**Research Note**

**Evaluation of a New One-Step Enrichment in Conjunction with a Chromogenic Medium for the Detection of *Cronobacter* spp. (*Enterobacter sakazakii*) in Powdered Infant Formula**

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**ABSTRACT**

The aim of the present study was to evaluate a new one-step enrichment protocol, consisting of a combined preenrichment and enrichment broth (*Cronobacter* Enrichment Broth [CEB]) used in conjunction with selective-differential agars ChromID Sakazakii, to facilitate a shortened 2-day cultural method for detection of *Cronobacter* spp. (*Enterobacter sakazakii*) in powdered infant formula (PIF). The CEB was evaluated using samples artificially inoculated with low concentrations of 10 lyophilized strains, representative of the genus *Cronobacter*. The detection of strains was compared in parallel with the enrichment medium from ISO/TS 22964 and a recently proposed differential screening broth for the detection of *Cronobacter*. All of the *Cronobacter* strains were recovered using the CEB, and a significantly higher final bacterial concentration was obtained with the CEB than with the other enrichment broths (P < 0.01). There was no significant difference between the cell concentrations for cultures grown in CEB at 37°C and those grown at 41.5°C. *Cronobacter* was recovered from both 1/10 (50 g:450 ml) and 1/5.5 (100 g:450 ml) sample-to-broth ratios, with no significant difference observed between the final bacterial concentrations obtained from the two ratios. Further studies on a wider range of PIFs, including naturally contaminated samples, are warranted to determine if the use of this protocol may facilitate the rapid release (within 40 to 48 h) of PIF.

*Cronobacter* is a novel genus of opportunistic pathogens, including *Enterobacter sakazakii*, which has been linked to meningitis, necrotizing enterocolitis, bacteremia, and sepsis in neonates (9, 14). In several of these cases contaminated powdered infant formula (PIF) was identified as the source of *Cronobacter* infection (1, 11, 17). In response to this hazard, the International Organization for Standardization (ISO) in conjunction with the International Dairy Federation, and in parallel with the U.S. Food and Drug Administration, has issued guidelines for the detection of *Cronobacter* spp. from PIF (2, 3). In the European context, ISO/TS 22964 underpins the implementation of the European Commission Regulation EC No. 2073/2005 (amended by EU Regulation No. 1441/2007), which requires the absence of *Cronobacter* in 30 10-g samples of PIF intended for consumption by infants younger than 6 months of age. However, it has now been established that some strains of *Cronobacter* do not grow well in the enrichment broths proposed in the current recommended methods (including *Enterobacteriaceae* enrichment broth and modified lauryl sulfate tryptose broth [mLST]), and this could lead to false-negative results (6, 8, 10, 12). Also, the incidence of *Cronobacter* spp. in PIF remains low, with reported levels being between 0.22 and 1.61 CFU/100 g of powdered sample (15). Furthermore, it is likely that the organism may be stressed or injured at the time of sampling. Therefore, it is desirable that any potential selective enrichment methods be capable of suppressing the growth of background microflora while allowing the target organism to recover and multiply to a detectable level in the shortest time possible. This study evaluates a new enrichment broth (*Cronobacter* Enrichment Broth [CEB]; bioMérieux, Marcy l’Etoile, France) for use in a simplified and shortened *Cronobacter* detection protocol compared with the current ISO/TS 22964:2006 protocol and a recently proposed differential screening broth method (7).

**MATERIALS AND METHODS**

**Test organisms.** The sensitivity of media was assessed using 10 strains of *Cronobacter* including five strains of *C. sakazakii* (ATCC 29544, ATCC 12868, SK90, E632, and Es626) and one strain each of *C. dublinensis* (CFS 237), *C. malonaticus* (CDC 1058-77), *C. muytjensii* (ATCC 51329), *C. turicensis* (3032), and *Cronobacter* genomospecies 1 (NCTC 9529). The collection included the type strains of the different species and strains that have previously been found to grow poorly in *Enterobacteriaceae* enrichment broth. The origins of the strains, where known, include PIF, environmental, and clinical samples (Table 1).
Lyophilization of test organisms. The strains were incubated at 37°C for 24 h on tryptone soya agar (TSA; Oxoid, Basingstoke, UK). Cells from several colonies were suspended in double-strength skim milk, and 1-ml aliquots of the suspension were dispensed into sterile glass ampoules (four ampoules per strain). The ampoules were lyophilized using a manifold freeze-dryer (EF03, Edwards, Sussex, UK) for 24 h. The ampoules were then sealed using a glass burner and stored at room temperature in the dark for 2 weeks prior to use. To estimate the number of CFU of lyophilized cells per gram, one freeze-dried ampoule per strain was rehydrated in 1 ml of buffered peptone water (BPW). Serial decimal dilutions in BPW were dispensed into columns of 96-well microtiter plates at 100 µl per well (8 wells per dilution). The most probable number was determined based on the number of wells positive for growth at each dilution (4).

Enrichment media. The following media were used for enrichment of Cronobacter spp.: BPW (Oxoid, Basingstoke, UK); CEB; mLST broth (comprising CM0451 [Oxoid] with 0.5 M NaCl and 10 mg liter⁻¹ vancomycin hydrochloride); and Cronobacter screening broth (CSB; comprising 10 g liter⁻¹ proteose-peptone from bovine meat, 5 g liter⁻¹ NaCl, 3 g liter⁻¹ yeast extract, 0.04 g liter⁻¹ bromocresol purple, and 10 g liter⁻¹ sucrose) (7).

Isolation from inoculated samples. Cartons of one brand of a milk-based infant formula intended for consumption by newborn infants were obtained from a retail outlet in France. Prior to the inoculation experiments, two cartons were opened and one-half of the contents were tested to ensure that no Cronobacter organisms were present. Portions of PIF (50 and 100 g) were aseptically added to 450 ml of BPW and CEB, and the samples were then inoculated with a single lyophilized Cronobacter strain. To imitate the isolation of the low numbers of Cronobacter usually found in PIF, lyophilized ampoules were rehydrated in 1 ml of BPW and immediately serially diluted in BPW to achieve an approximate concentration of 10² CFU ml⁻¹ (considering the 95% confidence intervals to be between 88 and 114 CFU ml⁻¹). The PIF enrichments (500 to 550 ml, depending on the amount of PIF) were immediately inoculated with 50 µl of 10² CFU ml⁻¹ to give approximately 5 to 10 CFU per sample. This is equivalent to an approximate contamination level of 0.01 to 0.02 CFU g⁻¹ of powdered formula. To determine the exact inoculation level, serial decimal dilutions in BPW were dispensed into the 8 wells in columns of 96-well microtiter plates at 100 µl per well and the most probable number per gram of lyophilized powder was determined based on the number of wells positive for growth at each dilution (4). Samples suspended in BPW were incubated for 24 h at 37°C, while samples in CEB were incubated for 24 h at 37 or 41.5°C. The enriched BPW and CEB samples were evaluated for the isolation of Cronobacter on ChromID Sakazakii plating medium (bioMérieux), and for endogenous flora they were evaluated by the Miles & Misra method as described below. Aliquots (100 µl) of the enriched BPW samples were also added to 10 ml of both CSB and mLST, which were subsequently incubated for 24 h at 41.5 and 44°C, respectively. The enriched CSB and mLST samples were also evaluated for the isolation of Cronobacter on ChromID Sakazakii plating medium and endogenous flora.

Miles & Misra plate count method. Enriched samples were evaluated using the Miles & Misra method (13). Briefly, decimal serial dilutions were made for each enriched suspension, ranging from 10⁻¹ to 10⁻⁸. Plates were divided into eight numbered
sectors. Serial dilutions were deposited as drops of 20 μl from a height of 2.5 cm onto ChromID Sakazakii to detect Cronobacter colonies, TSA (Oxoid) containing 10 mg liter⁻¹ vancomycin hydrochloride (TSA-V), and plate count agar (bioMérieux). TSA-V was included as a comparison medium to assess the effects of the ChromID Sakazakii agar on isolation after enrichment in the different broths, and plate count agar was included to detect the presence of mesophilic, aerobic, gram-positive organisms in each of the enrichment broths. Each serial dilution was plated in triplicate. Plates were allowed to dry for approximately 20 min, during which the droplets spread over an area of approximately 1.5 to 2.0 cm in diameter. The plates were inverted and incubated for 24 h at 37°C. Sectors with 2 to 20 colonies present, without any confluence, were enumerated, and the number of CFU per milliliter of enrichment broth was calculated.

**Real-time PCR confirmation.** All presumptive Cronobacter strains recovered during the course of this study were confirmed by real-time PCR using a primer set and a probe to target the dnaG gene on the macromolecular synthesis operon (5, 16).

**Statistical analysis.** All analyses were carried out in triplicate. Counts were transformed to log CFU per gram, and the mean log and standard deviations were calculated. The data shown in Table 1 are the averages of all repetitions. A paired Student t test (two samples for means) was used to test for significant differences between enrichments at a 0.05 level of confidence.

**RESULTS AND DISCUSSION**

The aim of the present study was to evaluate a new single broth (CEB) for the isolation of Cronobacter spp. from potentially contaminated PIF. The CEB is designed to reduce the level of nontarget microflora while supporting the growth of Cronobacter. The final concentrations (in CFU per milliliter) of target organisms recovered in 50-g and in 100-g inoculated samples in 450 ml of CEB were not significantly different. Both 50- and 100-g samples had been inoculated with the same concentration of lyophilized bacteria; therefore, this was the equivalent of the 100-g sample having one-half the concentration per gram of powder as the 50-g sample. As no significant difference was observed between the final counts for the two sample amounts, there is no evidence that the increased level of organic material in the 100-g samples has any detrimental effect on the active ingredients of the CEB that would affect the final concentration of the target organisms. Furthermore, no significant difference in final concentration was found between incubation at 37 or 41.5°C. As only one brand of PIF was used in the inoculation experiments, it is not known whether the same results would be obtained for other PIF products with different formulations and endogenous flora.

A comparison of the counts obtained from the individual enrichment broths shows that CEB promoted a significantly higher final concentration of bacteria (in CFU per milliliter) than the other broths (P < 0.01). CSB and mLST failed to recover one Cronobacter strain (E632) from the inoculated PIF samples. This strain has been reported to be particularly sensitive to antimicrobial agents used in selective enrichment media and is unable to grow in mLST (8, 10). It has been previously reported that for some Cronobacter strains a cell concentration of >10⁴ CFU ml⁻¹ in BPW is required to enable sufficient transfer of cells to CSB to effect a color change within 24 h. In this experiment for strain E632, the mean value (log CFU per milliliter) obtained in BPW was 2.8 to 4.5, which appears to be below the threshold for subsequent detection in CSB (7). The mean value (log CFU per milliliter) obtained for other strains ranged between 2.2 and 6.3, and all of these strains were subsequently detected in CSB (Table 1).

Interestingly, strain NCTC 9529 was not detected after preenrichment of 100 g of PIF in 450 ml of BPW but was subsequently detected after incubation of the sample in the secondary enrichment broths. Endogenous gram-positive organisms were observed in enriched BPW suspensions (but not in CEB), and it is reasonable to suggest that competition from endogenous gram-positive flora may affect the growth of target cells during preenrichment. The 100-g samples could potentially contain twice as much background flora as the 50-g sample, as well as twice as much organic material, and the selective nature of the secondary broth can inhibit competitors, allowing outgrowth of target cells. In this scenario, low cell numbers after preenrichment in BPW may have been below the theoretical detection limit for the plate count enumerations (16.7 CFU ml⁻¹) while still being transferred to the secondary enrichment broths (the limit for cell transfer to the secondary enrichment broth is theoretically 10 CFU ml⁻¹). This could explain the failed detection of NCTC 9529 after preenrichment, while allowing for the possibility that cells were transferred to CSB and mLST, where they could proliferate to exceed the detection limit.

The final concentrations obtained in the different enrichment media were enumerated using both the selective-differential agar, ChromID Sakazakii, and TSA-V. Only Cronobacter grew on these media, and the colony counts could be directly compared. Equivalence was shown between the colony counts from the two media (Table 1). This shows that the Cronobacter selective plating media used in conjunction with the CEB was not inhibitory to the growth of the target strains used in this study, while also having the ability to differentiate the organisms.

When used in conjunction with ChromID Sakazakii, CEB may have the potential to improve the detection of Cronobacter in PIF samples. Use of rapid identification methods, such as dnaG real-time PCR, directly from ChromID Sakazakii would facilitate further confirmation of any presumptive Cronobacter to be completed within two working days. Therefore, this new rapid and simplified detection protocol has the potential to facilitate the positive release of all PIF products within 40 to 48 h. Further studies on a wider range of PIF brands as well as naturally contaminated samples are required to support the results of this study.

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REFERENCES


