Screening for *Cronobacter* species in powdered and reconstituted infant formulas and from equipment used in formula preparation in maternity hospitals

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Abstract

Background/Aims: *Cronobacter* spp. have been identified as ~~being~~ of considerable risk to neonates. The occurrence of organism in infant formulas is therefore of considerable interest. ***Methods:*** The occurrence of *Cronobacter* spp. in infant feeds (formulas and fortified cow's milk) was determined using most probable number (MPN) analysis, and from formula preparation utensils. Ninety nine samples were analyzed, of which 42 were unopened cans of powdered infant formula (PIF), 25 reconstituted infant formulas in feeding bottles, 27 utensils used from the preparation of infant formula, and 5 samples of fortified cow's milk. Presumptive *Cronobacter* spp. isolates were identified using the 7 allele multilocus sequence typing (MLST) scheme**.** ***Results:*** *C. sakazakii, C. malonaticus* and *C. muytjensii* were recovered from PIF. Although the incidence of *Cronobacter* in PIF was 29% (12/42), the level was low with an average of 0.54 MPN/100g. According to MLST profiling, *C. sakazakii* was the most frequently isolated *Cronobacter* species, and *C. sakazakii* ST4 (associated with neonatal meningitis) was recovered from 2/42 PIF samples at 0.51 and 0.92 MPN/100g. ***Conclusions:*** *Cronobacter* spp. can be isolated from PIF and therefore strict hygienic practices during PIF preparation are important to minimize neonate exposure and reduce the risk of severe infections.

Introduction

Cronobacter spp. are Gram-negative bacterial pathogens that cause meningitis, septicaemia and necrotizing enterocolitis in newborn babies and infants [1]. Such infections have a high fatality rate of 40 to 80%, and survivors often suffer from severe neurological disorders [2]. The *Cronobacter* genus consists of seven species: *C. sakazakii*, *C. malonaticus*, *C. muytjensii*, *C. turicensis*, *C. dublinensis*, *C. universalis*, and *C. condimenti* [3,4]. *Cronobacter* spp., especially *C. sakazakii*, have been implicated in several outbreaks and sporadic cases of diseases involving mainly neonates [5,6,7]. This may be related to sialic acid metabolism which is only encoded on the *C. sakazakii* genome and none of the other six *Cronobacter* species [8]. This compound is found in breast milk, intestinal mucin, gangliosides and is added to powdered infant formula [9,10].

A MLST scheme has been established for the *Cronobacter* genus which is an open access database resource (www.pubMLST.org/cronobacter) hosted by the University of Oxford, UK. It is based on seven housekeeping genes; *atpD*, *fusA*, *glnS*, *gltB*, *gyrB*, *infB* and *ppsA* [11,12]. The total concatenated length of the 7 loci is 3036 nucleotides. Currently 136 sequence types (STs) have been identified in the *Cronobacter* genus of which 73 sequence types are in *C. sakazakii*. Genotyping of *Cronobacter* isolates using MLST has revealed that the majority of serious meningitis clinical cases in neonates over the past 30 years were caused by a single clonal lineage, clonal complex (CC4) of *C. sakazakii* and especially sequence type *C. sakazakii* ST4 [4,13]. The much publicized *Cronobacter* cases in the US in 2011 were also *C. sakazakii* CC4 [6,7]. The reason for the predominance is currently unclear, though recently it has been reported that a third of *Cronobacter* isolates recovered from milk powder processing factories are the *C. sakazakii* ST4 meningitic lineage [14].

Cases of neonatal *Cronobacter* infections can provoke strong public concern and despite thorough investigation their source cannot always be identified [6,15]. In several outbreaks PIF may have been the source of *Cronobacter* infection [16,17,18]. These food products are not commercially sterile products and even low contamination levels by *Cronobacter* spp. are considered a significant risk factor as the organism grows rapidly on reconstitution [19,20,21]. Nevertheless the organism has not been recovered from unopened cans of PIF at levels >1 cfu/g and therefore

hygienic practices and temperature abuse could considerably increase the risk of infection.

The source of *C. sakazakii* ST4 is therefore of considerable interest since controlling this lineage could reduce neonatal exposure to severe, life-threatening infections. Although *C. sakazakii* ST4 has been reported in PIF [11], due to the common practice of presence/absence testing, it has never been enumerated. *Cronobacter* is ubiquitous in the environment and therefore PIF is not the sole route of exposure or infection [1,22,23]. It has been isolated from the nasogastric feeding tubes of neonates not exposed to infant formula [24]. Therefore an informed assessment of neonatal exposure warrants further investigation for the prevalence of *Cronobacter* spp., especially CC4, in PIF and other sources. Previous studies of hospital practices following *Cronobacter* outbreaks have shown that equipment used for formula reconstitution and feeding practices can be significant risk factors [25,26,27,28].

Current *Cronobacter* detection methods use a pre-enrichment step in their initial isolation and therefore the organism is not enumerated in the sample. Given the importance of controlling neonatal exposure to the bacterium, this study used the most probable number (MPN) approach to enumerate the organism. In order to obtain a greater perspective on the routes of exposure to the bacterium in the hospital environment, the study included fortified cow's milk in infant feeding bottles and formula preparation equipment collected from three hospitals. This study has incorporated the recent taxonomic revisions to the *Cronobacter* genus, and the establishment of MLST for *Cronobacter* speciation and genotyping [4].

Materials and methods

Sample collection

Prepared infant feeds were obtained from four maternity hospitals. PIF samples were those commercially available in the city of Campinas (San Paulo, Brazil). In total, 99 samples were tested. This consisted of 14 PIF samples for premature or underweight newborn infants, 15 PIF for target age 0 to 6 months, 7 follow on formulas (target age 6 months to 1 year) and 6 PIF for nursing infants up to 1 year. The non-PIF samples from four hospitals were reconstituted infant formula in feeding bottles (n=25), bottles containing thickened cow's milk (n=5), used feeding bottles (n=7), bottle brushes (n=5), dosing cup (n=3), bottle storage (n=4) and blenders (n=8).

Isolation of **Cronobacter** *and Enterobacteriaceae*

Five hundred grams of each powdered formula, and 200 ml of each reconstituted infant formula and thickened cow's milk were analysed in 100g and 40 ml volumes appropriately. Samples were pre-enriched overnight at 37°C in buffered peptone water, before enrichment in modified lysine tryptose broth (with vancomycin (mLST-V). Preparation equipment was swabbed and used to inoculate 5ml mLST-V. Samples were analysed according to the BAX®-PCR System (DuPont Qualicon) included an additional cultivation in BHI broth at 37ºC for three hours before genotyping.

Identification of **Cronobacter** *isolates.*

Presumptive *Cronobacter* isolates were subcultured on TSA (25°C, 48-72h) before phenotypic identification using API20E and ID32E (BioMerieux-Brazil). *Cronobacter* species was assigned and the strains further profiled according to the 7 allele MLST scheme with reference to the open access database (http://www.pubMLST.org/cronobacter) [4,12].

Results and Discussion

Recovery of **Cronobacter** *spp.*

From a total of 42 PIF samples, 12 (29%) contained *Cronobacter* species; Table 1. The *Cronobacter* positive samples were PIF formulas from all infant age groups. In quantitative terms, the most frequent count was 0.51 MPN/100g with a mean of 0.54 MPN/100g. The highest value determined was 1.61 MPN/100g, which was found in a sample of formula for premature and/or underweight newborn infants. No *Cronobacter* spp. were isolated from the infant formula product designated for nursing infants up to 1 year in age. *Cronobacter* spp. were not recovered from any of the reconstituted infant formulas or fortified cow's milk samples collected from hospital nurseries and formula preparation units. Two hospitals used sterile water at room temperature to reconstitute powdered formula, and a third hospital used hot water (>70°C). Additionally, no *Cronobacter* species were detected on the utensils, brushes or empty feeding bottles collected from the hospitals.

Genotyping and phenotyping of **Cronobacter** *isolates*

Twelve PIF isolates were presumptively identified as being *Enterobacter sakazakii* (the former name for the *Cronobacter* genus) using API 20E and BAX® (Table 2). The ID32E phenotyping identified the strains as *E. sakazakii* except the *C. muytjensii* isolate (895) which was misidentified as *Pantoea* spp. The bioMerieux and DuPont Qualicon databases do not recognize the *Cronobacter* genus and were unable to identify the individual *Cronobacter* species. Eight strains were further analyzed using MLST (Table 2). Of these eight strains, six were identified using MLST as *C. sakazakii,* and the other two were *C. malonaticus* and *C. muytjensii*. Three of the six *C. sakazakii* strains were ST4, and had been isolated from follow on formulas for infants aged 6-12 months. No *Cronobacter* spp. were isolated from the infant formula product designated for nursing infants up to 1 year in age.

Isolation of other Enterobacteriaceae

Enterobacteriaceae other than *Cronobacter* were isolated from PIF samples. Two (out of 15) PIF products for infants aged 0 to 6 months contained *Pantoea* spp., *Escherichia vulneris* and *Enterobacter cloacae*. All seven follow on formulas contained Enterobacteriaceae, including *Pantoea* spp., *Enterobacter amnigenus*,

Klebsiella oxytoca, *Serratia rubidaea*, and *Pasteurella pneumotropical/haemolytica*. No Enterobacteriaceae were isolated from the 57 non-PIF samples.

Following the three FAO/WHO risk assessments [29,30,31], the Codex Alimentarius Commission [32,33] now recommends the absence of *Cronobacter* in PIF for infants less than 6 months in age, but this criterion is not applied to PIF products with intended use by older infants. Contamination of PIF, powdered infant drinks or other infant foods with *Cronobacter* spp. can occur during post-pasteurization processing, via the addition of dry ingredients as vitamins and minerals, or during packaging [34]. Unfortunately very few studies have enumerated the organism. Muytjens et al. [35] isolated *Cronobacter* from 20 of 141 (14.2%) PIF samples from 35 countries, and the highest concentration was <1 MPN/g. It is interesting to consider the levels post-2004 following the raised awareness and increased control of the organism. In this study *Cronobacter* spp. were detected in 29% (12/42) of PIF samples, yet none were >2 MPN/100g (Tables 1 and 2). Edelson-Mammel et al. [36] also reported that the concentration of presence of *Cronobacter* in US manufactured PIF was frequently below 1 MPN/100g, corroborating the results of the present study. Oonaka et al. [37] in Japan analyzed a total of 149 samples, of which 61 were of domestic production and 88 imported samples. Enterobacteriaceae were isolated from 36 (24.2%) samples. Nine (6%) of these, 4 domestic samples and 5 imported samples, were positive for *Cronobacter* spp. and the level was 0.36 - 91 MPN/100g. Palcich et al. [28] analyzed 186 PIF samples from Brazil with target age of infant 0-6 months in age. *Cronobacter* spp. and other Enterobacteriaceae were <0.03 MPN/100g and <5 MPN/g, respectively. These recent studies however did not identify the *Cronobacter* species or genotype.

The presence of *Cronobacter* spp. in follow on formula has not been so well documented in part due to the lack of a regulatory requirement for a microbiological criterion, and also because in some countries follow on formula as a defined product does not exist on the market. Chap et al. [38] analyzed infant formulas from 7 countries, of which 136 were follow on formulas, and 179 were other infant products. *Cronobacter* spp. was isolated from 1 sample of infant follow on formula (1%), and 22 (12%) of other infant products. However the level of *Cronobacter* was not determined due to the non-quantitative presence/absence testing of 25g quantities.

The presence of *Cronobacter* spp. in PIF is considered to be a risk due to the potential for multiplication of the microorganism in the reconstituted product. The

ingested level will be dependant on the time and temperature of cooling, storage, handling and preparation before consumption [31,39]. Hygienic practices including the control of the time/temperature regimes for the preparation of reconstituted formulae are important to minimize the risk of contamination and development microbial biofilms. Neonatal infections can be associated with the colonization of formula preparation equipment such as brushes, blenders and spoons by *Cronobacter* [16,25]. However in this study no *Cronobacter* or other Enterobacteriaceae were isolated from hospital equipment demonstrating a good level of hygiene control.

Cronobacter spp. are not the only Enterobacteriaceae isolated from PIF. The FAO/WHO [29,30] recommended that research should be undertaken to ascertain the presence of other Enterobacteriaceae in PIF. These organisms were termed 'Category B; plausible causing infections, but without supporting epidemiological evidence' by the expert committees. In this study the Enterobacteriaceae isolated from PIF were *Pantoea* spp., *Leclercia adecarboxylata, Klebsiella oxytoca*, *Serratia rubidaea*, *S. plymuthica,* and *Pasteurella pneumotropical/haemolytica*. *E. cloacae*, *Pantoea* spp. and *Klebsiella* have been associated with neonatal infections, however to date no NICU outbreaks have been attributed to this organisms through the consumption of reconstituted PIF. Nevertheless such surveillance data has been requested by FAO/WHO [29].

Discrepancies have previously been reported between the two phenotyping kits API20E and ID32E, both manufactured by bioMerieux with online databases [5,40]. Various other examples exist in the literature of organisms which have been mis-identified as *Cronobacter* [1,41]. Previously Baldwin et al. [11] demonstrated that using phenotyping to speciate *Cronobacter* isolates based on biotype was flawed as some biotype index strains had been assigned the incorrect *Cronobacter* species. As can be seen in Table 2, the biochemical profiles did not correspond with any particular *Cronobacter* species, or sequence type. Therefore phenotyping has limited value for profiling *Cronobacter* strains, and cannot be used to assign the isolate to any particular species within the *Cronobacter* genus, whereas the DNA sequence based MLST method is reliable and portable due to the open access database. Phylogenetic analysis showed the majority of isolates were *C. sakazakii* which corresponds with previous studies on prevalence [Fig 1]. Three of the six *C. sakazakii* strains were in the ST4 lineage which has a strong association with

neonatal meningitis and therefore a cause for concern [7,13]. Another isolated *C. sakazakii* strain was ST1. This is the same lineage as *C. sakazakii* BAA-894 which was isolated from the fatal Tennessee neonatal intensive care unit outbreak in 2001 and the genome of which has been sequenced [18,42]. The remaining *C. sakazakii* isolates were in sequence types which have not been linked to neonatal infections. However it is of interest to note that strains 892 and 894 (ST56 and ST113) were isolated from PIF for different age groups yet only differ in 2/3036 nucleotides. These were in the *fusA* loci (position 135 T:G and position 372 T:C; www.pubMLST.org/cronobacter). Therefore these strains are in the same clonal complex, CC11. Whether the PIFs were from the same manufacturer or had common ingredients is unknown. *C. malonaticus* was also isolated from one PIF sample for intended infant age 0-6months. This species has not been associated with neonatal outbreaks, but is more associated with adult infections [13]. The relevance of isolating *C. muytjensii* from PIF, with intended age of use 6-12 months, is uncertain as to date this species has not been associated with infant infections. Given that the two *Cronobacter* species *C. universalis* and *C. condimenti* were only formally recognized in 2011, previous PCR-based and MALDI-TOF detection methods for *Cronobacter* species may be inaccurate as reported by Cetinkaya et al. [43]. Hence the usefulness of the open access curated MLST database (www.pubMLST.org/cronobacter) which uses phylogeny to distinguish between the *Cronobacter* species and related organisms [4,12]. This level of discrimination and analysis is not available with previous genotyping methods for *Cronobacter* spp., such as pulsed-field gel electrophoresis and serotyping [44,45].

Although *Cronobacter* spp. are ubiquitous in the environment and therefore a source of neonatal exposure, it is evident that preventative measures in the preparation of infant feed are prudent to reduce neonatal exposure to this organism. Although in this study *C. sakazakii* ST4 and ST1 were not isolated from PIF intended for consumption by infants 0-6 months in age, nevertheless they were in follow on formula and represent a particularly severe, life-threatening form of *Cronobacter* infection. These two *C. sakazakii* sequence types were previously reported to be frequently (24 and 19% respectively) isolated from milk processing facilities [14]. The low level (<2 MPN/100g) of the organism in PIF and the lack of recovery from the hospital facilities probably reflects the microbiological monitoring by the PIF manufacturers and good hygienic practices in the hospitals.

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Tables

Table 1.

Most probable number (MPN) enumeration of *Cronobacter* spp. in powdered infant formula for various infant age groups

Product intended age group	Number of samples analysed	Number of positive samples	Most probable number (MPN/100g)
Premature and/or underweight newborn infants	14	3	1.61, 0.51, 0.22
0 to 6 months old children	15	3	0.51, 0.22, 0.22
6 months to 1 year old children	7	6	0.92, 0.51, 0.51, 0.51, 0.51, 0.22
PIF for nursing infants up to 1 year	6	0	<0.22
Total	42	12	

Table 2.

Identification of *Cronobacter* isolates from PIF samples

Strain	PIF intended age (months)	Isolate identification			
		API 20E (biochemical profile, %[a])	ID32E (biochemical profile, %)	BAX®-PCR	MLST (ST)[c]
894	0-6	*E. sakazakii*[b] (3305373, 98.4)	*E. sakazakii* (34274767050, 99.9)	*E. sakazakii*	*C. sakazakii* (113)
893	0-6	*E. sakazakii* (3207173, 96.8)	*E. sakazakii* (34274763251, 99.9)	*E. sakazakii*	*C. malonaticus* (7)
890	6-12	*E. sakazakii* (3305373, 98.4)	*E. sakazakii* (34274767050, 99.9)	*E. sakazakii*	*C. sakazakii* (4)
891	6-12	*E. sakazakii* (3305373, 98.4)	*E. sakazakii* (34276367250, 99.9)	*E. sakazakii*	*C. sakazakii* (4)
892	6-12	*E. sakazakii* (3305373, 98.4)	*E. sakazakii* (34274767250, 99.9)	*E. sakazakii*	*C. sakazakii* (56)
895	6-12	*E. sakazakii* (3305373, 98.4)	*Pantoea* spp. (00074703400, UA)[d]	*E. sakazakii*	*C. muytjensii*
896	6-12	*E. sakazakii* (3305373, 99.9)	*E. sakazakii* (34274767250, 99.9)	*E. sakazakii*	*C. sakazakii* (4)
897	6-12	*E. sakazakii* (3305173, 51.2)	*E. sakazakii* (34276763250, 99.9)	*E. sakazakii*	*C. sakazakii* (1)

a % match

b bioMerieux and BAX®-PCR databases give the former taxonomic name of *Enterobacter sakazakii* instead of *Cronobacter* genus.

c ST = Sequence type

d UA = Unacceptable profile

Figure 1. Maximum likelihood tree of the seven multilocus sequence typing loci (3036 base pair concatenated length) for the *Cronobacter* genus, showing the sequence type for strains isolated from PIF and type strains for the remaining *Cronobacter* species. The tree was drawn using MEGA5 (http://www.megasoftware.net/) with 1000 bootstrap replicates.