA that was supplemented with 4-methyl-umbelliferyl- α -D-glucoside (α -MUG). When *E. sakazakii* is grown on this medium, the substrate is metabolised by the enzyme to yield yellow pigmented colonies that are UV fluorescent. *Enterobacter sakazakii* is not the only yellow pigmented representative of the *Enterobacteriaceae* or non-*Enterobacteriaceae* present in IFM. Isolates of *Acinetobacter* spp., *Escherichia hermanii*, *Cedaceae lepagii*, *Leclercia adecarboxylata* and *E. agglomerans* also produce a yellow pigment on NA. However, these organisms are not fluorescent under UV-light. Some strains of *Escherichia asburiae* and *E. intermedium* grown on NA + α -MUG were fluorescent under UV-light, but do not produce yellow colonies. From the 58 products tested with this method, eight yielded positive results for *E. sakazakii*.

Similar to Leuschner *et al.* (2004), the fluorogenic substrate of α -glucosidase, 4-methyl-umbelliferyl- α -D-glucoside was used as a selective marker to develop a differential medium for *E. sakazakii* (Oh & Kang, 2004). A basal medium was used and different nitrogen sources were tested to reduce the ratio of fluorescent colonies versus total colonies and tryptone yielded the lowest. The optimal growth conditions for differentiation were also determined and 24 h incubation at 37°C was found to be optimum since more fluorescent colonies were observed after incubation at 37°C than 30°C. When tested with a culture mixed cocktail (including *E. sakazakii*), distinct fluorescent colonies appeared when exposed to long wavelength UV-light. A total of 48 fluorescent colonies were examined and all colonies were verified as *E. sakazakii*, while none of the 44 non-fluorescent colonies were identified as *E. sakazakii*.

Guillaume–Gentil *et al.* (2005) developed a method to detect and identify *E. sakazakii* in environmental samples. The method is based on selective enrichment at $45^{\circ} \pm 0.5^{\circ}\text{C}$ in lauryl sulfate tryptose broth supplemented with 0.5 M NaCl and 10 mg.l^{-1} vancomycin (mLST) for 22 to 24 h followed by streaking on TSA with bile salts. When exposed to light during incubation at 37°C , *E. sakazakii* produces yellow colonies within 24 h. All of the *E. sakazakii* strains tested ($n = 99$) were able to grow in mLST at $45^{\circ} \pm 0.5^{\circ}\text{C}$, whereas 35 of 39 strains of potential competitors, all belonging to the *Enterobacteriaceae*, were suppressed. A survey was carried out with 192 environmental samples from four different milk powder factories. *E. sakazakii* could be isolated from almost 40% of the samples, using this new protocol whereas the reference procedure (enrichment in buffered peptone water, isolation on VRBGA, and biochemical identification of randomly chosen colonies) only yielded 26% positive results. This selective method is very useful for the rapid and reliable detection of *E. sakazakii* in environmental samples.

There has been a significant increase in the use of chromogenic substrates in isolation media during the past decade (Manafi, 2000). A major advantage of these chromogenic substrates is that strong colours are produced that do not diffuse out of the colonies and even small positive colonies are visible in the presence of numerous competitors. It has been reported that all strains of *E. sakazakii* tested ($n = 129$) were positive for α -glucosidase and that all other *Enterobacter* isolates ($n = 97$) tested negative for this enzyme (Muytjens *et al.*, 1984). The indolyl substrate 5-bromo-4-chloro-3-indolyl- α -D-glucopyranoside (XaGlc) was thus added to a basal medium to differentiate *E. sakazakii* colonies from other members of the *Enterobacteriaceae* (Iversen *et al.*, 2004b). *Enterobacter sakazakii* hydrolyses this substrate to an indigo pigment, producing blue–green colonies on this medium. This new DFI chromogenic medium can be used to detect *E. sakazakii* two days earlier than when using the conventional method. This medium also shows a higher sensitivity (87.2%) and specificity (100%) than the current FDA method used for the detection of *E. sakazakii*.

Little information exists on the characterization and typing of *E. sakazakii*. Nazarowec–White & Farber (1999) investigated phenotypic (biotyping and

antibiograms) and genotypic (ribotyping, randomly amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE)) methods for their ability to discriminate between *E. sakazakii* isolates. Two primers, UBC 245 (5'-CGC GTG CCA G-3') and UBC 282 (5'GGG AAA GCA G-3') were selected for PCR amplification reactions using RAPD. These primers were chosen based on their performance in trial experiments to produce reproducible RAPD patterns. The restriction endonucleases used during this study for PFGE was chosen based on the recognition site of the enzyme and the 57% G + C content reported for *E. sakazakii* (Farmer *et al.*, 1980). The restriction enzymes *Xba*I (recognition site 5'-TCTAGA-3') and *Spe*I (recognition site 5'-ACTAGT-3') produced the clearest and most discriminating banding patterns. From the results obtained they found that both clinical and food isolates of *E. sakazakii* were genetically heterogeneous. The most discriminatory typing methods were RAPD and PFGE and this was followed by ribotyping, biotyping and antibiograms. They also found that with the correct primers, RAPD was quicker and easier to perform than PFGE. It was further recommended that in an outbreak biotyping should be used as a screening tool and that in addition, either RAPD or PFGE should be used for the best discrimination.

In view of the fact that *E. sakazakii* has been associated with milk powder based formulas, it is critical to know whether and where *E. sakazakii* occurs in food manufacturing environments (Kandhai *et al.*, 2004a). A simple detection method was developed based on two features of *E. sakazakii*, namely the production of yellow colonies when grown on TSA and its constitutive α -glucosidase, which is detected in a 4 h colorimetric assay (Kandhai *et al.*, 2004b). Samples were collected from environmental sources such as floor sweepings, dust, vacuum cleaner bags and spilled product near equipment. *Enterobacter sakazakii* was detected by both pre-enrichment and direct plating of diluted samples on VRBGA. *Enterobacter sakazakii* strains were isolated from 18 of the 152 environmental samples and from three individual factories. This method is useful for routine screening of environmental samples taken from milk powder factories and most likely also for dry milk powder based formulas. Optimisation of the enrichment step to detect low numbers of *E. sakazakii* is necessary (Kandhai *et al.*, 2004b).

F. INFECTIOUS DOSE

Enterobacter sakazakii has been shown to cause disease in all age groups, but it is the immunocompromised and low birth weight infants that are at particular risk. IFM is a source of nutrition for many infants; consequently 5 – 6 servings will be consumed by an infant each day (Anon., 2004b).

Only one or a few organisms in rehydrated IFM could cause illness. Some abuse allowing for growth to high levels in reconstituted formulas would have to occur since the minimum infectious dose for infants seems to be fairly high (Nazarowec–White *et al.*, 2003). Although there is no epidemiological evidence for a value of the infectious dose (the amount of agent that must be consumed to give rise to symptoms of foodborne disease), 1 000 *E. sakazakii* cells is used as the first approximation (Iversen & Forsythe, 2003). This is similar to the infectious dose of other pathogenic bacteria such as *Neisseria meningitides*, *Esherichia coli* O157 and *Listeria monocytogenes* 4b. Various factors affect the infectious dose, such as the microorganism's history, host health status and the food matrix (Iversen & Forsythe, 2003).

Using the growth rate of the organism, the time required for the organism to multiply through 14 generations to an infectious dose of 1 000 cells at different incubation times can then be calculated (Table 3). *Enterobacter sakazakii* is stressed in the case of IFM, since it has been spray dried (Iversen & Forsythe, 2003). Furthermore, since the milk is in liquid form, it will quickly pass through the stomach into the small intestines. The infectious dose is assumed to be due to 1 000 *E. sakazakii* cells ingested as a single dose and not due to cumulative exposure. However, it should be recognised that babies receive 5 – 6 feeds in a 24 h period. It is evident that IFM at the normal low levels (≤ 1 *E. sakazakii* cells.100 g⁻¹) is unlikely to cause infection unless there has been gross temperature abuse or contamination via poor hygiene preparation such as a contaminated blender or mixing spoon. These poor hygiene practices have probably been the cause of outbreaks (Clark *et al.*, 1990).

Table 3. Time required for infectious dose (1 000 cells) to be reached in reconstituted infant formula milk (Iversen & Forsythe, 2003).

Temperature (°C)	Doubling time (h)	Time required to reach infectious dose (14 generations)
10	13.6	7.9 d
18	2.9	1.7 d
21	1.3	17.9 h
37	0.5	7 h

Calculations assume an average of less than 1 *E. sakazakii* cells.100 g⁻¹ IFM powder and that a single feed is 18 g powder (reconstituted to 115 ml) with no microbial deaths during preparation or any multiplication in the stomach. Lag time at 10°C was 2 h, for all other temperatures the lag time was not significant. The infectious dose is assumed to be due to 1 000 *E. sakazakii* cells being ingested as a single dose and not due to cumulative exposure. It should be recognised that babies receive 5 – 6 feeds in a 24 h period.

Nazarowec–White & Farber (1997c) showed that even very low counts of 1 cfu.ml⁻¹ can increase to levels as high as 10⁷ per serving of 100 ml in bottles when kept at room temperature for 10 h, which correlates with the predictive model. It is thus clear that potentially hazardous levels of *E. sakazakii* would be reached even sooner in formula held at 35° – 37°C.

G. PUBLIC HEALTH CONCERN AND MEDICAL SIGNIFICANCE OF ENTEROBACTER SAKAZAKII INFECTIONS

Enterobacter species have only recently become important causes of nosocomial infections (Sanders & Sanders, 1997). *Enterobacter sakazakii* has been isolated from a variety of sterile environments, including human blood and cerebrospinal fluid with clinical conditions consistent with Gram–negative infections (CAC, 2004). *Enterobacter sakazakii* has also been shown to cause disease in all age groups and it can be deduced from the age distribution of reported cases that infants (children less than 1 year old) are at particular risk (WHO, 2004). Among infants, those at greatest risk for *E. sakazakii* infection are neonates (up to 4 weeks of age) (WHO, 2004). Approximately 75% of these infected infants had a low birth weight (<2 500 g) and 75% were premature (born at less than 37 weeks gestation) or were immunocompromised.

Mortality rates from *E. sakazakii* infection have been reported to be more than 50% (CAC, 2004), but this figure has declined to less than 20% in recent years. The disease is usually responsive to antibiotic therapy, but increasing antibiotic resistance has been reported for initial treatment of suspected *Enterobacter* infections (Lai, 2001).

Pagotto *et al.* (2003) evaluated clinical and foodborne isolates of *E. sakazakii* for enterotoxin production by using the suckling mouse assay. The pathogenesis and virulence factors of *E. sakazakii* were studied and 18 *E. sakazakii* strains were isolated of which four tested positive for enterotoxin production. During this study, suckling mice were challenged both orally and intraperitoneally. All the strains of *E. sakazakii* were lethal to suckling mice at 10⁸ cfu per mouse by intraperitoneal

injection. Two strains also caused death by the peroral route suggesting that there are apparent strain differences in virulence. This may in part be related to the organism's ability to survive the acidic conditions present in the stomach.

H. INFANT MILK FORMULA PRODUCTION

Since the beginning of the 20th century, there has been a steady increase in the production of dried IFM from cow's milk. Human and cow's milk differ in the relative content and chemical composition of macronutrients (Nazarowec–White & Farber, 1997b). Cow's milk must be modified in order to simulate breast milk and this includes reduction of the protein and mineral content, increasing the amount of whey protein, increasing the carbohydrate content, and increasing the Ca/P ratio. Vitamins are also added and the fat is modified.

Powdered IFM is produced from ingredients that may include milk, milk derivatives, soy protein isolates, carbohydrates, fats, minerals, vitamins and some food additives (Anon., 2004b). Different procedures are used including the “dry procedure” and “wet procedure” (Nazarowec–White & Farber, 1997b). The main ingredients, either in liquid or powdered form are mixed with water to form a liquid (Fig. 1). The liquid mix is pasteurised at either 71°C for 15 s or 74.4°C for 25 s for products containing starches or thickeners or at higher temperatures (105° – 125°C) for at least 5 s. After this, the product is homogenized, and in some cases evaporated and stored in large chilled holding tanks. Prior to the spray drying process the vitamins are added. The product is then spray dried to a powder with an $a_w \leq 0.3$. Unlike liquid formula products, the dried products are not treated with high temperatures for sufficient time to make the final packaged product commercially sterile (Farber, 2004).

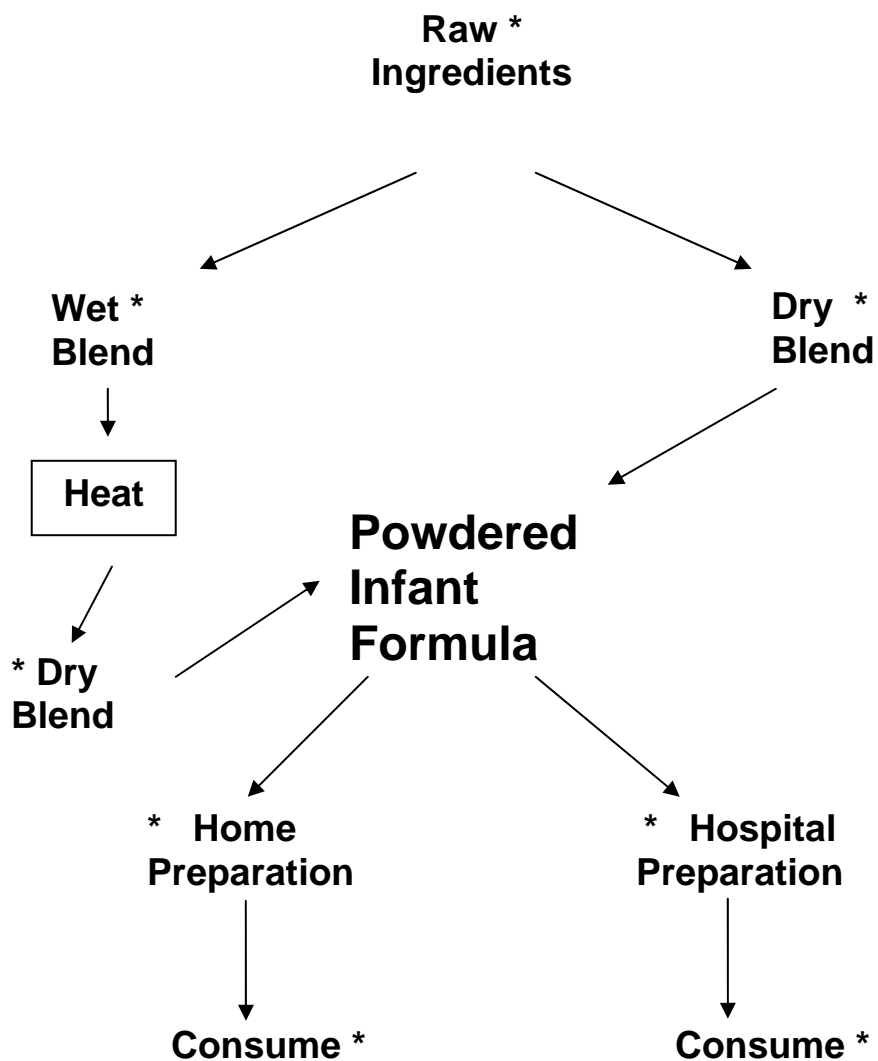


Figure 1. Flow chart of the production and use of powdered IFM. The heat step during wet blending is assumed to effectively eliminate *Enterobacteriaceae* (Adapted from Anon., 2004b).

*Potential sites for environmental contamination.

There are three routes by which *E. sakazakii* can enter IFM: through the raw material used for producing the formula; through contamination of the formula or other dry ingredients after pasteurisation; and through contamination of the formula during reconstitution by the care giver/mother just prior to feeding (WHO, 2004). A difficulty that needs to be considered when evaluating potential treatment options for inactivation of microbial pathogens in powdered IFM is the behaviour of vegetative cells in dried products. For example, the cells often show an increased heat resistance (Anon., 2004b).

A primary means of reducing the risk associated with foodborne pathogens is thermal treatment of foods prior to consumption (Edelson–Mammel & Buchanan, 2004). This has been identified as a useful way of reducing the risk of *E. sakazakii* in rehydrated IFM (Muytjens & Kolleé, 1990; Nazarowec–White *et al.*, 2003). The effective use of thermal treatment should be sufficient to inactivate the microorganism of concern, while minimising the loss of nutrients. Sterilisation of the final product in its dry form in a processing environment in cans or sachets seems only possible using irradiation. The irradiation doses that are likely to be required to inactivate *E. sakazakii* in the dry state do not appear to be feasible due to organoleptic deterioration. Other potential techniques include ultra–high pressure and magnetic fields. Since these technologies are at an early stage of development, none of these are currently suitable for dried foods.

I. POLYMERASE CHAIN REACTION AND MOLECULAR DETECTION

The use of the PCR reaction in microbial diagnostics has been established in research laboratories as a valuable alternative to traditional detection methods (Malorny *et al.*, 2003). Some of the most important advantages include a low detection limit, selectivity, specificity, sensitivity and potential for automation. Major reasons for the delay in acceptance of this technique include the technological newness of this process, the high investment cost, and the lack of officially approved standard regulations and instructions.

A frequent problem encountered with PCR is due to its high sensitivity, false positive and misleading results commonly caused by carry-over or cross-contamination may occur (Belák & Ballagi-Pordány, 1993). The most common sources of ineffective amplification resulting in negative results are the inhibitory effect of certain substrate ingredients and/or pipetting errors. A wide range of PCR inhibitors exist and include factors found in body fluids, food, soil, bacterial cells, non-target DNAs, culture medium, DNA extraction solutions and laboratory plastic ware (Rossen *et al.*, 1992).

An assay was developed for the specific detection of *E. sakazakii* in infant formula in a study done by Seo & Brackett (2005), using an application of the fluorogenic 5' nuclease assay (TaqMan). By using the *E. sakazakii* partial macromolecular synthesis operon (the rpsU gene 3' end and the primase (dnaG) gene 5' end) a set of primers and probe was designed. The sequence of the primers was as follows (5' to 3'): GGGATATTGTCCCCTGAAACAG (forward primer) and CGAGAATAAGCCGCGCATT (reverse primer), while the sequence of the probe was 6-FAM-AGAGTAGTAGTTGTAGAGGCCGTGCTTCCGAAAG-TAMRA.

The assay was specific enough to discriminate *E. sakazakii* from all other *Enterobacter* and non-*Enterobacter* strains tested. The developed real-time PCR assay could save up to 5 days and eliminate the need for plating samples on selective or diagnostic agars and for biochemical confirmation steps. The real-time PCR assay could be used to rapidly screen infant formula samples for *E. sakazakii* and would be beneficial to food industries and regulatory agencies.

Lehner *et al.* (2004) designed specific *E. sakazakii* 16S rRNA gene targeting primers Esakf (5'GCT YTG CTG ACG AGT GGC GG 3') and Esakr (5' ATC TCT GCA GGA TTC TCT GG 3') which binds to conserved regions (*E. coli* position 88 – 107 (Esakf) and 1017 – 998 Esakr)) in the 16S rRNA gene sequences giving an amplicon of 929 bp. These primers can be used to identify *E. sakazakii* from both phylogenetic distinct lineages within the *E. sakazakii* species.

Most published diagnostic PCR protocols do not contain an internal amplification control (IAC) (Hoorfar *et al.*, 2004). In PCR diagnostics, internal controls are required in order to prevent false negative results (Pallen *et al.*, 1992) that may be

caused by PCR inhibitors. In contrast to an external positive control, an IAC is a non-target DNA sequence present in the same sample tube, which is co-amplified concurrently with the target sequence. An internal standard is more reliable than an external one, since it can detect inhibition in each reaction tube (Ballagi–Pordány & Belák, 1996). The presence of the PCR control product in the absence of the target PCR products shows that the amplification conditions were appropriate, but target DNA was absent.

In some PCR systems, the IAC and the diagnostic target are amplified with the same primers. In other PCR assays, two pairs of primers are applied, one pair complementary to the diagnostic target and the other complementary to the reporter DNA sequence (Gilliland *et al.*, 1990). Some disadvantages of this approach include the possibility of heteroduplex formation during PCR due to sequence similarity of the target and control DNA, as well as the risk of contaminating the control DNA with target DNA used for the preparation of the control DNA.

J. CONCLUSIONS

The growing number of reports of *E. sakazakii* infections and its role as an emerging foodborne pathogen are of concern to clinicians, the food industry and the consumers (Hamilton *et al.*, 2003). Since *E. sakazakii* is primarily associated with IFM causing disease in infants, it is important to be able to detect the organism and prevent contaminated products from being distributed (Anon., 2004b). The production of dry IFM requires particular attention and adherence to strict hygiene conditions in order to prevent contamination (Nazarowec–White *et al.*, 2003). The potential for post process recontamination with low levels of *E. sakazakii* exist. Given that powdered formula is not a sterile product, risk management strategies have to be developed in order to address the presence of *E. sakazakii* in the food product (Anon., 2004b).

It is important that test methods for the detection of *E. sakazakii* give prompt and accurate results. To date no standardised/validated or official method exists for the direct isolation of *E. sakazakii* from foods (Nazarowec–White *et al.*, 2003). Since current test methods for the detection of *E. sakazakii* in IFM are not selective enough

for only detecting *E. sakazakii* and are also time consuming. Leuschner *et al.* (2004) and Iversen *et al.* (2004b) developed a presumptive medium and a selective differential medium, respectively, for the selective detection of *E. sakazakii*. Molecular techniques such as PCR also provide an accurate and highly specific alternative for the identification of microorganisms in food. PCR could thus serve as a reliable tool for the detection of *E. sakazakii* in IFM.

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CHAPTER 3

ISOLATION AND PCR DETECTION OF *ENTEROBACTER SAKAZAKII* IN SOUTH AFRICAN FOOD PRODUCTS, SPECIFICALLY INFANT FORMULA MILKS

ABSTRACT

Enterobacter sakazakii is a member of the *Enterobacteriaceae* and has been identified as a possible human pathogen. Current isolation and detection methods for *E. sakazakii* involve various time-consuming culture-dependent steps. The aim of this study was to develop a polymerase chain reaction (PCR) method for the detection of *E. sakazakii* present in South African infant formula milks (IFM) and other baby food products and to isolate this bacterium from these products on selective growth media. DNA was isolated from pre-enriched and enriched samples of 22 different South African IFM and baby foods. An 850 base pair (bp) PCR amplification product of part of the 16S ribosomal RNA (rRNA) gene was utilized to indicate the presence of *E. sakazakii* in a product. The detection limit for *E. sakazakii* using the developed method was $1\ 000\ \text{cfu}\cdot\text{ml}^{-1}$ in IFM. An internal amplification control (IAC) was also developed in order to prevent false negative results. This new method was compared to the current isolation methods to determine its effectiveness. *Enterobacter sakazakii* was detected in four of the products tested using culture-dependent methods, as well as the developed PCR detection method. A number of other pathogens, including *Escherichia coli* and *Klebsiella pneumoniae* were isolated from four of the 22 (18%) food products tested. This PCR method proved valuable for the detection of *E. sakazakii* within three days and can be used for the rapid and reliable detection of this potential human pathogen in food products.

INTRODUCTION

Enterobacter sakazakii is a member of the family *Enterobacteriaceae* (Nazarowec–White & Farber, 1997a). The organism is a Gram–negative, peritrichous motile rod and was referred to as the “yellow pigmented *Enterobacter cloacae*” until 1980, when it was renamed *E. sakazakii* (Farmer *et al.*, 1980). This microorganism is a rare, but an important cause of life–threatening neonatal meningitis and sepsis (Nazarowec–White & Farber, 1997a). The mortality rates from *E. sakazakii* infections can be as high as 50% or more, but in recent years this figure has declined to less than 20% (Anon., 2004a). A review of cases in infants reported in literature from 1961 to 2003 revealed only 48 cases of *E. sakazakii* induced illness among infants.

Although the environmental origin of *E. sakazakii* is not known, IFM has been identified as a potential source of this microbial pathogen (Nazarowec–White & Farber, 1997a). It is thus likely to be present in both food manufacturing facilities, as well as domestic environments. Factors contributing to some cases of *E. sakazakii* contamination include improper handling and the use of contaminated utensils, such as food mixers (Biering *et al.*, 1989; Simmons *et al.*, 1989). Studies on the contamination of IFM have shown an occurrence of 0 to 12% at very low levels (less than 70 cfu.100 g⁻¹) in samples positive for *E. sakazakii* (Muytjens *et al.*, 1988; Nazarowec–White & Farber, 1997b). Nazarowec–White & Farber (1997b) also found that when prepared bottles are stored overnight at room temperature, levels of *E. sakazakii* could reach more than 10⁵ cfu.ml⁻¹.

The current Food and Drug Administration (FDA) method for the detection of *E. sakazakii* is very time consuming and includes a pre–enrichment, enrichment in *Enterobacteriaceae* enrichment (EE) broth, culturing on violet red bile glucose agar (VRBGA) and subsequent subculturing onto TSA (Nazarowec–White & Farber, 1997a; Anon., 2002). *Enterobacter sakazakii* produces characteristic yellow colonies on TSA. New selective growth media were developed to rapidly detect *E. sakazakii*. This included the chromogenic *E. sakazakii* agar for the selective detection of the bacterium two days faster than when using the conventional method (Iversen *et al.*, 2004). A differential–elective medium based on α –glucosidase for the presumptive

isolation of *E. sakazakii* (Leuschner *et al.*, 2004) and a differential medium for *E. sakazakii* (Oh & Kang, 2004) has also been developed. More recently, Guillaume–Gentil *et al.* (2005) developed a method that includes selective enrichment at $45^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ in lauryl sulphate tryptose broth supplemented with 0.5 M NaCl and 10 mg.l^{-1} vancomycin (mLST) for 22 to 24 h, followed by streaking on TSA with bile salts. Seo & Brackett (2005) developed a real–time PCR assay that could be used to rapidly screen IFM samples for *E. sakazakii*. This real–time PCR assay can save up to 5 days and eliminate the need for plating samples on selective or diagnostic agars and for biochemical confirmation steps.

The introduction of PCR detection to facilitate microbial diagnostics has been established as a valuable alternative to traditional detection methods (Malorny *et al.*, 2003). A frequent problem encountered with the PCR method is false positive and misleading results commonly caused by carry–over or cross–contamination (Belák & Ballagi–Pordány, 1993). In order to monitor the presence of inhibitors and thus avoid false negative results during PCR amplifications, an internal amplification control (IAC) ought to be included in PCR detection reactions (Hoorfar *et al.*, 2004a). The aim of this study was to develop a PCR method that includes an IAC for the detection of *E. sakazakii* in IFM and other baby food products and to compare the results with current culturing techniques using selective growth media.

MATERIALS AND METHODS

Products evaluated

A total of 22 different commercially available IFM and other baby food products were obtained from local supermarkets (Table 1). The products included cow’s milk, as well as soya milk substitutes and one goat’s milk product. The products were evaluated for the presence of *E. sakazakii* using the current recommended FDA culturing techniques (Anon., 2002) and the PCR detection method developed in this study.

Table 1. List of the different South African products tested for the presence of *Enterobacter sakazakii*, a description of each product, and the microbial content before and after overnight incubation.

Company	Product number	Description	Before pre-enrichment (cfu.g ⁻¹)		After pre-enrichment (cfu.g ⁻¹)		<i>Enterobacter sakazakii</i> present/absent (PCR and culture media)	Other microorganisms
			PCA	MRS	PCA	MRS		
A	1	Starter infant formula with iron	2.00 x 10 ²	7.00 x 10 ¹	5.60 x 10 ⁷	9.90 x 10 ⁷	-	Not determined
	2	Milk and lactose free infant starter formula with iron (soya based)	9.00 x 10 ²	<10 (Spreader)	5.80 x 10 ⁸	6.00 x 10 ⁸	+	Not determined
	3	Follow-on infant formula with iron	No growth	<10 (Spreader)	7.80 x 10 ⁸	1.38 x 10 ⁸	-	Not determined
	4	Infant formula with iron for babies with reflux	<10 (Spreader)	<10 (Spreader)	4.60 x 10 ⁶	1.08 x 10 ⁷	-	<i>Chryseomonas luteola</i> <i>Bacillus licheniformis</i>
	5	Lactose free infant formula	<10 (Spreader)	<10 (Spreader)	1.10 x 10 ⁸	9.50 x 10 ⁷	-	<i>Bacillus stearothermophilus</i> <i>Klebsiella pneumoniae</i>
B	6	Infant formula with iron	<10 (Spreader)	<10 (Spreader)	Not determined	Not determined	-	Not determined
	7	Infant formula with iron	<10 (Spreader)	<10 (Spreader)	<10 000 (Spreader)	<10 000 (Spreader)	-	Not determined
	8	Milk and lactose free with iron (soya product)	No growth	<10 (Spreader)	1.04 x 10 ⁸	1.01 x 10 ⁸	-	Not determined

Table 1. Continued.

Company	Product number	Description	Before pre-enrichment (cfu.g ⁻¹)		After pre-enrichment (cfu.g ⁻¹)		<i>E. sakazakii</i> present/absent (PCR and culture media)	Other microorganisms
			PCA	MRS	PCA	MRS		
C	9	Infant formula with AA, DHA and iron	<10 (Spreader)	<10 (Spreader)	<10 000 (Spreader)	<10 000 (Spreader)	-	Not determined
	10	Starter infant formula with iron	<10 (Spreader)	<10 (Spreader)	<1 000 000 (Spreader)	<1 000 000 (Spreader)	-	Not determined
	11	Acidified infant formula with iron	No growth	No growth	Not determined	Not determined	-	Not determined
	12 ^a	Probiotic milk for young children with bifidus and honey	1.35 x 10 ⁷	1.30 x 10 ⁷	1.32 x 10 ⁹	1.04 x 10 ⁹	-	Not determined
	13	Hypoallergenic, reduced risk of allergic reaction	<10 (Spreader)	<10 (Spreader)	5.00 x 10 ⁵	1.48 x 10 ⁶	-	<i>Klebsiella pneumoniae</i>
	14	Starter infant formula with iron	<10 (Spreader)	<10 (Spreader)	Not determined	Not determined	-	Not determined
	15	Follow-up formula with iron and prebiotics	No growth	No growth	5.00 x 10 ⁵	1.48 x 10 ⁶	-	Not determined
	16	Full cream instant milk powder	<10 (Spreader)	<10 (Spreader)	4.30 x 10 ⁸	3.90 x 10 ⁸	+	Not determined
17	Lactose free infant formula with iron	No growth	No growth	9.10 x 10 ⁷	9.30 x 10 ⁷	-	Not determined	

Table 1. Continued.

Company	Product number	Description	Before pre-enrichment (cfu.g ⁻¹)		After pre-enrichment (cfu.g ⁻¹)		<i>E. sakazakii</i> present/absent (PCR and culture media)	Other microorganisms
			PCA	MRS	PCA	MRS		
D	18	UHT Growing up milk rich in calcium with added vitamins and minerals	No growth	No growth	2.90 x 10 ⁷	2.20 x 10 ⁵	-	Not determined
E	19	Soy infant formula	No growth	No growth	No growth	No growth	-	Not determined
F	20	Premium soya milk powder	<10 (Spreader)	<10 (Spreader)	5.10 x 10 ⁷	3.40 x 10 ⁷	+	<i>Klebsiella pneumoniae</i>
G	21	Goats milk	3.80 x 10 ⁴	3.30 x 10 ⁴	4.50 x 10 ⁸	1.09 x 10 ⁸	-	<i>Escherichia coli</i>
H	22	Infant cereal	<10 (Spreader)	<10 (Spreader)	<10 000 (Spreader)	<100 000 (Spreader)	+	<i>Raoultella terrigena</i> (<i>Klebsiella terrigena</i>)

^aContains *Streptococcus thermophilus* and *Bifidobacterium lactis* at levels of 10⁶ cfu.g⁻¹.

+: *Enterobacter sakazakii* detected by PCR and culture techniques.

-: *Enterobacter sakazakii* absent using both PCR and culture techniques.

PCA: Plate Count Agar.

MRS: DeMann, Rogosa and Sharpe Agar.

DNA extraction

Samples were prepared for DNA extraction by aseptically placing 100 g of the food product into sterile containers and adding 900 ml sterile distilled water at 45°C. This mixture was incubated overnight at 37°C after which triplicate 2 ml samples were removed and subjected to DNA extractions. For the UHT milk, the container was directly incubated overnight at 37°C and sampled for DNA extraction. Ten ml of the incubated sample was also transferred to EE broth (Oxoid) and incubated overnight at 37°C, after which triplicate samples were removed for DNA extraction.

DNA extractions were done by the modified method of Van Elsas *et al.* (1997). Before DNA extraction the 2 ml samples were centrifuged at 5 900 x g for 10 min and the supernatant discarded. Sterile glass beads (0.6 g) (0.2 – 0.3 mm diameter) (Sigma) were added to the pellet, as well as 800 µl phosphate buffer (1 part 120 mM NaH₂PO₄ (Merck) and 9 parts 120 mM Na₂HPO₄ (Merck)), 700 µl phenol (Fluka) and 100 µl 20% (m/v) sodium dodecyl sulphate (SDS) (Merck). This mixture was vortexed for 2 min and then incubated at 60°C for 20 min. This step was repeated twice. After incubation, the sample was centrifuged for 5 min at 1 500 x g. The aqueous phase was collected and the proteins were extracted with 600 µl phenol (Fluka). Further extraction was performed with a 600 µl phenol:chloroform: isoamylalcohol (25:24:1) mixture until the interphase was clean. DNA was precipitated with 0.1 volume 3 M sodium acetate (NaOAc) (pH 5.5) (Saarchem) and 0.6 volume isopropanol (Saarchem). The samples were kept on ice for approximately 1 h and then centrifuged for 10 min at 15 000 x g. The pellets were washed with 70% (v/v) ethanol, centrifuged for 5 min at 15 000 x g, air dried and redissolved in 100 µl TE (10 mM Tris (Fluka), 1 mM EDTA (Merck); pH 8).

PCR amplification

The primer pair Esak2 (5' CCC GCA TCT CTG CAG GAT TCT C 3') and Esak3 (5' CTA ATA CCG CAT AAC GTC TAC G 3') was developed for the specific amplification of *E. sakazakii* (Keyser *et al.*, 2003). PCR detection reactions were performed in 25 µl reaction volumes containing 1 U (0.35 µl) *Taq* DNA polymerase (Promega), 2.5 µl magnesium free buffer supplied with the enzyme, 2 mM (1.5 µl) MgCl₂ (Promega), 1 µl dimethyl sulfoxide (DMSO) (Merck), 1 mM (1 µl) dNTPs

(Promega), 0.5 μ M (0.5 μ l) of each primer and 750 μ g/ml (1 μ l) DNA template. One μ l of a DNA extraction from a pure culture of *E. sakazakii* (1039, University of Stellenbosch Food Science Culture Collection) was added as a positive control and as a negative control, 1 μ l sterile distilled water was added replacing the DNA template. PCR amplification reactions were performed using a Mastercycler (Eppendorf, Germany) and the amplification conditions were as follows: the reaction mixture was kept at 95°C for 2 min for the initial denaturation; this was followed by 35 cycles of 35 sec at 95°C for further denaturation, 1 min at 61°C for primer annealing, and 1 min at 72°C for chain elongation; and a final chain elongation for 10 min at 72°C. The PCR products were separated on a 1% (m/v) agarose gel containing ethidium bromide in 0.5 x TBE electrophoresis buffer. The separated PCR fragments were visualised under UV light (Vilber Lourmat).

Construction of an internal amplification control

A PCR reaction using the primer pair Esak2 and Esak3 was performed on DNA extracted from pure *E. sakazakii* cells grown on TSA. Ten μ l of the 850 bp PCR amplicon was digested with 1 U (1 μ l) of the restriction enzyme *AluI* (Promega), in the presence of 1 x (1.5 μ l) restriction buffer and 2.5 μ l sterile distilled water. The reaction mixture was then incubated overnight at 37°C. The digested product was electrophoresed on a 3% (m/v) agarose gel containing ethidium bromide and visualised under UV light.

The two resulting fragments containing the binding sites of the respective primers were excised from the agarose gel and cleaned using the High Pure PCR Product Purification Kit (Roche) according to the manufacturer's specification (Fig. 1). After clean-up, 10 μ l of each fragment were ligated using 1 U (1 μ l) T4 DNA ligase (Promega). A PCR amplification reaction was performed on the ligated fragment using the PCR primers Esak2 and Esak3 as described. The resulting PCR product was cloned using the InsT/AcloneTM PCR product cloning kit (Fermentas). Transformed cells were screened for the correct sized insert using the primers T7 (5'-GTA ATA CGA CTC ACT ATA GGG-3') and SP6 (5'-TAC GAT TTA GGT GAC ACT ATA G-3'). PCR was performed in a total reaction volume of

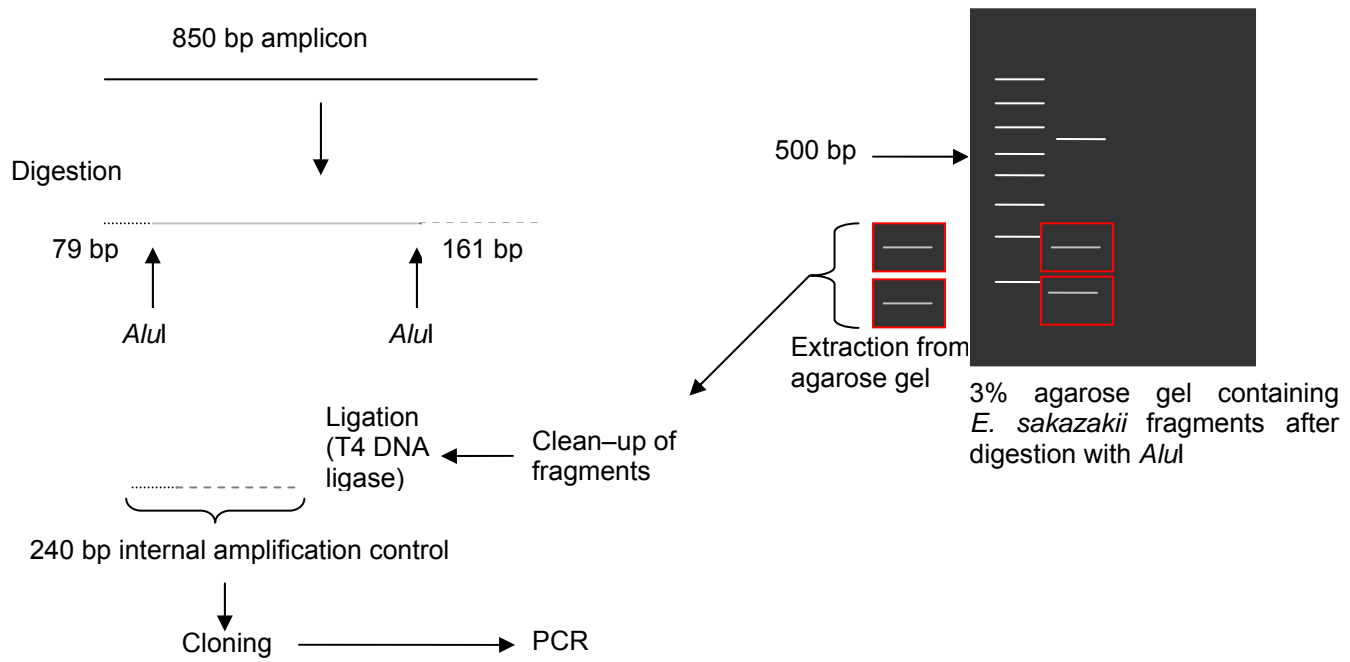


Figure 1. Schematic representation of how internal amplification control was constructed.

50 µl containing 0.5 µM (2µl) of each of the primers, 0.5 mM (2µl) dNTPs (Promega), 1 U (1.5 µl) *Taq* DNA polymerase (Promega), the buffer containing MgCl₂ (5 µl) supplied with the enzyme and the transformed cells.

Plasmid DNA was isolated from the recombinant strain using the Qiagen Midi Plasmid kit (Qiagen). The plasmid DNA was used as an IAC in all the PCR reactions for the detection of *E. sakazakii* in South African IFM and baby foods. The constructed IAC was sequenced with the primer Esak3 (Keyser *et al.*, 2003) using the ABI PRISM 377 DNA sequencer (PerkinElmer) at the DNA Sequencing Facility at Stellenbosch University. This was done to confirm the presence of the two primer binding sites and the correct size of the IAC.

After determining the DNA concentration of the isolated plasmid DNA fluorometrically (DyNA Quant 200 Fluorometer, Hoefer Pharmacia Biotech Inc, USA), the optimum concentration that produced a positive signal for both the *E. sakazakii* DNA and the IAC was determined. Aliquots of 1 µl of the IAC were added to all PCR reactions performed, excluding the positive and negative control.

Detection limit

The lowest number of *E. sakazakii* cells that could be detected in IFM using the developed PCR detection method was determined by growing a pure culture of *E. sakazakii* (1039) in MRS broth. Serial dilutions (10^{-1} to 10^{-8}) were done in sterile saline solution (SSS) (0.85% (m/v) NaCl) (Merck) and samples were taken to determine the optical density at 500 nm, using a spectrophotometer (Spectronic 20, Genesys, Spectronic Instruments, USA). The serial dilutions were plated on MRS agar using the pour plate method. After incubation at 37°C for 24 h the number of colonies was determined. A standard curve was then plotted of the optical density (OD) versus the number of colonies (cfu.ml⁻¹). This was used to obtain a solution containing approximately 1×10^6 cfu.ml⁻¹ of which a serial dilution series to 1×10^0 cfu.ml⁻¹ was prepared in SSS (0.85% (m/v) NaCl (Merck)) and in IFM. One ml of each sample was subjected to DNA extraction and subsequently to the PCR amplification of *E. sakazakii* as previously described.

Standard culturing techniques and identification

To determine the reliability of the PCR detection method, the results were compared to the standard culture techniques for the isolation of *E. sakazakii*. A

100 g of the product was aseptically removed from the IFM container and 900 ml sterile distilled water at 45°C was added. This mixture was gently shaken by hand until the powder was dissolved, followed by overnight incubation at 37°C. Ten ml was then removed and transferred to 90 ml EE (Oxoid) and incubated overnight at 37°C. After incubation, 0.1 ml of each enrichment culture was placed onto duplicate VRBGA (Oxoid) plates and evenly distributed onto the surface using a sterile glass rod. A loop (10 µl) of enrichment culture was also streaked on duplicate VRBGA. When high numbers of bacteria were suspected, the samples were diluted in SSS (0.85% (m/v) NaCl (Merck)) before plating on VRBGA. All the VRBGA plates were incubated overnight at 37°C and the presumptive *E. sakazakii* colonies were subcultured onto TSA plates (Oxoid). These plates were incubated at 25°C for 48 – 72 h after which the plates were visually evaluated for the presence of yellow colonies. Isolates were then streaked on TSA to obtain single, pure colonies, after which the isolates were Gram stained, and subjected to catalase and oxidase tests. The isolates were identified using the API 20E system (API System S.A., La Balme le Grottes, 38390, Montalieu Vercieu, France).

A serial dilution of IFM in SSS (0.85% (m/v) NaCl) (Merck) before and after overnight incubation at 37°C was prepared and 1 ml of each dilution plated on both MRS (Merck) and plate count agar (PCA) (Merck) in order to determine the microbiological quality of each product. Plates were incubated at 37° and 30°C for 24 h, respectively and counted. Isolates were evaluated using the API 50CHB system (API System S.A., La Balme le Grottes, 38390, Montalieu Vercieu, France) (MRS isolates), Gram stains and the catalase and oxidase tests.

RESULTS AND DISCUSSION

Internal amplification control

A PCR IAC is a DNA fragment co-amplified with the target PCR product (Sachadyn & Kur, 1998) that differs in size from the target product. Initially attempts were made to produce an IAC by adding two different primer pairs (Esak2 and Esak3 (Keyser *et al.*, 2003) and F341 and R534 (Muyzer *et al.*, 1993)) to the same reaction tube. However, it was observed in this study, that the simultaneous use of both sets of primers resulted in a shift of the 850 bp band of

E. sakazakii to a band of approximately 700 bp in size. It has been shown in the literature that multiple sets of primers may interfere with the amplification of one or both of the target genes due to differences in the primer sequences (Hoorfar *et al.*, 2004b). This approach, therefore, was unsuccessful in developing an IAC in this study.

The composite primer technique (Siebert & Larrick, 1992) was then used to develop an IAC. With the use of this technique, the target and the IAC are amplified with one common set of primers under the same conditions and in the same PCR tube. After cleaving the 850 bp *E. sakazakii* PCR detection fragment with *AluI*, the two fragments containing the binding sites of the primer pairs (79 and 161 bp in size) were ligated to yield a fragment of 240 bp, 610 bp smaller than the *E. sakazakii* PCR fragment. The ligated fragment was cloned and the isolated plasmid DNA was used as template in the PCR amplification reactions. DNA sequence data of the cloned product indicated that both primer binding sites are present and confirmed that the fragment is 240 bp in size.

Since an IAC is an indication of PCR failure, a positive band should be produced by both the IAC and target DNA to indicate that the PCR amplification reaction was successful. If a signal is produced by target DNA, but not the IAC the positive result is valid because the target DNA is present in proportionally greater amounts. Competition often occurs between the target DNA and an IAC, when the IAC is present at high concentrations (Abdulmawjood *et al.*, 2002). Also, the detection limit can be negatively influenced by high concentrations of the IAC template. It is, therefore, important to determine the lowest reproducible IAC template concentration in the PCR detection reaction. A positive band for both the target DNA and IAC was obtained simultaneously after a minimum concentration of 0.72 pg.ml⁻¹ of plasmid DNA was added to the PCR reaction mixture. Although Brightwell *et al.* (1998) and Abdulmawjood *et al.* (2002) reported that an IAC of less than 500 bp does not influence the PCR sensitivity, it was found that the initial concentration of the plasmid DNA (0.72 ng.ml⁻¹) resulted in no amplification of the target DNA (Fig. 2).

PCR detection limit

A part of the 16S rRNA gene of *E. sakazakii* was successfully amplified from the DNA isolated from a pure culture grown on TSA (Fig. 3). Strain purity

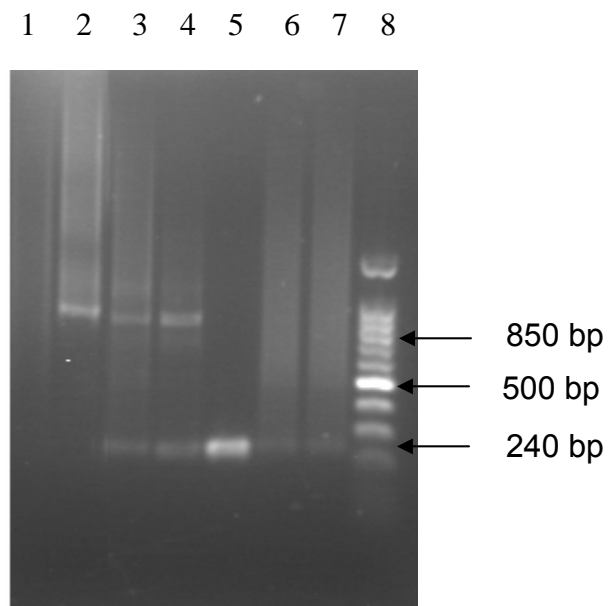


Figure 2. PCR amplification products obtained after co-amplification of the newly constructed IAC and DNA isolated from the products tested. Lane 1: negative control (containing no added DNA); Lane 2: positive control (containing only *E. sakazakii* DNA); Lane 3 and 4: products testing positive for *E. sakazakii* with IAC included; Lane 5: IAC; Lane 6 and 7: products testing negative for *E. sakazakii*; Lane 8: 100 bp molecular marker (Promega).



Figure 3. Characteristic yellow colonies of *Enterobacter sakazakii* grown on tryptone soy agar (TSA).

was confirmed using the API 20E test system, which identified the organism as *E. sakazakii* with 99.9% certainty. The strain was also confirmed to be oxidase negative, Gram-negative rods. PCR detection resulted in an 850 bp amplification product. *Enterobacter sakazakii* was also successfully amplified from the DNA isolated from the pure isolate of *E. sakazakii* inoculated and diluted in SSS and IFM. The detection limit using this PCR detection method was found to be 100 *E. sakazakii* cells.ml⁻¹ in SSS and 1 000 *E. sakazakii* cells.ml⁻¹ in IFM. Since normal levels of *E. sakazakii* found in foods are very low (less than 70 cfu.100 g⁻¹) (Muytjens *et al.*, 1988; Nazarowec-White & Farber, 1997b) an enrichment step is necessary to allow low levels of *E. sakazakii* to be detected in IFM and baby foods.

PCR detection of E. sakazakii

Isolates from IFM could be successfully amplified using the primers Esak2 and Esak3, with no amplification of non-*E. sakazakii* isolates (Table 1). Since *E. sakazakii* is present in very low numbers, enrichment of the product is necessary to allow the low numbers of the microorganism to multiply and to be detected in IFM using the developed PCR method. *Enterobacter sakazakii* was not detected in any of the products after overnight incubation and it was needed to further enrich in EE broth. Enrichment in EE broth could result in other *Enterobacteriaceae* overgrowing *E. sakazakii*, leading to few *E. sakazakii* colonies on VRBGA and a reduced chance of picking the organism onto TSA (Iversen & Forsythe, 2004).

Product 1 tested negative for the presence of *E. sakazakii* using current culture techniques, as no growth was observed on the VRBGA plates. This was confirmed by the developed PCR method, since no positive band was observed after PCR amplification of isolated DNA.

Product 2 was a soya-based starter formula of which the first sample gave a positive PCR band indicating the presence of *E. sakazakii* (Table 1). *Enterobacter sakazakii* was detected when using both PCR detection and isolation on selective growth media. Colonies from the VRBGA was selected and streaked on TSA. The yellow pigmented isolates on TSA were identified using the API 20E test system as *E. sakazakii* (97.7%). PCR detection of *E. sakazakii* in a different batch

of the same product also revealed a positive 850 bp band, which indicated the presence of *E. sakazakii*.

When Product 3 was plated on VRBGA plates after enrichment no colonies were observed and the product tested negative for *E. sakazakii*. This was confirmed by the absence of an amplification product.

When streaking colonies from the VRBGA plates on TSA Product 4 produced yellow colonies, but these were not the characteristic *E. sakazakii* colonies. Identification of this isolate using API 20E showed the isolate to be *Chryseomonas luteola*. This microorganism is an aerobic, oxidase–negative, Gram–negative rod that produces a yellow pigmented colony (Anon., 2004b). It is widely distributed in both nature and in the hospital environment. This species has been associated with nosocomial infections in humans (Anon., 2004b), but has rarely been reported as a human bacterial pathogen (Chihab *et al.*, 2004). No PCR band was observed, confirming that *E. sakazakii* was not present in Product 4.

Two different batches of Product 5 were evaluated. One of the three samples tested from the second batch showed microbial growth on VRBGA. The PCR detection reaction was negative after DNA extraction from this product. The microorganism present in this product was Gram–negative rods, identified as *Klebsiella pneumoniae* subsp. *pneumoniae* (97.6%) using the API 20E test system. *Klebsiella pneumoniae* is clinically the most important species of the genus *Klebsiella* (Anon., 2005a) causing pneumonia and urinary tract infections.

PCR–based detection of *E. sakazakii* in Product 6 did not reveal a positive band for *E. sakazakii*. This correlated with the results observed using conventional methods when colonies selected from the VRBGA, streaked on TSA resulted in no yellow colonies.

No positive PCR detection and no growth were observed on VRBGA plates for Products 7, 8, 9, 10, 11, 14, 15 and 19. Product 12, a probiotic product for young children also showed no positive growth on VRBGA and tested negative for the presence of *E. sakazakii*, using the PCR detection method.

Product 13 produced white colonies on TSA and tested negative for the presence of *E. sakazakii*. The white colonies present on TSA were identified as *Klebsiella pneumoniae* subsp. *pneumoniae* (97.7%) using the API 20E test system.

Product 16 was a full cream instant milk powder that is not modified for infant feeding and found to contain both white and yellow colonies on TSA. The yellow colonies were identified as *E. sakazakii* (99.9%) using the API 20E test system. The product also tested positive for *E. sakazakii* when using the PCR detection method, indicating that although white colonies were present, *E. sakazakii* was still positively identified using the PCR detection method. When another batch of the same product was tested, there was no growth on VRBGA, and no positive band could be identified when using the PCR detection method. This shows that the organism is present in low numbers and in heterogeneous distribution in IFM (Leuschner *et al.*, 2004).

Two of the three samples tested for Product 17 produced white colonies on TSA, but the third revealed no growth on VRBGA. *Enterobacter sakazakii* could not be detected using the PCR method.

In order to determine the quality of other products available on the South African market for infants and children, Product 18, a long-life UHT product (sterile and ready to drink, with no need for preparation) was evaluated. No growth was found on VRBGA after incubation and *E. sakazakii* could not be detected in this food product.

Two batches of Product 20, a soya drinking milk were evaluated. The first batch tested positive for the presence of *E. sakazakii* using both culturing and PCR detection, while the second batch tested negative for the presence of *E. sakazakii*. However, *Klebsiella pneumoniae* was present in the second batch (Table 1).

The goat's milk product (Product 21) is not formulated milk product. Although this product is sold with IFM, it is not suitable for babies, since some of the proteins in goat's milk are similar to those in cow's milk (Anon., 2005b). Most babies that react to cow's milk protein are also likely to react to goat's milk protein. The lactose levels are also similar, so it is also unsuitable for babies who are lactose intolerant. After DNA extraction and PCR detection, no positive PCR band was visible on the agarose gel. Colonies isolated from the VRBGA plates were identified as *Escherichia coli* (96.3%) with the API 20E and the isolate was oxidase negative, catalase positive, Gram-negative rods (Table 1). *Escherichia coli* is part of the human intestinal tract and an indicator of fecal contamination (Anon., 2005c).

Product 22 was a baby cereal product and the first batch tested positive for *E. sakazakii*. This was confirmed using the API 20 E test system (98.4%). The second batch tested positive for *Raoultella terrigena* (*Klebsiella terrigena*), but with a low discrimination percentage (68.9%). It has been isolated from soil and water and its clinical importance in humans is unknown (Drancourt *et al.*, 2001).

Enterobacter sakazakii was detected in all the products using the developed PCR detection method that tested positive using the culture techniques (Table 1). The results further showed that *E. sakazakii* could only be successfully detected and isolated from products after enrichment in EE broth. It is suggested that the first incubation period (pre-enrichment) should be shortened since *E. sakazakii* has a doubling time of 40 min at 23°C (Nazarowec-White *et al.*, 2003) and this combined with enrichment in EE broth could further reduce the time needed for detection of this human pathogen. It was found that there is a heterogeneous distribution of potentially pathogenic organisms in IFM, since some of the products had samples that tested positive, while other samples from the same batch tested negative. Consequently, it is necessary to evaluate more than one sample and use a sample size as large as possible and even test different batches in order to ensure that low numbers of the organism will be detected.

Microbial quality of products tested

During the evaluation of the microbial quality of the IFM samples plated on MRS and PCA, a large number of the products had a spreader that covered the surface of the plate and the viable counts could not be determined for these products (Table 1). Products 1, 2 (PCA only), and 21 had viable counts that ranged between 70 and 3.80×10^4 cfu.ml⁻¹. Stringent microbiological criteria have been established for total viable counts, which are used as a hygiene indicator (out of 5 samples, three must have $< 10^3$ cfu.g⁻¹, the other two $< 10^4$ cfu.g⁻¹) by Codex Alimentarius (Nazarowec-White *et al.*, 2003). The viable counts of product 1 and 2 are lower than 10^3 cfu.ml⁻¹ which is within these specifications. Although only two samples were evaluated during this study, product 21 had counts higher than the specified number and this could be an indication that hygiene practices are not sufficient. The high total viable count for product 12 was expected since this product contains *Streptococcus thermophilus* and *Bifidobacterium lactis* at levels of 10^6 cfu.g⁻¹ as specified on the label.

Products 11, 15, 17, 18, 19 had no growth, while Products 3, 4, 5, 6, 7, 8, 9, 10, 13, 14, 16, 20, 21 and 22 all had a spreader, with growth characteristic of *Bacillus*. All of these isolates were Gram-positive and catalase positive, characteristic of *Bacillus* species. Isolates from three of the products (product 4, 5 and 21) were identified using the API 50 CHB to identify the *Bacillus* species present. In products 4 and 21 the microorganisms were identified as *Bacillus licheniformis* and in product 5 as *B. stearothermophilus*. *Bacillus licheniformis* grows in foods, especially if held between 30° and 50°C and its spores are present in soil (Todar, 2003). *Bacillus stearothermophilus* has an upper temperature of 65°C for growth. Sources of this bacterium include soil, hot springs, desert sand, Arctic waters, ocean sediment, food and compost.

After overnight incubation (pre-enrichment) of the products, microbial counts varied between 10^7 and 10^9 cfu.ml⁻¹. This is an indication that temperature abuse such as leaving prepared IFM overnight at room temperature or keeping prepared formula in a bottle warmer (Nazarowec-White & Farber, 1997c) could lead to the multiplication of microorganisms to high numbers in short periods. Potential pathogens could thus reach high numbers and cause infections when ingested.

CONCLUSIONS

The PCR detection method used during this study proved to be effective and *E. sakazakii* was detected in four of the 22 (18%) products tested, but only after enrichment in EE broth. The PCR-based method developed can be used to detect *E. sakazakii* in three days without the need of using API or time consuming culture methods. This includes the first and second enrichment steps, DNA extractions and PCR amplification. The IAC was included in order to avoid false negative results and thus serving as control to indicate PCR failure.

Prepared formula is an excellent culture medium for pathogenic organisms and bacterial contamination and growth during the preparation of IFM must be avoided (Muytjens & Kollée, 1990). *Enterobacter sakazakii* is an environmental organism, and is likely to be present in both manufacturing facilities and at home. This may lead to frequent contamination and multiplication in prepared formula,

especially at temperatures of between 25° – 45°C (Nazarowec–White & Farber, 1997c).

The improvement of hygiene practices, monitoring of the food processing environment and end–product testing should be made obligatory (Lehner & Stephan, 2004). Also, safety instructions supplied with the product should be carefully followed, since this reduces the risk of pathogenic organisms multiplying to very high levels. The storage period of IFM (< 24h) should on the other hand, not facilitate the growth of *E. sakazakii* to an infectious level (1 000 cells based on *Escherichia coli* O157:H7 and *Listeria monocytogenes*). However, where the product is subjected to severe temperature abuse or poor hygiene practices (Iversen & Forsythe, 2004) it may become a problem. It is also unlikely that sufficient multiplication will occur under refrigeration temperatures to cause an infection.

Molecular assays for the detection of microorganisms have often proved to be useful as they offer an alternative means to rapidly and specifically identify organisms from a variety of sources (Lehner *et al.*, 2004). The PCR detection method used during this study, including the IAC could thus serve as a valuable method for the detection of *E. sakazakii* in IFM products.

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CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

Enterobacter sakazakii, the cause of meningitis in neonates, has been identified as a contaminant of powdered infant formula milk (IFM). Current identification methods for the detection of *E. sakazakii* are very time consuming, taking up to seven days (Nazarowec–White & Farber, 1997a; Anon., 2002). A need, therefore, exists for a rapid and reliable method for the detection of *E. sakazakii* present in IFM.

A PCR–based method was developed for the detection of *E. sakazakii* from South African IFM and other baby food products. An internal amplification control (IAC) was also constructed using the restriction endonuclease *AluI*. After digesting the 850 base pair (bp) *E. sakazakii* PCR product, the fragments containing the primer binding sites were ligated to give a 240 bp IAC which was then used in all the PCR detection reactions. The IAC was developed in order to prevent false negative results (Hoorfar *et al.*, 2004) and a positive band for both the target DNA (850 bp) and the IAC (240 bp) was obtained after a minimum concentration of 0.72 pg.ml⁻¹ of the IAC was added to the PCR reaction mixture. The IAC is therefore of value so as to prevent false negative results and would also serve as an indicator of PCR failure. In this study, the presence of an 850 bp PCR amplification product indicated the presence of *E. sakazakii*, while a band at 240 bp indicated that the PCR amplification was successful.

DNA was isolated from 22 different food products, both after pre–enrichment and enrichment in *Enterobacteriaceae* enrichment broth. Data from literature showed that the concentration of *E. sakazakii* in IFM is generally low, typically less than 70 cfu.g⁻¹ (Muytjens *et al.*, 1988), making it necessary to use both a pre–enrichment and enrichment step, as well as culturing on violet red bile glucose agar and tryptone soy agar to isolate the organism. The data from this study showed that four of the 22 different products tested (18%) did contain *E. sakazakii* (confirmed by both PCR and culturing techniques). One product contained *Escherichia coli*, three products *Klebsiella pneumoniae*, one product *Raoultella terrigena* (“*Klebsiella terrigena*”) and one product *Chryseomonas*

luteola. The identity of each species was confirmed using the API 20E test system.

It should be taken into consideration that *E. sakazakii* was isolated from four of the 22 different products tested, but this was only after a pre-enrichment and enrichment step. These enrichment steps allow the organism to multiply and reach higher numbers. The low numbers of *E. sakazakii* in IFM can still be the cause of serious infections in vulnerable infants, specially when allowed to multiply to high numbers when abuse such as leaving prepared IFM overnight at room temperature or keeping prepared formula in a bottle warmer (Nazarowec-White & Farber, 1997b) takes place. The standard period of storage (<24h) should however, not permit the growth to an infectious level (the infectious dose is based on 1 000 *E. sakazakii* cells used as the first approximation (Iversen & Forsythe, 2003)). This infectious dose is similar to the infectious dose of other pathogenic bacteria such as *Neisseria meningitides*, *Escherichia coli* O157:H7 and *Listeria monocytogenes* 4b.

CONCLUDING REMARKS

The presence of *Enterobacter sakazakii* in powdered IFM should be considered a serious risk given the potential of the organism to multiply during the preparation and holding time prior to the consumption of the reconstituted formula product (Anon., 2004). *Enterobacter sakazakii* is an environmental organism and thus it is recommended that both the raw materials and the final products be evaluated for the presence of this bacterium in order to prevent contaminated products from being distributed. Even low levels of *E. sakazakii* contamination may pose risks to infants and it is, therefore, important to educate parents and child/infant caretakers of the potential risk associated with the inappropriate handling of IFM.

The PCR-based method developed in this study proved to be effective in the detection of *E. sakazakii* in IFM products, and results obtained were similar to results reported using current culturing techniques (Nazarowec-White & Farber, 1997a; Anon., 2002). The PCR detection method, including the IAC served and in future could serve as a rapid and accurate method for the detection of *E. sakazakii* in IFM products.

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