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3 **_Cronobacter_ species as emerging causes of healthcare-associated infection**
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Summary

Background

Until recently members of the *Cronobacter* genus (formerly known as *Enterobacter sakazakii*) were a relatively unknown cause of hospital infections. However their association with infant infections and in particular through the consumption of contaminated reconstituted infant formula in neonatal intensive care units, has resulted in international efforts to improve neonatal healthcare.

Aim

This review considers the current status of our understanding of this emergent group of bacterial pathogens and the steps taken to reduce neonatal infection.

Methods

A literature review was undertaken to collate our current knowledge of the *Cronobacter* genus, with respect to recent taxonomic revisions, sources and clinical relevance.

Findings

The majority of severe neonatal meningitis infections are associated with only one of the ten *Cronobacter* species, and in particular the clonal complex known as *C. sakazakii* ST4. International efforts by FAO-WHO to reduce the risk of neonatal infection by this organism have resulted in improved microbiological safety of powdered infant formula (PIF) and revised guidelines for feeding practices have been problematic. However the majority of infections are in the adult population, the sources of which are unknown.

Conclusion

International improvements in the microbiological safety of PIF and advice on feeding practices have been directed towards improving neonatal healthcare following the heightened awareness of *Cronobacter* infections in this particular age group. While these are likely to also reduce neonatal exposure to other opportunistic bacterial pathogens, nevertheless a number of unresolved issues remain with respect to the practicalities of feeding premature neonates safety while following WHO advice.

1 **General Introduction**
2 This review concerns the bacterial genus *Cronobacter* which can cause severe illness in the highly
3 vulnerable neonates, infants and the elderly. The organism has come to prominence due to its
4 association with severe though rare neonatal infections leading to necrotizing enterocolitis,
5 septicaemia and meningitis, which can be fatal. As neonates are frequently fed reconstituted
6 powdered infant formula (PIF), which is not a sterile product, this potential vector has been the focus
7 of attention for reducing infection risk to neonates as the number of exposure routes is limited.
8 Meanwhile, the majority of *Cronobacter* infections occur in the adult population but are less severe.
9 Cases of *Cronobacter* infection in all age groups are probably under-reported for a number of reasons
10 such as misidentification as *Enterobacter cloacae*.[1] Fortunately our understanding of *Cronobacter* has
11 grown considerably in recent years. In part this is due to new developments and applications in
12 clinical microbiology of Next Generation Sequencing methods, which have led to rapid improvements
13 in our understanding of this organism; changing our perspective of the former *Enterobacter sakazakii*
14 species into a genus composed of 10 species, with high clonality and host-adaption, along with
15 improved methods of identification and typing. This review aims to bring together our current
16 knowledge on the clinical aspects of *Cronobacter*, and consider unresolved issues concerning the
17 hygienic preparation of powdered infant formula.
18
19 ***Cronobacter* taxonomy and phylogeny:**
20 The bacterial genus *Cronobacter* was formerly known as *Enterobacter sakazakii*, and was first defined
21 as a new genus in 2007.[2] It is a member of the *Enterobacteriaceae* family and is closely related to the
22 *Enterobacter* and *Citrobacter* genera. In recent years the *Cronobacter* genus has undergone a
23 number of revisions and currently contains 10 species.[3,4] The formally recognised species are *C.*
24 *sakazakii*, *C. malonaticus*, *C. universalis*, *C. turicensis*, *C. muytjensii*, *C. dublinensis, C. condimenti, C.*
25 *helveticus, C. pulveris* and *C. zurichensis*; Figure 1. These include the former bacterial species
26 *Enterobacter sakazakii, E. helveticus, E. pulveris* and *E. turicensis*. Subsequently it is uncertain which
27 specific *Cronobacter* species were referred to in many pre-2007 publications. The species can be
28 grouped, with the mostly clinically relevant being Group1: *C. sakazakii* and *C. malonaticus* which form
29 the majority of clinical isolates, and Group 2: *C. turicensis* and *C. universalis* which have been less
30 frequently reported. The close relatedness of these species is shown in Figure 1. The other species
31 are primarily environmental commensals and are probably of little clinical significance. According to
32 phylogenetic analysis these major divisions formed about 41 million years ago and further host-
33 adaptation has occurred as will be considered later with the specific case of *C. sakazakii* sequence
34 type 4.[5]
35
36 **Cronobacter physiology**
37 *Cronobacter* spp. can grow over a wide temperature range. The lowest being near refrigeration
38 temperatures (~5°C), the optimal ~37-39°C, with the maximum growth temperature is 44-47°C.[6] The
39 organism's tolerance to desiccation is well recognised. It can survive for two years desiccated in
40 infant formula and then rapidly grow on reconstitution.[7] The organism often produces a capsule which

can be so copious that on milk agar plates the colonies drip onto the lid of inverted Petri dishes.[8] *Cronobacter* spp. are able to adhere to silicon, latex and polycarbonate, stainless steel, glass and polyvinyl chloride.[6,9] These materials are commonly used for infant-feeding and food preparation equipment and, if contaminated, may increase the risk of infection. This capsular material may facilitate the organism forming biofilms that are resistant to cleaning and disinfectant agents.[10] The organism has been isolated as part of the mixed flora biofilm in enteral feeding tubes of neonates.[11]

Virulence traits

The *Cronobacter* are opportunistic pathogens though few virulence factors have been identified to date. Some strains can invade human intestinal cells, replicate in macrophages, and invade the blood brain barrier.[12] Based on the analysis of archived strains and clinical outcome of the 1994 outbreak in France, it was proposed that certain strains of *C. sakazakii* were more virulent, and this has been confirmed by multilocus sequence typing (MLST).[13,14] The route of infection is probably through attachment and invasion of the intestinal cells, and therefore genes encoding surface appendages such as pili (fimbriae) have been studied. A number of fimbriae clusters were identified in the genomes of *Cronobacter* species.[1,15-17] Many are common to all species, though there are some interesting variations. *C. sakazakii* is the only *Cronobacter* species encoding for β-fimbriae, whereas the genomes of the other species encode for curli fimbriae.[1,16] This may reflect evolution to the host ecosystem.

Since *Cronobacter* is associated with neonates and infants, the utilization of iron from breast milk and formula could be an important virulence trait. A number of iron assimilation mechanisms have been found in *Cronobacter* species.[1,15-18] Type VI secretion system (T6SS) is a newly described system that may be involved in adherence, cytotoxicity, host-cell invasion, growth inside macrophages and survival within the host. Five putative T6SS clusters have been identified *Cronobacter* spp. genomes.[1,17-19] It remains to be determined whether they encode functional type VI secretion systems. *Cronobacter* produce an enterotoxin, and as with neonatal meningitic *E. coli*, the outer membrane proteins ompA and ompX possibly have roles in the organism penetrating the blood brain barrier. The mechanism(s) leading to the destruction of the brain cells in unknown and could in part be a host response. The organism also encodes for a number of haemolysins.[1,19]

C. sakazakii is unique in the *Cronobacter* genus in its utilization of exogenous sialic acid, and this may have clinical significance. The ability to utilise sialic acid could be a major evolutionary host-adaptation since the compound is found in breast milk, mucin and gangliosides.[20] Sialic acid is also an ingredient in powdered infant formula due to its association with brain development. *C. sakazakii* is also able to grow on the ganglioside GM1 as a sole carbon source.[20]

High levels of heat-stable lipopolysaccharide (LPS, also known as endotoxin) in infant formula enhances the translocation of *Cronobacter* across both the intestines and the blood–brain barrier, and therefore increase the risk of bacteraemia in neonates.[23,24,25] Kim and Loessner speculated that frequent LPS contamination of PIF (known to disrupt tight junctions) might contribute to the invasiveness of *Cronobacter* across the blood-brain barrier.[24] It is known that the levels of LPS in PIF

vary 500-fold.[25] In addition, the oligopolysaccharide component of the LPS layer can serve as a basis for serotyping and other characterisation methods.[21,22]

Cronobacter spp. tend to be more sensitive to most antibiotics compared to other *Enterobacteriaceae,* though resistance to ampicillin has developed. In 1980, all strains tested were susceptible to ampicillin, whereas in 2001 Lai described five cases of *Cronobacter* infection in which one or more of the isolates were resistant to ampicillin and most cephalosporins of 1st and 2nd generation.[26,27] In 2001, Lai reported increasing β-lactamase production among *Cronobacter* strains.[27] Similarly, Block and colleagues reported that all *Cronobacter* isolates tested were β-lactamase positive.[28] Caubilla-Barron et al. in a retrospective study of a NICU outbreak in 1994, reported two neonatal deaths from ESBL-encoding *C. sakazakii* strains.[8] It is of interest that indistinguishable pulsetype strains were non-ESBL, indicating the possible acquisition of the ESBL genes from the individual neonatal intestinal flora during the infection period.

Non-human sources of *Cronobacter* spp.

Cronobacter spp. has been isolated from a range of foods including cheese, meats, milk powder, powdered infant formula, weaning foods, and a large portion of food ingredients; Table 1.[29,30] Although the bacterium is isolated from many foods, no foodborne infections have not been reported. A productive source of *Cronobacter* strains are fresh or dried herbs and spices with ~30% incidence[29]. Rats, flies and cockroaches may be additional sources of contamination.[31,32,33]

The bacterium has been isolated from the home environment; household dust, vacuum cleaning bags and also from utensils used for the reconstitution of powdered infant formula; Table 1.[34] Hence contamination of reconstituted infant formula can be intrinsic or extrinsic in origin. The bacterium has also been isolated hospital environments, as well as various areas in milk powder and PIF processing plants; roof, shoes, and roller driers.[35,36,37,38]

The organism has been recovered from previously unopened tins of powdered infant formula indicating intrinsic contamination; Table 1.[39,40,41] Accurate enumeration is difficult but is generally in the order of <1 bacterial cells/100g. The intrinsic prevalence of *Cronobacter* in powdered infant formula has been determined a number of times and varies between 2-14%, with no published reports exceeding 1 cell/g. Hence the consideration of opportunities for extrinsic bacterial contamination, and multiplication, especially temperature abuse following reconstitution as it would allow the bacterium to multiply and increase the risk of infection. Currently microbiological criteria for *Cronobacter* spp. are required for infant formulas with an intended target age <6 months.[42] A presence/absence test is applied to large volumes due to the low incidence of the organism in the product. Although the organism has been recovered from follow up formulas (infant formulas with intended target age >6 months) and weaning foods, there is currently insufficient epidemiological evidence to support the implementation of criteria for these products.

A limited number of studies have shown that *Cronobacter* is waterborne.[3,43,44] This important issue has not received as much attention as bacterial contamination of PIF. As will be considered later, since powdered infant formula is not a sterile product one method of reducing neonatal exposure to *Cronobacter* has been the recommendation to reconstitute with water >70°C to kill any vegetative

bacteria present.[39,40,45] This advice has not been adopted by all countries. It should be noted that detailed microbiological examination of the USA December 2011 infant infection cases revealed the presence of *C. sakazakii* in the PIF reconstitution water which was a close match (differed by 35/3036 nucleotides of the MLST alleles) to that recovered from the cerebral spinal fluid of the associated infant with meningitis.[14]

Human and clinical sources of *Cronobacter* spp.

Asymptomatic human carriage of *Cronobacter* spp. has been reported in a few studies with recovery from mouth, skin and faeces; Table 1.

Cronobacter spp. have been isolated from various hospital environmental and clinical samples; cerebrospinal fluid, blood, bone marrow, sputum, urine, inflamed appendix, breast abscess, and conjunctivae.[3,5,8,13,16,27,46,47] Nazarowec-White and Farber studying three isolates obtained from one hospital over 11 years showed that they were indistinguishable.[48] Smeets *et al.* showed that isolates from a contaminated dish brush used for cleaning bottles in a hospital and the isolates from three patients were identical making an epidemiological connection likely.[49] The organism has also been isolated from a doctor’s stethoscope and from nursery food preparation equipment such as spoons and a blender.[26,50,51,52] The organism has been found as part of the mixed flora biofilm in enteral feeding tubes of neonates not fed PIF.[11] Related to this, laboratory studies have shown that one contaminated feed passing through the feeding tube would subsequently contaminate further feeds due to bacterial attachment to the inner tube wall and multiplication.[53]

Isolation, identification and typing methods

As the organism has only been reported at low numbers (<1cfu/g) in PIF, a large volume of material needs to be tested in microbiological analysis. Therefore presence/absence testing of bacteria in PIF is applied rather than direct enumeration. This includes the use of chromogenic agars, along with DNA-based identification and fingerprinting techniques. Although it is generally possible to differentiate *Cronobacter* species by biochemical profiling, molecular methods are increasingly used as a more rapid and reliable tool to study bacterial genomic diversity and to track sources of infection. Since the organism is ubiquitous, typing schemes are required both for epidemiological and environmental investigation. For epidemiological analysis (ie. tracing source and dissemination during an outbreak), PFGE with two restriction enzymes (*Xba*1 and *Spe*1) is the most common method.[8] The technique is widely employed and can be used for transnational investigations, as per PulseNet, since the gel results can be electronically analyzed (http://www.cdc.gov/pulsenet/). The method is limited however as not all strains can be typed, non-identical strains can give the same PFGE profile and the method does not give the relationship between strains.[35,54]

On a larger scale, multilocus sequence typing (MLST) is increasingly being applied to understand the evolution and diversity of bacterial pathogens, for example *E. coli* ST131, MRSA-15 ST22 and *Klebsiella pneumoniae* ST258.[55] The method defines sequence types (ST) based on 7 allelic profiles and clonal complexes based on relatedness of the sequence types (1-3 loci differences). The MLST scheme for *Cronobacter* has been established and is available online with approximately 600 strains

profiles (www.pubMLST.org/cronobacter/).[5,56,57] The site also includes open access for the further analysis of all published *Cronobacter* genome sequences using the 'Bacterial Isolate Genomes Sequence Database' (BIGSdb) facility.[58] The web site contains the MLST protocols, as well as >200 DNA sequence defined profiles for strains which have been collected from various sources and countries over a 60 year period. The *Cronobacter* MLST analysis is based on 7 housekeeping genes; *atp*D, *fus*A, *gln*S, *glt*B, *gyr*B, *inf*B and *pps*A. The 7 sequenced alleles can be concatenated together to give 3036 nucleotide sequence for phylogenetic analysis (Fig 1). This analysis has revealed a remarkably strong clonal nature in the *Cronobacter* genus.[5,57] These clones may reflect different ecologies of the organism. The clonal complex *C. sakazakii* ST4 is a DNA sequence defined evolutionary lineage for the causative agent of neonatal meningitis among the *Cronobacter* isolates.[5,13,14,57] This remarkable discovery gives a clear direction for further meningitis research with the bacterium. *Neisseria meningitidis* also shows clonality of meningitis infection. To date there does not appear to be such a clear link between sequence type and other *Cronobacter* infections, such as necrotizing enterocolitis.

It should be noted that 16S rDNA sequence analysis of archived strains has revealed that the use of phenotyping in early studies led to a number of mis-identifications in the literature.[16] These include:

1. An independent fatal case of neonatal sepsis due to *E. cloacae* during a *C. sakazakii* outbreak in a neonatal intensive care unit.[8]
2. Neonatal intensive care unit outbreak attributed to *E. sakazakii*, reidentified as *E. hormaechei*.[59]
3. A reported quinolone-resistant *E. sakazakii* strain, reidentified as *E. hormaechei.*[60]
4. *E. sakazakii* strain used for oligo-polysaccharide structure determination, re-identified as *E. ludwiggi*.[61]

Such mis-identifications are likely to continue given that the database for a commonly used phenotyping based method does not recognised the 2007 taxonomic revision of *Cronobacter* and continues to use the old *E. sakazakii* nomenclature, and three of the most recently defined *Cronobacter* species (*C. helveticus*, *C. pulveris* and *C. zurichensis*) are not recognised even as *E. sakazakii.*

Cronobacter spp. infections

A number of *Cronobacter* infection incidents have been reported as outbreaks.[8,39,40] In the USA, the reported *Cronobacter* infection incidence rate is 1 per 100 000 infants. This incidence rate increases to 9.4 per 100 000 in infants of very low birth weight, i.e. <1.5 kg.[40] Fatal *Cronobacter* infections of infants have followed cases of necrotising enterocolitis (NEC), septicaemia and meningitis.[62,63] Infections in older age groups are principally bacteraemia as well as urosepsis and wound infections. Infants can be colonized by more than one strain of *Cronobacter*, and therefore multiple isolates need to be characterized in epidemiological investigations.[8]

NEC is non-invasive (as well as multifactorial), whereas in septicaemia and meningitis the organism has attached and invaded presumably through the intestinal epithelial layer. NEC is a common gastrointestinal illness in neonates and can be caused by a variety of bacterial pathogens. It

is characterized by ischaemia, bacterial colonisation of the intestinal tract, and increased levels of proteins in the gastrointestinal lumen. The incidence of NEC is 2-5% of premature infants and 13% in those weighing <1.5kg at birth. It is 10 times more common in infants fed formula compared with those fed breast milk. NEC has a high mortality rate; 15-25% of cases. *Cronobacter* has been implicated as a causative agent of NEC, but its role in the pathogenesis of the disease is somewhat unclear. There are reports of *Cronobacter* isolation from babies who developed NEC and these strains were indistinguishable by PFGE from those isolated from meningitis cases.[8] This suggests that there is an association between *Cronobacter* occurrence and NEC, although until recently, the organism has not been conclusively proven to cause the disease.

Cronobacter-related meningitis is characterized by a mortality rate of 40-80 % and generally a very poor clinical outcome. The bacterium causes cystic changes, abscesses, fluid collection, brain infarctions, hydrocephalus, necrosis of brain tissue and liquefaction of white cerebral matter. This pathogenesis is different to that caused by both *Neisseria meningitidis* and neonatal meningitic *E. coli*. Some reports suggest a similarity between the tropism of *Cronobacter* and the closely related organism *Citrobacter koseri* for invasion and infection of the central nervous system. Patients surviving *Cronobacter*-related meningitis often suffer from severe neurological sequelae, such as hydrocephalus, quadriplegia and retarded neural development.[16,62,63] The infection usually arises between the fourth and fifth day after birth and it can be fatal within hours to days following the first clinical signs. Compared with patients suffering from *Cronobacter*-induced enterocolitis, infants in whom meningitis developed tend to have normal gestational age and birth weight.

In December 2011, there was considerable publicity concerning neonatal *Cronobacter* infections in the United States.[64] All but one isolate from the meningitic cases were in the *C. sakazakii* ST4 clonal complex.[14]

Infections caused by *Cronobacter* in adults comprise a wide range of symptoms from conjunctivitis, biliary sepsis, urosepsis and appendicitis to wound infection and pneumonia. Adult patients at increased risk include those previously treated with antibiotics, immuno-compromised and elderly patients, those with medical implants or with acute, chronic, or serious illnesses. *C. sakazakii* can cause urinary tract infections, though to date this aspect has not been studied in any detail. The only published age-profiled data is for 819 *Cronobacter* spp. bacteraemia cases reported for England and Wales between 1992 and 2007.[41] In this report, the majority (91%) of bacteraemia cases were patients >15 years in age.

Sources of infection

While the source of contamination in *Cronobacter*-related outbreaks has not always been confirmed, breast milk substitutes (one group of PIF products) have been epidemiologically or microbiologically established as the source of infection in a number of cases.[8,39,40,41] Although an outbreak in a NICU in Tennessee in 2001 is often cited as a strong link between the presence of *Cronobacter* in powdered infant formula and *Cronobacter* infection, it is overlooked that the formula fed to the infant in Tennessee was in fact a non-infant formula and was not intended to be consumed by neonates.[65]

As already covered, the *C. sakazakii* clonal complex ST4 is strongly associated with cases of meningitis and it is notable that this clonal complex has been reported to be frequently isolated from milk powder factories, powdered infant formula (PIF) processing plants and from PIF in Ireland, Switzerland, Germany and Australia.[36,37,38] Sonbol et al. reported that 24% of strains isolates from the environment of 6 milk powder manufacturing plants in Australia and Germany were *C. sakazakii* ST4.[38]

Infections which have been directly linked to reconstituted PIF may have been the result of intrinsic or extrinsic contamination during preparation and administration. A common feature in some of these outbreaks is the opportunity for temperature abuse of the prepared feed, which would permit bacterial growth. In reported outbreaks in France and USA, the neonates were fed using perfusion devices where the reconstituted PIF is slowly pumped over several hours at ambient temperature into the neonate's stomach through an enteral feeding tube.[8,65] Using this procedure there is the possibility of bacterial multiplication in the syringe leading to the ingestion of large numbers of *Cronobacter* by the neonate.

The neonate has an immature immune system and a low intestinal microflora density. Consequently, if a large number of *Cronobacter* cells were ingested they would not be outcompeted by the resident intestinal flora. Following invasion of the intestinal cells, the lack of a developed immune system could make the neonate more prone to systemic infection. No infectious dose has been determined for neonates. Animal studies by Pagotto and others have used large numbers of *Cronobacter* cells ($\sim 10^8$) for infection studies.[66] Whether this number is reflective of that necessary for neonate infection is uncertain, but it does contrast with the number of cells reported in contaminated PIF (<1 cfu/g), and may therefore indicate the significant role of temperature abuse in enabling bacterial multiplication.

It is pertinent to note that *Cronobacter sakazakii* has been isolated from the tracheae, and sputum as well as from the feeding tubes of neonates fed breast milk and ready-to-feed formula, not infant formula; Table 1.[8,11,67] Therefore wider sources of the organism during an outbreak need to be investigated, not just the use of PIF. The 1994 NICU outbreak in France showed that infants can be colonized by more than one strain of *Cronobacter,* and therefore multiple isolates need to be genotyped in epidemiological investigations to increase the chance of tracing probable source.[8]

Bowen and Bradden reported that there are a number of neonatal cases which have no links with the ingestion of reconstituted infant formula.[63] Pertinent to this, is the recognition of bacterial colonisation of the nasogastric enteral feeding tube, and the isolation of *C. sakazakii* from tubes from neonates feed breast milk, and (sterile) ready to feed formula. Breast milk can contain the bacterium, and the *C. malonaticus* type strain (LMG 23826^{T}) was isolated from a breast abscess. In some countries breast milk from mothers with mastitis is still fed to the neonate. Breast milk has been a suspect source in two meningitis cases.[68,69] *Cronobacter* infections of babies with breast milk as sole source of feed have been reported in US and Israel; Block *pers. comm.*[28,70]

Cronobacter species have also been isolated from hospital air, dust, human intestines and throats. Hence control of microbiological content of PIF will not necessarily totally remove the risk of neonate infection by this bacterium.

Consequences and current issues.

To date the raised awareness of the organism has focussed on infant infections and resulted in changes in the microbiological criteria for PIF and reconstitution procedures.[39,40,41,42] Those identified as being at high risk of *Cronobacter* infection are neonates (especially low birth weight) for whom their source of nutrition will be limited to breast milk, fortified breast milk, or breast milk replacement. Hygienic preparation of feed is essential due to their immature immune system and lack of competing intestinal flora. Key advice from these FAO-WHO risk assessments was that PIF should be reconstituted with water >70°C, minimise any storage time by not preparing in advance and if storage for short periods is necessary then the temperature should be <5°C. The high water temperature will drastically reduce the number of vegetative bacteria present, and minimising the storage period will reduce the multiplication of any surviving organisms. These recommendations have been well addressed by the WHO 'Guidelines for the safe preparation, storage and handling of powdered infant formula'.[42] However this has subsequently caused a number of additional issues for weaning foods, follow-on formulas, fortified breast milk, and probiotic-supplemented PIFs as consider below.

As referred to above, the WHO guidelines for hygienic preparation of PIF are aimed at reducing the number of bacteria in the reconstituted product by using hot water and limiting the time available for any survivors to multiply.[42] However a wider perspective is that neonates are frequently feed via enteral feeding tubes. These tubes are in place for prolonged periods (even several days) to reduce distress to the neonate by the gagging reaction. However *Cronobacter*, and other opportunistic pathogens can attach and colonise these tubes which are at 37°C, and at regular intervals receive fresh feed.[11] This scenario is applicable to all neonates with nasogastric tubes, and not only those on reconstituted PIF. In fact *Cronobacter* and other *Enterobacteriaceae* have been isolated from such tubes in intensive care units from neonates receiving breast milk and various other feeding regimes at levels up to 10^7 cfu per tube.[11] Therefore hygienic practices and avoidance of temperature abuse are vitally important regardless of the type of feed.

So where are we now?

1. Ironically despite the international changes, the original Tennessee outbreak which precipitated the FAO-WHO risk assessments and WHO preparation of PIF guidelines could still occur. Why? Because the outbreak was due to the use of formula that was not intended for consumption by infants, therefore the product is currently not subject to the revised microbiological criteria.
2. The FAO-WHO recommended reconstitution of powdered infant formula with water >70°C is not followed in all countries such as the US, although it is supported by the CDC. Dipping a thermometer into reconstituted formula would have its own inherent problems of contamination and so the advice has been to use water which has been boiled in a kettle and left to cool for 30 minutes. Aside from the variation in cooling curves according to volume of water and type of kettle, this is impractical for premature babies who require only small volumes of formula, and are fed at 2 hourly intervals. This practice may result in staff being

taken away from bedside care to oversee feed preparation. The term 'powdered infant formula' includes 'breast milk fortifiers'. These products are added to supplement the nutritional value of mother's milk. They are not reconstituted with water and cannot receive the heat-treatment to kill intrinsic bacteria. The use of high-temperature for reconstitution precludes the inclusion of probiotic bacterial cultures (such as *Lactobacillus fermentum* and *L. reuteri*) in PIFs, as marketed in some countries.

3. Most *Cronobacter* infections are in adults, possibly primarily due *C. malonaticus*.[5,16] The source of infection maybe through ingestion as the organism is ubiquitous in food, however it is also plausible that it is nasopharyngeal (like *Neisseria meningitis*) which could explain the cases of pneumonia and isolation from sputum.
4. The source of *C. sakazakii* ST4 is of considerable interest since controlling this lineage could reduce neonatal exposure to severe, life-threatening infections. An informed assessment of neonatal exposure warrants further investigation for the prevalence of *Cronobacter* spp., especially ST4, in hospitals, PIF and other sources such as human carriage.[47]

Late onset Gram negative bacterial sepsis remains a significant cause of neonatal morbidity/mortality and infections on NICUs are predominantly due to *Enterobacteriaceae*, whereas non-fermenting bacteria (ie. *Pseudomonas* spp.) predominate in other ICU outbreaks.[71,72] A recent UK neonatal unit outbreak has lead to development of specific national guidelines (under consultation) on the prevention and management of Gram negative sepsis in neonates.[73] The incidence of sepsis in premature babies in England is 8/100 live births, and 71/1000 neonatal admissions.[74] It should be noted that *Cronobacter* spp. are not the only *Enterobacteriaceae* isolated from PIF, and that the FAO-WHO recommended that research should be undertaken into the other *Enterobacteriaceae* and *Acinetobacter* spp. in PIF.[39,40] These organisms were termed '*Category B; plausible causing infections, but without supporting epidemiological evidence'* by the expert committees, whereas *Cronobacter* spp. and *Salmonella* serovars were '*Category A: Clear evidence of causality'*. A wide range of *Enterobacteriaceae* can be present in PIF, and are the same species as occur in neonatal infections. However cases linking isolates from infants and PIF have not be substantiated.[8,59,75]

Therefore, despite considerable improvement in our understanding of the emergent bacterial pathogen now known as *Cronobacter*, there remains a number of practical issues concerning the hygiene feeding of neonates, and the possible significance in adult infections which are not studied to date.

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