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***Enterobacter sakazakii*: an emerging microbe with implications for infant health**

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Introduction

Enterobacter sakazakii is a motile peritrichous, Gram-negative non-spore forming facultative anaerobic bacillus belonging to the family Enterobacteriaceae, genus *Enterobacter*. The microbe was listed as a new species in 1980 [1]. Using DNA-DNA hybridisation and biochemical tests Farmer et al. defined the species and identified fifteen distinct biogroups[1] with a new biogroup being added recently[2]. Based on genotyping and biotyping *E. sakazakii* represents a genetically diverse and taxonomically ill-defined species[2-5].

Enterobacter sakazakii is recognised as an emerging opportunistic pathogen and is the aetiological agent in rare cases of meningitis, necrotising enterocolitis and bacteremia in infants[6-15]. Since the first case of meningitis in an infant iwas described in England in 1958[16], neonatal infections involving *E. sakazakii* have been reported from all parts of the developing world with many of these resulting in the deaths of more than seventy infants **Table 1**.

Sources of *E. sakazakii* illness have not been extensively investigated and therefore the origin of the bacterium remains unknown. However, several reports have implicated contaminated powdered infant formula (PIF) as a source of *E. sakazakii* in neonatal infection[8, 14, 17, 18]. In 2004, several high profile recalls of PIF contaminated with *E. sakazakii* from Asia, South America and Europe **(WHO/FAO, 2006)** occurred.

These developments have focused attention on this pathogen, by both the PIF manufacturers and the health professionals. A revision of the microbiological standards applied by PIF producers has taken place, with the implementation of updated testing requirements, underpinned by legislation, aimed at reducing the risk of dissemination of the pathogen through powdered products. Attention has also focused on the development of guidelines for the correct preparation of PIF in hospitals and in homes.

This review will describe the microbiology of *E. sakazakii,* provide an understanding of its epidemiology and examine its role in public health. We will also aim to highlight the issues surrounding the proper reconstitution of PIF prior to the feeding of infants.

Taxonomy and background of *Enterobacter sakazakii*

Enterobacter sakazakii was originally referred to as a "yellow-pigmented" *Enterobacter cloacae.* It was subsequently re-defined as a new bacterial species in 1980[1]. Farmer et al.,[1] originally described 15 biogroups including 57 strains, based on biochemical profiles with five strains used to define the species by DNA-DNA hybridisations. These data suggested that the biogroups may represent more than one species and that *E. sakazakii* was 41-54% related to *Enterobacter* and *Citrobacter* species. Detailed phylogenetic analysis of 16S rDNA and *hsp60* genes confirmed the diverse heterogeneity that existed between strains and further analysis of these gene sequences showed the presence of four distinct genetic clusters, all of which

contained strains of clinical origin[3-5]. In a recent extension of the initial study [1], Iversen et al.,[2] analysed 189 strains using geno- and phenotypic methods and showed that a new biogroup (known as biogroup 16) could be defined. Further analysis determined that biogroup 1 included 32% of all known strains, whilst genogroup 1 was the largest and most genetically diverse cluster containing 90% of the studied strains and representing biogroups 1-5, 7-9, 11, 13 and 14.

No evidence has been presented to suggest that there may be differences in the virulence capacity of any one genomo- or biogroup. Therefore for the purposes of diagnosis, all 16 biogroups should be considered as potential threats to infant health.

Infection and epidemiology

Infections resulting from *E. sakazakii* are rare. Nevertheless, they are a serious cause of fatal meningitis, necrotising enterocolitis and bacteremia in infants. Interestingly, although *E. sakazakii* infections (including osteomyelitis and bacteremia) have been reported in immunocomprimised elderly patents[19, 20], infants appear to be the group at particular risk with neonates, especially those born prematurely or of low birth weight (< 2,500 g) reported to be in the highest risk category[20, 21]. In a recent risk analysis of 46 clinical cases two groups were identified,

(a) those infants born close to gestation age and of normal birth weight, and who developed meningitis in the neonatal period and

(b) premature infants, who developed bacteremia after one month of age[22]. The differences in the apparent timing of disease onset was likely due to the infant feeding practices wherein, premature infants are normally fed using sterile formula only, whilst full term babies consume prepared PIF. However, this may be an over simplification, since, immuno-compromised infants up to 10 months have developed blood stream infections and formerly healthy infants have developed meningitis after the neonatal period. Furthermore asymptomatic infants have demonstrated stool carriage for up to 18 weeks[9]. Based on current estimates, one in 100,000 births may be infected with *E. sakazakii.*

The clinical outcomes of infants with meningitis compared those with bacteremia differs as the later generally result in a better prognosis (mortality rate of 10%)[22]. Conversely, those with meningitis often develop clinical complications including ventriculitis, seizures, brain abscess, cerebral infarction and cyst formation. Mortality rates of up to 80% have been reported[20] and these infections often associated with significant morbidity. Ninety-four % of meningitis survivors can develop neurological sequalae, including hydrocephalus, quadriplegia, impaired sight and hearing, and retarded neural development. Early intervention is critical, and computed tomography or magnetic resonance imaging is recommended to identify cerebral abnormalities in suspected cases of meningitis.

Origin and transmission of *E. sakazakii*

Enterobacter sakazakii is a ubiquitous organism found in a variety of foods. It has been cultured from a range of foods including; dried baby food, milk powder, cheese, sausage meet, vegetables, dry food ingredients, rice, lettuce, sour tea, bread and herbs and spices and PIF[21, 23, 24]. The bacterium has also been isolated from both environmental and clinical sources. Documented environmental sources include house holds, food factories and PIF manufacturing facilities[23, 25]Mullane in submission]. Microbiological analysis of surface water, rotting wood, soil, grain, bird droppings, domestic animals, livestock and cows milk failed to yield a positive *E. sakazakii* [26]. Clinical sources have provided culture positive samples from cerebrospinal fluid (CSF), blood, bone marrow, septum, urine, inflamed appendix tissue, intestinal and respiratory tracts, eye, ear, wounds, stools and from a physicians stethoscope [Adamson 1981, Farmer 1980, [11, 21].

The existence of a primary reservoir (if any) remains to be determined although it has been postulated that the bacteriums natural habitat may be associated with plant material, based on some of the physiological characteristics of the bacterium, such as its yellow pigment, that can afford a measure of protection against harmful ultra-violet light [24]Mullane, 2006).

In epidemiological studies, PIF has been acknowledged as the vehicle of transmission for *E. sakazakii*. A strong association has been described evident between the consumption of contaminated PIF and infection in infants[8, 14, 17, 27] France 2004]. Prevalence studies in PIF have been

performed[24, 28] FDA, 2003]. Muytjens et al. [28] inspected 141 PIF's from 35 countries and isolated *E. sakazakii* at a frequency of 14.2% representing 13 countries. The organism was recovered from powdered samples at levels ranging from 0.36 through 0.66 CFU in 100 g samples . More recently the WHO reported a prevalence of <1.8% in 6 out of the 12 unrelated studies (WHO, 2006).

The first cases of *E sakazakii* infection linked to the consumption of reconstituted PIF were reported in 1988 and involved four infants [REF]. Since then sporadic cases and outbreaks have been attributed to the consumption of contaminated PIF **(Table 1).** A review of 48 *E. sakazakii* cases since 1961 revealed that at least 25 were directly linked to the consumption of PIF, and PIF was also identified in 85% of these cases as the potential source (WHO, 2004). Similar investigations found *E. sakazakii* in a blender used for the preparation of PIF (Noriega 1990). In 1983, six infants died following the ingestion of contaminated PIF. Importantly, in this case, the organism was cultured from the PIF previously fed to these infants, along with a dish brush used for bottle cleaning and from a spoon used for preparation[13].

An outbreak in Tennessee in 2001 was the first occasion whereby infection was attributed and linked to previously unopened cans belonging to the same batch of powder[27]. In Belgium in 2002, an infant died of *E. sakazakii* meningitis following consumption of contaminated PIF (FDA). A follow up investigation identified low cell numbers of *E. sakazakii* and the implicated

formula subsequently was withdrawn. In New Zealand in 2004, a premature infant died following *E. sakazakii* meningitis, the bacterium was isolated from four asymptomatic infants in the same neonatal intensive ward and PIF was identified as the source of infection. In December 2004, contaminated PIF triggered a worldwide product recall following a possible link to two fatal cases of *E. sakazakii* meningitis in France (Wan, 2004).

More recently------Niall I'll chat to you about this one!

Resistance to antimicrobials

When a case of *E. sakazakii* infection is identified, it is important to treat the infection as quickly as possible. Successful chemotherapeutic intervention requires that the target orgsniasm is susceptible to the antimicrobial agents used. Similar to other members of *Enterobacteriaceae E. sakazakii* is susceptible to a range of antimicrobials of different classes, including: aminoglycosides, anti-folates, chloramphenicol, carbapenems, nitrofurantoin, tetracyclines and ticarcillin [1]. Some *Enterobacteriaceae* can exhibit resistance to benzylpenicillin, clindamycin, glycopeptides, lincosamides, oxacillin, all macrolide antibiotics, rifampicin, and streptogramins[2]. Mullane et al[unpublished] tested XX strains against a panel of 25 antibiotics and found that the majority were highly susceptibile. *Enterobacter sakazakii* have been reported to be capable of inactivating cephalothin, sulfamethoxazole, and third generation cephalosporin antibiotics. Resistance to antibiotics like ampicillin has arisen from the production of β-lactamases and also the ability of many *Enterobacteriaceae* to alter membrane permeability through the

production of membrane porins, thereby reducing the intracellular concentration of the drugs [3]. Multidrug treatments should be taken into consideration for treatment of infections, while newer antibiotics such as trimethoprim-sulfamethoxazole have been found to be successful [5].

Disinfection, we need a few lines to review the AEM findings in reklation to the efficacy of disinfectants in terms of cells in planktonic state, dried onto food preparation surfaces and in biofilms.

Virulence and pathogenicity

The pathogenisis of *E. sakazakii* meningitis is currently poorly defined. It is thought that infection occurs by translocation of the bacterium through the *cordus plexus* followed by invasion into the previous sterile nutrient rich cerebral matter [Iversen, 2003]. No dose-response relationships have been published but it is probable that disease progression is host dependant requiring a large initial inoculum. *In vitro* studies in suckling mice showed that of the strains tested, all were lethal at 1x 10^8 cfu [Pagotto et *al.*]. The infectious dose in humans is unknown. These authors also examined the production of enterotoxin. Of the 18 strains tested, 4 were found to produce enterotoxin in suckling mice.

A few lines reviewing the cell adhesion work-showing adherence to varions cell lines, including Caco-2 intestinal cells and a cerebral epithelial cell line.

Recent studies indicate that the risk of infection with *E. sakazakii* can increase due to the presence of an endotoxin (bacterial derived lipopolysaccharide) in PIF. This endotoxin is heat stable and survives through the PIF production process. It is postulated that ingestion of the toxin increases the permeability of the intestinal epithelium leading to the translocation of *E. sakazakii* across the epithelium barrier [1].

Another putative virulence factor of *E. sakazakii* that has been suggested, is the production of exopolysaccharide capsular proteins (EPS), which aid in the adherence of cells to a variety of surfaces. Cells have been found attached to stainless steel surfaces in hospital environments and on the utensils being used for the PIF preparation [2]. Biofilm formation by a number of strains has also been reoprted and this is a defence mechanism that promotes resistance to disinfectants and sanitizing agents. Recent studies investigating the efficacy of different a range of chemical-based disinfectants used in the clinical setting have found that all were effective against planktonic cells. When cells were dried onto food preparation surfaces, the efficacy of some was reduced and when the cells were contained as opart of a biofilm, none of these agents were effective. These findings suggest that the choice of sanatising agent and where it is to be applied, may have serious consequences for the dissemination of this organisms in manufacturing, health care and home settings. Chlorine, chlorine dioxide and Tsunami 200 were all found to reduce *E. sakazakii* numbers [3]. Quaternary ammonium-based compounds were the best and these contained the active agent, n-Alkyl dimethyl (60% C14, 30% C16, 5% C12, 5% C18), benzyl ammonium

chlorides and n-Alkyl dimethyl ethybenzyl ammonium chlorides. Environments of high alkalinity can lyse the membrane of Gram-negative bacteria, which results in leakage of the cytoplasm and death of the cell. Quaternary ammonium compound disinfectants could be used on all surfaces and preparation equipment for PIF [4].

Successful adherence to a target cell is likely the first step in the infection pathway. Mange *et al.,* investigated the ability of different *E. sakazakii* strains to colonize two intestinal epithelial cell lines, Hep-2 and Caco-2 and the microvascular endothelial cell line HBMEC. Adherence to these cells was maximal at late exponential phase, with a 10-fold increase reported during this period of growth [Mange et *al.*].

All *Enterobacteriaceae* possess efflux pumps that can extrude a range of xenobiotic compounds from the cell cytoplasm. In *Salmonella*, *Campylobacter* and other enteric organisms, bile acids are a natural substrate for these membrane bound complexes. Active efflux is a recognised virulence mechanism which contributes to the survival of these pathogens in the host gastrointestinal tract. Further, they also contribute to resistance facilitating the bacteriums survival in the presence of sanitizers and disinfectants [4]. Little is known about the involvement of efflux in *E. sakazakii.*

Laboratory identification of *E. sakazakii*

Suitable detection methods are required to reliably isolate and distinguish between *E. sakazakii* and other closely related Enterobacteriaceae that can

be found in PIF (REF). At present there is no internationally validated protocol for the specific detection of *E. sakazakii*. However, selective microbiological culture media has been formulated based on the inclusion of either a chromogenic or flurogenic substrate for an enzyme α-glucosidase[29-33] http://www.aeslaboratoire.com/cgi-bin/go_produits.pl, ISO). Currently an ISO method (DTS 22964) which is being evaluated, incorporates one such selective agar. Initially, a pre-enrichment step is carried out, whereby the PIF is enriched in buffered peptone water, followed by enrichment in modified lauryl sulphate broth (mLST). A 100 ml volume is then surface plated onto a selective agar. Confirmation is performed by **(a)** streaking presumptive colonies to TSA and examining them for their characteristic "yellow pigment" production and **(b)** biochemical confirmation using the API20E or API32E identification galleries(bioMérieux, France. This approach has been adopted and is now being implemented as part of the revised EU microbiological criteria (EC 852/2004) for PIF. However, it should be noted that α-glucosidase negative *E. sakazakii* strains have been identified[32] and there are also other α-glucosidase positive Enterobacteriaceae[24] both of which could result in errors in diagnosis. Furthermore, Iversen at al. (2006) recently reported that 2% of *E. sakazakii* strains surveyed failed to produce a yellow pigment on TSA[34] while others questioned the reliability of API test systems[29, 32].

As an aid to facilitate the confident identification of *E. sakazakii,* several molecular-based methods have been published molecular based. Some of these are based on the detection of conserved regions of the *E. sakazakii*

genome [35-37]Hassan 2006, Malorny, 2005]. One such method reported by Seo and Brackett, 2005 targeted the macromolecular synthesis (MMS) operon and discriminated *E. sakazakii* from 68 *Enterobacter* and 55 non-*Enterobacter* strains tested[37].

Currently the Codex Alimentarius Commission is conducting a risk assessment on the microbiological safety and safe preparation of infant formula. This Commission is reviewing the code of hygienic practice for foods for infants and children, and a microbiological criteria has been set wjich requires the absence of *E. sakzakii* in 10 g of powder has been introduced by the European Commission (EC 2073:2005) (WHO, 2004&2006, http://www.fsai.ie/legislation/food/eu_docs/Food_hygiene/Reg2073_2005.pdf L338/11). These microbiological criteria provide product specification guidance to PIF manufacturers on the acceptability of their product and should form part of good hygienic practice regime during manufacturing, handling and distribution processes.

Molecular surveillance of *E. sakazakii*

Molecular typing methods have long been used to fingerprint clinical Enterobacteriaceae isolates associated with nosocomial infections. Application of these methods aided our understanding of bacterial epidemiology, and are regarded as useful tools to monitor food borne disease. The basis of these sub-typing methods relies on the fact that identical isolates share highly similar or indistinguishable DNA profiles or fingerprints, to link or rule out particular strains in outbreak investigations.

Pulsed-field gel electrophoresis (PFGE) has long been recognized as a gold standard in molecular sub-typing. PFGE has been used to investogate the relationship among recovered *E. sakazakii* strains isolated from formula to those cultured from infected infants[9] (**and smeets 1998**). Other typing protocols, including ribotyping and random amplification of polymorphic DNA (RADP) have also been used to similar effect[38]. Despite the frequent use of PFGE in clinical settings, the food industry has been slower to embrace this approach. Nevertheless, PFGE is now beginning to from an important part of the surveillance plan, wherein it can provide a useful backup to HACCP plans aimed at limiting transmission of spoilage and pathogenic food borne organisms. Add two lines to update your findings.

Food Safety and Public Health Implications of *E. sakazakii*

It is important to understand that PIF is not a microbiologically sterile product. Reconstitution and subsequent handling and strorage must recognise the importance of maintining temperature control, preventing the rapid grpwth of potential contaminating *E. sakazakii*. However, commercially available ready-to-feed liquid infant formula is sterile. PIF (like dried bovine milk and milk products) has been previously documented as a potential source of bacterial pathogens such as *Bacillus* spp*., Clostridium* spp., *Staphylococcus* spp. and Enterobacteriaceae, notably *E. sakazakii,* (Townsend *et al.* 2006; Forsythe, 2005).

The Codex Alimentarius Commission standards recommend that infants who are not breast-fed should be provided with a suitable breast milk substitute

that is properly formulated. PIF has a water activity (a_w) of ca. 0.2 and this carefully prepared product has been formulated so as to mimic the nutritional profile of human milk. Studies have shown that *E. sakazakii* can persist for very long periods of time in a low a_w environment which would normally exist in finished PIF product. Moreover, *E. sakazakii* was reported to be more persistent in PIF than other Enterobacteriaceae including *Salmonella* and *E. coli* (Caubilla-Barron *et al.* 2004), highlighting the fact that *E. sakazakii* is more adapted to drying stress than other genera in this family. While the frequency of intrinsic *E. sakazakii* in powdered milk is of concern, the intrinsic concentration of *E. sakazakii* in PIF typically appears to be low. It is for this reason that the prevention of possible proliferation of this pathogen in *situ* in PIF is critical. It is now recognised that PIF is epidemiologically linked to neonatal infections (Bar-Oz *et al.* 2001; Nazarowec-White and Farber, 1999; Nazarowec-White and Farber, 1997). Guidelines have been developed by FAO/WHO aimed at health care givers and those in the home, alerting them to the proper protocols for reconstututing PIF . These guidelines highlight the importance of rapid cooling and feeding of reconstituted PIF where possible. It actively discourages prolonged storage and rewarming of reconstituted formula. This approach is supported by scientific evidence which clearly shows that improper storage of contaminated reconstitued PIF (ie: temperature abuse), supports the rapid growth of *E. sakazakii*. Hygenic preparation measures therefore remain a significant critical control point in the prevention of infection of *E. sakazakii* from reconstitution PIF. A summary of these general guidelines for the reconstitution of PIF in hospitals and homes is given in Table 2.

E. sakazakii remains an issue of global concern to the infant formula food industry. Manufacturers are being encouraged to produce a greater range of commercially sterile formula products (particularly for high risks groups). Efforts are also being made, to reduce the concentration and prevalence of *E. sakazakii* in the manufacturing environment. This is being done through in-house environmental monitoring systems and through research programmes. The intial control of bacterial populations by the infant food industry and the prevention of any potential post process contamination during the production of PIF, should have a positive effect on the control of *E. sakazakii* in the food industry.

Conclusions

Enterobacter sakazakii is a clinically significant pathogen of public health concern to neonates and infants. Its significance has been demonstrated by its addition to the list of notifiable diseases in New Zealand (New Zealand Ministry of Health, 2005). While the role of the broader food chain as a possible source of contamination of this pathogen remains unknown, preventative measures by carers, neonatal units and the infant food industry will remain of prominent importance in the prevention of *E. sakazakii* related infections in the near future.

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