Screening for Cronobacter Species in Powdered and Reconstituted Infant Formulas and from Equipment Used in Formula Preparation in Maternity Hospitals

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Key Words
Cronobacter · Powdered infant formula · Preparation

Abstract
Background/Aims: Cronobacter spp. have been identified as being of considerable risk to neonates. The occurrence of organisms in infant formulas is therefore of considerable interest. \textbf{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 in the preparation of infant formula and 5 samples of fortified cow’s milk. Presumptive Cronobacter spp. isolates were identified using the \textit{MLST} scheme. \textbf{Results:} \textit{C. sakazakii}, \textit{C. malonaticus} and \textit{C. muytjensii} were recovered from PIF. Although the incidence of \textit{Cronobacter} in PIF was 29% (12/42), the level was low with an average of 0.54 MPN/100 g. According to MLST profiling, \textit{C. sakazakii} was the most frequently isolated \textit{Cronobacter} species, and \textit{C. sakazakii} ST4 (associated with neonatal meningitis) was recovered from 2/42 PIF samples at 0.51 and 0.92 MPN/100 g. \textbf{Conclusions:} \textit{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

\textit{Cronobacter} spp. are Gram-negative bacterial pathogens that cause meningitis, septicemia and necrotizing enterocolitis in newborn babies and infants [1]. Such infections have a high fatality rate of 40–80% 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 mainly involving neonates [5–7]. This may be related to the 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 and gangliosides, and is added to powdered infant formula (PIF) [9, 10].

A multilocus sequence typing (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 3,036 nucleotides. Currently, 136 sequence types have been identified in the Cronobacter genus, of which 73 sequence types are in C. sakazakii and none of the other six Cronobacter species [8].

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The source of 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 [16–18]. These food products are not commercially sterile and even low contamination levels by Cronobacter spp. are considered a significant risk factor as the organism grows rapidly on reconstitution [19–21]. 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 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]. 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 [25–28].

Current Cronobacter detection methods use a pre-enrichment step in their initial isolation, meaning 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, Brazil. In total, 99 samples were tested. This consisted of 14 PIF samples for premature or underweight newborn infants, 15 PIF for target age 6–6 months, 7 follow-on formulas (target age 6 months to 1 year) and 6 PIF for nursing infants up to 1 year of age. 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 cups (n = 3), bottle storage equipment (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 analyzed in 100-gram and 40-ml volumes, respectively. 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 5 ml of mLST-V. Samples were analyzed according to the BAX®-PCR System (DuPont Qualicon), including an additional cultivation in BHI broth at 37°C for 3 h before genotyping.

Identification of Cronobacter Isolates
Presumptive Cronobacter isolates were subcultured on trypticase soy agar (25°C, 48–72 h) before phenotypic identification using API 20E and ID32E (bioMerieux). Cronobacter species was

DOI: 10.1159/000353137
Ann Nutr Metab 2013;63:62–68

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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].

Table 1. MPN enumeration of Cronobacter spp. in PIF for various infant age groups

| Product intended age group                          | Samples analyzed, n | Positive samples, n | MPN/100 g          |
|-----------------------------------------------------|---------------------|---------------------|--------------------|
| Premature and/or underweight newborn infants        | 14                  | 3                   | 1.61, 0.51, 0.22   |
| Children 0–6 months old                             | 15                  | 3                   | 0.51, 0.22, 0.22   |
| Children 6 months to 1 year-old                     | 7                   | 6                   | 0.92, 0.51, 0.51, 0.51, 0.51, 0.22 |
| PIF for nursing infants up to 1 year old            | 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, %)¹ | ID32E (biochemical profile, %)¹ | BAX®-PCR | MLST (ST) |
|--------|--------------------------|------------------------|----------------------------------|---------------------------------|----------|-----------|
| 894    | 0–6                      | E. sakazakii (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) | 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) |

ST = Sequence type; UA = unacceptable profile.

¹ % match.

² bioMerieux and BAX®-PCR databases give the former taxonomic name of Enterobacter sakazakii instead of Cronobacter genus.

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/100 g with a mean of 0.54 MPN/100 g. The highest value determined was 1.61 MPN/100 g, 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 of 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 for 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 of age.

Isolation of Other Enterobacteriaceae

Enterobacteriaceae other than Cronobacter were isolated from PIF samples. Two (out of 15) PIF products for infants aged 0–6 months contained Pantoea spp., Escherichia vulneris and E. cloacae. All seven follow-on formulas contained Enterobacteriaceae, including Pantoea spp., E. ammigenus, Klebsiella oxytoca, Serratia rubidaea, and Pasteurella pneumotropicalis/haemolytica. No Enterobacteriaceae were isolated from the 57 non-PIF samples.

Following the three FAO/WHO risk assessments [29–31], the Codex Alimentarius Commission [32, 33] now recommends the absence of Cronobacter in PIF for infants younger than 6 months of 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 postpasteurization 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/100 g (Table 1, 2). Edelson-Mammel et al. [36] also reported that the concentration of Cronobacter present in US manufactured PIF was frequently below 1 MPN/100 g, corroborating the results of the present study. In Japan, Oonaka et al. [37] 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 0.36–0.91 MPN/100 g. Palcich et al. [28] analyzed 186 PIF samples from Brazil with a target age of infants of 0–6 months. Cronobacter spp. and other Enterobacteriaceae were <0.03 MPN/100 g 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 nonquantitative presence/absence testing of 25-gram 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 dependent 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 of microbial biofilms. Neonatal infections can be associated with the colonizion 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 infection’, but without supporting epidemiological evidence’ by the expert committees. In this study, the Enterobacteriaceae isolated from PIF were Pantoea spp., Leclercia adecarboxylata, K. oxytoca, S. rubidaea, S. plymuthica and P. pneumotropicalis/haemolytica. E. cloacae, Pantocea spp., and Klebsiella have been associated with neonatal infections; however, to date no NICU outbreaks have been attributed to these 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 bioMérieux with online databases [5, 40]. Various other examples exist in the literature of organisms which have been misidentified 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

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Ann Nutr Metab 2013;63:62–68
DOI: 10.1159/0003533137
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 that 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, is 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 2/3,036 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 0–6 months. 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 has been 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, which therefore presents 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 of age, they were detected 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/100 g) 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.

Acknowledgements

The authors wish to thank bioMerieux and Du Pont Qualicon of Brazil, and Nottingham Trent University for their support.

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