Phenotypic profiles and detection of target genes by PCR in isolates from different sources and reference strains, identified as *Cronobacter* spp. (*Enterobacter sakazakii*)

Perfis fenotípicos e detecção de genes alvo por PCR em isolados de diferentes origens e cepas de referência identificados como *Cronobacter* spp. (*Enterobacter sakazakii*)

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Márcia Barbosa WARNKEN[1]*, Marcelo Luiz Lima BRANDÃO[1], Aline Encarnação SOUZA[1], Célia Maria Carvalho Pereira Araulo ROMÃO[1], Ana Cristina Martins Almeida NOGUEIRA[2], Maria Teresa DESTRO[3]

Endereço para correspondência: [1]Departamento de Microbiologia, Instituto Nacional de Controle de Qualidade em Saúde (INCQS), Fiocruz, Av. Brasil, 436, Manguinhos, Rio de Janeiro, RJ, 21040-360. (21) 3865-5161/5151, ramal 5161. E-mail: marcia.warnken@incqs.fiocruz.br; mwarnken@terra.com.br

[2]Departamento de Imunologia, Instituto Nacional de Controle de Qualidade em Saúde (INCQS).

[3]Departamento de Alimentos e Nutrição Experimental, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo.

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ABSTRACT

Cronobacter, formerly known as *Enterobacter sakazakii*, is a novel genus of the *Enterobacteriaceae* family recognized as a cause of high number of fatal cases in neonates, after consuming infant formula**.** The conventional methods for detecting these organisms are time-consuming and lack sensitivity. The ISO/TS 22964:2006 is the most recently standardized methodology for detecting *Cronobacter* in powdered infant formula. This study aimed at confirming the Brazilian isolates previously identified as *E. sakazakii* as *Cronobacter* spp. by biochemical assays, and also to compare characteristics of 37 *Cronobacter* and non-*Cronobacter* isolates; and the miniaturized kits and the ISO/TS methodology were evaluated. A conventional PCR protocol targeting *dna*G was also developed and a previously described *glu*A targeting protocol was used. The majority of the Brazilian isolates were not confirmed as *Cronobacter* spp., and the selective enrichment step of ISO/TS methodology was inhibitory to some *Cronobacter* strains. The ID 32E was the most reliable kit. The PCR protocol targeting *glu*A showed consistent results with ID 32E and the developed *dna*G PCR protocol was 100% sensitive and specific. Thus, the PCR protocols targeting *glu*A and *dna*G might be used to complement the *Cronobacter* spp. detection or identification after performing the conventional isolation and identification methods.

Keywords. *Cronobacter* spp., *Enterobacter sakazakii*, ISO/TS 22964:2006, PCR, phenotypic characterization

RESUMO

Cronobacter (*Enterobacter sakazakii*) é um novo gênero da família *Enterobacteriaceae*, reconhecido como responsável pelo elevado número de casos fatais em neonatos após consumo de fórmulas infantis. Os métodos convencionais de detecção desses micro-organismos são demorados e de sensibilidade inadequada. A ISO/TS 22964:2006 é a metodologia padronizada mais recentemente para detecção de *Cronobacter*. Este estudo teve como objetivo identificar como *Cronobacter* spp. os isolados brasileiros previamente classificados como *E. sakazakii*, comparar as características de 37 culturas de *Cronobacter* e não-*Cronobacter* e avaliar os *kits* miniaturizados e a ISO/TS 22964:2006. Foi também desenvolvido um protocolo convencional de PCR com alvo em *dna*G. A maioria dos isolados brasileiros não foi confirmada como *Cronobacter* spp., e o enriquecimento seletivo da metodologia ISO foi inibitório para algumas cepas de *Cronobacter*. O kit ID 32E demonstrou desempenho mais confiável. O protocolo de PCR com alvo em *glu*A apresentou resultados consistentes com o ID 32E, e o protocolo com alvo em *dna*G foi 100% sensível e específico. Portanto, os protocolos de PCR com alvo em *glu*A e *dna*G podem ser usados para complementar a detecção e identificação de *Cronobacter* spp. após o emprego de métodos de isolamento e identificação convencionais.

Palavras-chave. *Cronobacter* spp., *Enterobacter sakazakii*, ISO/TS 22964:2006, PCR, caracterização fenotípica.

INTRODUCTION

Cronobacter is a novel genus of the *Enterobacteriaceae* family comprising six species and three subspecies[1,2]. These microorganisms, formerly known as *Enterobacter sakazakii,* have been considered emerging food pathogens associated with meningitis, necrotizing enterocolitis and sepsis in neonates[3]. Case fatality rates vary from 40% to 80%, and survivors may suffer from severe neurological sequelae such as hydrocephalus, quadriplegia and retarded neural development[4]. In several of these cases, infection was linked to infant formula consumption[4,5,6,7,8]. The source of reconstituted infant formula contamination may be intrinsic, arising from the manufacturing environment and/or addition of raw ingredients or extrinsic, via catheters, nipples or utensils used to reconstitute infant formula[6,9,10]. There have been many reported cases of infection related to powdered infant formula (PIF) contaminated with *Cronobacter* spp.[3,11]. Concern over this problem led to several voluntary recalls of PIF contaminated with *Cronobacter* spp. worldwide, resulting in a collective effort among manufacturers and government bodies to reduce health risks associated with the consumption of contaminated PIF[3,12,13].

The methods currently used for the detection and identification of *Cronobacter* spp. have been reported as inadequate and controversial[14]. The traditional methods[15,16] for the detection of these organisms are time-consuming and labor-intensive, taking 5-7 days for a final result. Moreover, some culture media do not support the growth of all *Cronobacter* strains, and several commercial identification kits have been found unreliable[17-20]. The sensitivity and the specificity of culture media and kits used for the detection and identification of *Cronobacter* are important features that directly affect the cost of production of PIF because they can reduce or increase the number of rejected batches during routine surveillance. In a public health context, culture media and commercial kits are important tools for the correct identification of the etiological agent of infection. Reliable methods are also important for monitoring food safety. To minimize the hazards due to the *Cronobacter* spp. contamination of PIF, the use of accurate, sensitive and specific detection and identification methods for these microorganisms is of utmost importance.

Development of molecular methods such as polymerase chain reaction (PCR) with different target genes have been described and provide powerful tools for rapid and specific detection and identification of *Cronobacter* spp[21]. The United States Food and Drug Administration (USFDA) has developed a new method for the detection of *Cronobacter* spp. in PIF. This method incorporates the chromogenic agars Brilliance Enterobacter Sakazakii Agar (Druggan-Forsythe-Iversen formulation) (DFI; Oxoid) and Enterobacter Sakazakii Plating Medium (ESPM; R&F Products Inc.) as well as the real-time PCR (qPCR) assay developed by Seo and Brackett[22] targeting the *dna*G from the macromolecular synthesis operon (MMS). This protocol successfully passed an AOAC pre-collaborative study and is in the process of further validation for the inclusion into the FDA's *Bacteriological Analytical Manual*[23]. Although it is expected that laboratories from different countries will adopt this new protocol for PIF analysis, some laboratories may not have access to real-time PCR technology.

This study aimed at detecting the presence of *Cronobacter* spp. among Brazilian isolates previously identified as *E. sakazakii* by biochemical assays, comparing characteristics of 37 *Cronobacter* and non-*Cronobacter* isolates from different origins and evaluating the ISO/TS 22964:2006 methodology. This study also developed a conventional PCR protocol based on the pair of primers designed by Seo and Brackett[22]. This new protocol was evaluated for the identification of *Cronobacter* by including *Cronobacter* target as well as non-target strains and by comparison with the PCR protocol targeting *glu*A[18].

MATERIALS AND METHODS

Bacterial strains

A total of 37 strains were used in this study (Table 1 and 2). They were obtained from various sources and included isolates from clinical samples, PIF, PIF raw ingredient, PIF production environment, PIF handlers' hands and nipple, as well as reference strains of *Cronobacter* spp. and isolates previously identified as *E. sakazakii*/C*ronobacter* spp. from culture collections. Nineteen additional reference strains were used as negative controls in the PCR assays (Table 3). Stock cultures were maintained at -20 °C in brain heart infusion broth (BHI; Merck) containing 20% glycerol (Merck). Inocula for each experiment were prepared by transferring one loopful from the stock tube into 3 mL-fresh BHI broth and incubating at 35 °C for 24 hours, unless otherwise stated. For daily use, cultures were maintained at 4 °C on nutrient agar (Merck).

Table 1. Origin and phenotypic and molecular characterization of *Cronobacter* strains.

Supplying labs	Original code	Original identification	Source	Phenotypical behaviour						Detection by PCR of				Final identification
				Conventional biochemical tests	API 20E (% match)	Vitek 32 (% match)	ID 32 (% match)	Biogroup	ZYM type[a]	*glu*A		V1/ V3	*dna*G	
										1680 pb	105 pb	952 pb	78 pb	
UCD[b]	CDC 1058-77	*C. malonaticus* (T)[c]	Clinical	*C. malonaticus*	*E. sakazakii* (51.1)/ *E. amnigenus* 1 (31.7)	*E. sakazakii* (61)/ *Kluyvera* sp. (22)	*E. sakazakii* (99.9)	9	1	+	+	+	+	*C. malonaticus*
UCD	3032	*C. turicensis* (T)	Clinical	*C. turicensis*	*E. sakazakii* (98.4)	*E. sakazakii* (98)	*E. sakazakii* (99.9)	16	2	+	+	+	+	C. turicensis
UCD	E 922	*C. sakazakii*	Clinical	C. sakazakii	*E. sakazakii* (51.1)/ *E. amnigenus* 1 (31.7)	*E. sakazakii* (98)	*E. sakazakii* (99.9)	2	3	+	+	+	+	C. sakazakii
UCD	E 785	*C. sakazakii*	Clinical	*C. sakazakii*	*E. sakazakii* (98.4)	*E. sakazakii* (98)	*E. sakazakii* (99.9)	1	4	+	+	+	+	*C. sakazakii*
UCD	CDC 996-77	*C. sakazakii*	Clinical	*C. sakazakii*	*E. sakazakii* (98.4)	*E. sakazakii* (98)	*E. sakazakii* (99.9)	3	5	+	+	+	+	*C. sakazakii*
UCD	CDC 3128-77	*C. sakazakii*	Clinical	*C. sakazakii*	*E. sakazakii* (51.1)/ *E. amnigenus* 1 (31.7)	*E. sakazakii* (97)	*E. sakazakii* (99.9)	11	6	+	+	+	+	*C. sakazakii*
UCD	HPB 2856	*C. sakazakii*	Clinical	*C. sakazakii*	*E. sakazakii* (98.4)	*E. sakazakii* (98)	*E. sakazakii* (99.9)	1	7	+	+	+	+	C. sakazakii
INCQS[d]	00597(ATCC[e] 29544)	*C. sakazakii* (T)	Clinical	*C. sakazakii*	*E. sakazakii* (98.4)	*E. sakazakii* (99)	*E. sakazakii* (99.9)	1	9	+	+	+	+	C. sakazakii
INCQS	00583(ATCC BAA 894)	*C. sakazakii*	Unknown	*C. sakazakii*	*E. sakazakii* (51.1)/ *E. amnigenus* 1 (31.7)	*E. sakazakii* (99)	*E. sakazakii* (99.9)	2	9	+	+	+	+	C. sakazakii
HCSC[f]	HPB 2871	*Cronobacter* spp.	Milk powder	*C. sakazakii*	*E. sakazakii* (98.4)	*E. sakazakii* (99)	*E. sakazakii* (99.9)	1	8	+	+	+	+	*C. sakazakii*
USP[g]	2 A	*Cronobacter* spp.	Milk powder	*C. malonaticus*	*E. sakazakii* (51.9)/ *E. amnigenus* 1 (30.8)/ *E. cloacae* (17)	*E. sakazakii* (96)	*E. sakazakii* (99.9)	9	9	+	+	+	+	*C. malonaticus*
USP	4 A	*Cronobacter* spp.	Milk powder	*C. sakazakii*	*E. sakazakii* (98.4)	*E. sakazakii* (99)	*E. sakazakii* (99.9)	1	9	+	+	+	+	C. sakazakii
HCSC	HPB 2875	*Cronobacter* spp.	Milk powder	*C. sakazakii*	*E. sakazakii* (51.9)/ *E. amnigenus* 1 (30.8)	*E. sakazakii* (98)	*E. sakazakii* (99.9)	2	9	+	+	+	+	*C. sakazakii*
HCSC	HPB 2876	*Cronobacter* spp.	Milk powder	*C. sakazakii*	*E. sakazakii* (51.9)/ *E. amnigenus* 1 (30.8)	*E. sakazakii* (98)	*E. sakazakii* (99.9)	2	9	+	+	+	+	*C. sakazakii*
UCD	E 782	*C. sakazakii*	Milk powder	*C. sakazakii*	*E. sakazakii* (98.4)	*E. sakazakii* (98)	*E. sakazakii* (99.9)	1	5	+	+	+	+	*C. sakazakii*
UCD	CFS 10	*C. sakazakii*	Raw ingredient	*C. sakazakii*	*E. sakazakii* (98.4)	*E. sakazakii* (99)	*E. sakazakii* (99.9)	8b	4	+	+	+	+	*C. sakazakii*
USP	3 A	*Cronobacter* spp.	Environmental	*C. sakazakii*	*E. sakazakii* (98.4)	*E. sakazakii* (99)	*E. sakazakii* (99.9)	1	9	+	+	+	+	*C. sakazakii*
UCD	CFS 237	*C. dublinensis* (T)	Environmental	*C. dublinensis*	*E. sakazakii* (99.9)	*P. agglomerans* (95)/ *E. sakazakii* (3)	*E. sakazakii* (99.9)	12	11	+	+	+	+	*C. dublinensis*
UCD	CFS 101	*C. sakazakii*	Environmental	*C. sakazakii*	*E. sakazakii* (51.1)/ *E. amnigenus* 1 (31.7)	*E. sakazakii* (74)/ *E. cloacae* (24)	*E. sakazakii* (99.9)	2	6	+	+	+	+	C. sakazakii
UCD	NCTC 9529	*C.* gemonospecies 1 (T)	Environmental	*C.* gemonospecies 1	*E. sakazakii* (98)	*E. sakazakii* (99)	*E. sakazakii* (99.9)	16c	12	+	+	+	+	*C.* gemonospecies 1

FUNED[h]	1390	*C. sakazakii*	Utensil (nipple)	*C. sakazakii*	*E. sakazakii* (98.4)	*E. sakazakii* (99)	*E. sakazakii* (99.9)	3	10	+	+	+	+	*C. sakazakii*
HCSC	HPB 2887	*Cronobacter* spp.	Unknown	*C. sakazakii*	*E. sakazakii* (98.4)	*E. sakazakii* (96)	*E. sakazakii* (99.9)	1	9	+	+	+	+	*C. sakazakii*
HCSC	HPB 2891	*Cronobacter* spp.	Unknown	*C. sakazakii*	Unacceptable profile	*E. sakazakii* (95)	*E. sakazakii* (99.9) (doubtful profile)	1	9	+	+	+	+	*C. sakazakii*
IOC[i]	IOC-PC	*Cronobacter* spp.	Unknown	*C. sakazakii*	*E. sakazakii* (98.4)/ *E. cloacae* (1.5)	*E. sakazakii* (99)/ *E. cloacae* (1)	*E. sakazakii* (99.9)	3	9	+	+	+	+	C. sakazakii
INCQS	00579(ATCC 51329)	*C. muytjensii*	Unknown	*C. muytjensii*	*E. sakazakii* (99.9)	*E. sakazakii* (99)	*E. sakazakii* (99.9)	15	10	+	+	+	+	C. muytjensii
INCQS	00115(ATCC 29044)	*C. sakazakii*	Unknown	*C. sakazakii*	*E. sakazakii* (97.3)	*E. sakazakii* (98)	*E. sakazakii* (99.9)	1	10	+	+	+	+	C. sakazakii
INCQS	00580(ATCC 12868)	*C. sakazakii*	Unknown	*C. sakazakii*	*E. sakazakii* (98.4)	*E. sakazakii* (98)	*E. sakazakii* (99.9)	1	9	+	+	+	+	C. sakazakii

[a] - code of enzymatic profile in API ZYM; [b] -S. Fanning, Centre for Food Safety, University College Dublin, Belfield, Dublin 4, Ireland; [c] - type strain; [d] – Coleção de Culturas do Instituto Nacional de Controle de Qualidade em Saúde (INCQS), Fiocruz, Rio de Janeiro, Brazil; [e] - American Type Culture Collection; [f] – J. M. Farber, Bureau of Microbial Hazards, Health Canada, Ottawa, Canada; [g] - M. T. Destro, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Brazil; [h] - M. C. C. Silva, Laboratório Central de Saúde Pública, Minas Gerais, Brazil; [i] - M. D. Asensi, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, Brazil.

Table 2. Origin, phenotypic and molecular characterization of the Brazilian non-*Cronobacter* strains.

Suppliyng labs	Original code	Original identification	Source	Phenotypical behavior				Detection by PCR of				Final identification
				Conventional biochemical tests	API 20E (% match)	Vitek 32 (% match)	ID 32 (% match)	*glu*A		V1/V3	*dna*G	
								1680 pb	105 pb	952 pb	78 pb	
UERJ[a]	A 1	*Cronobacter* spp.	Clinical	*E. cloacae*	*E. sakazakii* (51.1)/ *E. amnigenus* 1 (31.7)	*E. cloacae* (98)	*E. cloacae* (99.9)	-	-	-	-	*E. cloacae*
IOC[b]	D 2	*Cronobacter* spp.	Clinical	*E. cloacae*	*E. sakazakii* (99.9)	*E. cloacae* (97)/ *E. sakazakii* (2)	*E. cloacae* (99.9)	-	-	-	-	*E. cloacae*
IOC	501 M	*Cronobacter* spp.	Clinical	*E. cloacae*	*E. sakazakii* (98.4)	*E. cloacae* (97)/ *E. sakazakii* (2)	*E. cloacae* (99.9)	-	-	-	-	*E. cloacae*
IOC	8 F	*Cronobacter* spp.	Clinical	NC[c]	*Bordetella*/ *Alcaligenes*/ *Moraxella* spp.	*Acinetobacter lwoffii/junii* (99)	NC	-	-	-	-	NC
IOC	57 L	*Cronobacter* spp.	Clinical	*Enterobacter* spp.	*E. sakazakii* (86.6)/ *E. gergoviae* (11)	*E. cloacae* (70)/ *E. sakazakii* (15)	*E. gergoviae* (99.9)	-	-	-	-	*Enterobacter* spp.
UERJ	M 1	*Cronobacter* spp.	PIF[d] handler's hand	*Enterobacter spp.*	*Enterobacter asburiae* (82.3)	*E. cloacae* (98)	*E. asburiae* (99.9)	-	-	-	-	*Enterobacter* spp.
UERJ	M 3	*Cronobacter* spp.	PIF handler's hand	*E. cloacae*	*E. cloacae* (90.9)	*E. cloacae* (99)	*E. cloacae* (54.4)/ *E. asburiae* (45.4)	-	-	-	-	*E. cloacae*
UERJ	M 12	*Cronobacter* spp.	PIF handler's hand	*Enterobacter spp.*	*Enterobacter asburiae* (82.3)	*E. cloacae* (98)	*E. asburiae* (99.9)	-	-	-	-	*Enterobacter* spp.
USP[e]	5 A	*Cronobacter* spp.	Environmental	*E. cloacae*	*E. cloacae* (95)/ *E. sakazakii* (3)	*E. cloacae* (94)	*E. cloacae* (60.2)	-	-	-	-	*E. cloacae*
USP	N 1	*Cronobacter* spp.	Environmental	*E. helveticus*	*Escherichia vulneris* (66.2)/ *Buttiauxella agrestis* (44.2)/ *Pantoeae* spp. (6.3)	*E. cancerogenus* (88)/ *P. agglomerans* (4)	*Escherichia vulneris*(66.2)/ *Buttiauxella agrestis* (44.2)/	-	-	-	-	*E. helveticus*

[a] - A. C. F. Almeida & M. L. P. Queiroz, Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Brazil; [b] - M. D. Asensi, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, Brazil; [c] -Not Classified; [d] – Powdered Infant Formula; [e] - M. T. Destro, Faculdade de Ciências Farmacêuticas, Universidade de Sao Paulo, Brazil.

Table 3. Reference strains used as negative controls in PCR assays.

INCQSa Code	Original code	Microorganism
00073	ATCCb 8090	*Citrobacter freundii*
00145	ATCC 13048	*Enterobacter aerogenes*
00146	ATCC 23355	*Enterobacter cloacae*
00074	ATCC 13047	*Enterobacter cloacae*
00586	CDCc 6453	*Enterobacter gergoviae*
00310	ATCC 11775	*Escherichia coli*
00033	ATCC 25922	*Escherichia coli*
00507	CTCd 0182/CIP 79.32	*Klebsiella oxytoca*
00083	ATCC 4352	*Klebsiella pneumoniae*
00147	ATCC 13883	*Klebsiella pneumonia subsp. pneumonia*
00201	ATCC 14029	*Plesiomonas shigelloides*
00332	ATCC 29906	*Proteus mirabilis*
00106	ATCC 13315	*Proteus vulgaris*
00323	ATCC 29944	*Providencia rettgeri*
00113	ATCC 15175	*Pseudomonas putida*
00028	ATCC 10708	*Salmonella Cholerasuis*
00258	ATCC 13076	*Salmonella Enteritidis*
00150	ATCC 14028	*Salmonella Typhimurium*
00100	ATCC 13880	*Serratia marcescens*

[a] – Instituto Nacional de Controle de Qualidade em Saúde; [b] - American Type Culture Collection; [c] - Center for Disease Control and Prevention; [d] - Coleção de Culturas Tropical.

Identification of *Cronobacter* spp. by PCR target genes detection

The study was first conducted using two groups of organisms, one identified as *Cronobacter* spp. (*E. sakazakii*) plus *Cronobacter* reference strains (Table 1); the other group was used as negative controls (Table 2).

The PCR protocols used were that from Hassan et al.[24] targeting the V1/V3 hypervariable region of the 16S rRNA gene and those from Lehner et al.[25] and Iversen et al.[18] targeting *glu*A. It was also tested a conventional PCR method developed by this study using the qPCR primers designed by Seo and Brackett[22] targeting *dna*G. For this new protocol, DNA extraction was performed according to Kothary et al.[26] with modifications. Briefly, a single colony from a Tryptone Soy Agar (TSA; Difco) plate was transferred to 5 mL BHI broth and incubated at 37 °C on a shaker (150 rpm; Orbit-Shaker/Lab-Line) to obtain approximately 6 x 10^8 CFU/mL (as previously determined by optical density experiments). From each culture, 2 mL-aliquots were centrifuged at 6,000 x g for 10 minutes, and pellets were re-suspended in 500 µL of sterile distilled water and heated at 100 °C for 15 minutes in a heating block (Dri-bath type 17600, Barnstead/Thermolyne). The aliquots were immediately cooled and stored at -20 °C for further use. The PCR reactions (Invitrogen reagents) were carried out in a total volume of 50 µL containing 3 µL of DNA template, 1 x *Taq* polymerase buffer, 2.0 mM $MgCl_2$, 100 µM of each dNTP, 5 pmol of each primer and 2 U *Taq* polymerase. Reactions were carried out in a Peltier Thermal Cycler (MJ Research) using an initial denaturation step at 94 °C for 2 minutes, followed by 30 cycles of 94 °C for 30 seconds, 60 °C for 30 second, 72 °C for 30 second and a final elongation step at 72 °C for 4 minutes. PCR products were applied to a 2% TAE (wt/vol) agarose (Sigma) gel, and electrophoresis was carried out (PowerPacBasic/BioRad) at 80 V. After ethidium bromide staining, images were digitized with the Video Documentation System (Pharmacia Biotech) and analyzed with ImageMaster software (Amersham Pharmacia Biotech). All of the experiments were performed in duplicate and repeated on separate days.

Evaluation of *Cronobacter* spp. behavior using the ISO/TS 22964:2006 methodology

All 37 cultures were grown in 10 mL-buffered peptone water (BPW; Merck) at 37 °C for 18 hours, and an aliquot of 0.1 mL from each culture was inoculated into modified Lauryl Sulfate Tryptose broth with vancomycin (mLST/v)[16] and incubated at 44 °C for 24 hours according to the ISO protocol. After incubation, the cultures were streaked onto Enterobacter Sakazakii Isolation Agar (ESIA; AES Chemunex), the chromogenic agar described in the current ISO technical specification for detection of *E. sakazakii*. Similarly, the cultures were also applied to Brilliance Enterobacter Sakazakii Agar (Druggan-Forsythe-Iversen formulation) (DFI; Oxoid) and Enterobacter Sakazakii Plating Medium (ESPM; R&F Products Inc.). The strains that showed no turbidity on mLST/v were streaked onto the surface of the solid media by a loopfull of growth from BPW. ESIA plates were incubated at 44 °C for 24 hours, and DFI and ESPM were incubated at 35 °C for 24 hours. Typical and atypical colonies from ESPM were streaked on Enterobacter Sakazakii Screening Medium (ESSM; R&F Products Inc.) to test for acid production from melibiose and sucrose. ESSM plates were incubated at 35 °C and observed after 6 and 24 hours. Tests for yellow pigment production were performed by streaking a loopful of the cultures onto Tryptone Soy Agar (TSA; Difco) plates. After incubation at 25 °C for 48 hours, plates were maintained at 25 °C under illumination for additional 72 hours.

Phenotypic tests

Phenotypic characterization was conducted according to Farmer et al.[27] and Iversen et al.[28]. The biochemical tests performed were: motility at 37 °C, acid production from carbohydrate (D-sucrose, D-melibiose, D-raffinose, D-sorbitol, L-rhamnose, D-cellobiose, adonitol, myo-inositol, dulcitol and methyl-α-D-glucopyranoside), gas production from glucose and methyl-α-D-glucopyranoside, utilization of citrate as a sole carbon source, malonate utilization, MR-VP test, indole production and nitrate reduction. Presumptive *Cronobacter* speciation of strains received as *E. sakazakii* was conducted through the identification of corresponding biogroups, according to Iversen et al.[1]. The miniaturized kits API 20E, API ZYM, ID 32E and the Vitek 32 system (bioMérieux) were also used according to the manufacturer's instructions.

Evaluation of sensitivity and specificity of the identification systems

The sensitivity and the specificity of the identification systems were determined by comparing results with the *glu*A PCR results[18], which were considered in this study as a gold standard for *Cronobacter* spp. identification. Sensitivity and specificity were calculated according to Greenhalgh[29]. Since *Cronobacter* and *E. sakazakii* are contaxic, the new nomenclature (*Cronobacter*) was used when comparing the results.

RESULTS

Identification of *Cronobacter* spp. by PCR target genes detection

Among the 37 cultures from the first group (Tables 1 and 2), 27 produced the expected fragment of 78 bp that is characteristic for *Cronobacter* spp.[22] when the PCR protocol developed by this study was used (Table 1). However, along with the negative control strains, strains A1, D2, 501M, 8F, 57L, M1, M3, M12 5A and N1 (isolated in Brazil and sent to our laboratory after being biochemically identified as *E. sakazakii*) did not produce the expected amplicon (Table 2). Similar results were obtained for *glu*A using both pairs of primers[18,25] and for the V1/V3 hypervariable region of the 16S rRNA gene[24], indicating consistency between the reference methods and the protocol proposed in this study.

The sensitivity and the specificity of the proposed protocol were 100% (Table 4). The same values were observed for the protocol of Hassan et al[24]; however, this protocol showed repeatability problems in our hands (data not shown).

***Cronobacter* behaviour using ISO/TS 22964:2006 methodology**

ISO/TS 22964:2006[16] is the technical specification for the detection of *Cronobacter* spp. in milk powder, PIF and environmental samples collected from milk powder or infant formula plants. This method was published before the reclassification of *E. sakazakii* as *Cronobacter* spp. and is currently in use by several researchers and companies across the globe. In this study, test and reference strains of *Cronobacter* spp. were inoculated in mLST/v broth, and the ISO protocol was followed. Surface plating was performed on ESIA, DFI and ESPM agars; these media all exploit the α-glucosidase activity of *Cronobacter* spp. Typical and atypical colonies from ESPM were tested for acid production from melibiose and sucrose on the surface of ESSM, according to Restaino et al.[30]. *Cronobacter* cultures are expected to give positive results for both carbohydrates.

In this study, all but two *Cronobacter* strains were able to grow well in the selective enrichment broth mLST/v (Table 4). Strains HPB 2856 and NCTC 9529 showed a very weak and almost imperceptible growth. Some non-*Cronobacter* strains were also able to grow in the selective enrichment broth, but only strain N1 (*Enterobacter helveticus*) produced characteristic colonies on the three chromogenic agars tested (Table 4). However, this isolate did not produce acid from sucrose on the ESSM medium. All *Cronobacter* spp. isolates and reference strains presented typical colonies (blue-black colonies with or without a clear halo) after a 24-hour incubation at 35 °C on ESPM medium, although the type strain of *Cronobacter* genomospecies 1 (NCTC 9529) showed poor growth with few small, blue-grey colonies with clear halos (data not shown). In this study, the 6-hour incubation period proposed by Restaino et al.[30] was not sufficient to detect the production of acid from melibiose on ESSM medium by six *Cronobacter* spp. strains (*C. sakazakii* ATCC 29544, ATCC 29004, CFS 10, HPB 2856, *C. turicensis* 3032 and *C. dublinensis* CFS 237), and the acid production from sucrose by five other isolates (*C. sakazakii* 4A, HPB 2887, ATCC 12868, IOC-PC and *C. malonaticus* CDC 1058-77) was very weak. This result can generate misidentification of an isolate. After additional 18 hours of incubation, all isolates

Table 4. Sensitivity and specificity of modified Lauryl Sulfate Tryptose broth/vancomycin (mLST/v), yellow pigment production on Tryptone Soy Agar (TSA), isolation culture media, phenotypic and molecular assays

Species	Growth in mLST/v[a]	Culture media				Production of yellow pigment on TSA[b]	Phenotypic assays				Molecular assays		
		DFI	ESIA	ESPM	ESSM		Conventional biochemical tests	API 20E	VITEK 32	ID 32E	*glu*A PCR	*dna*G PCR	V1/V3 PCR
All *Cronobacter* spp. (positive results/ tested strains)	25/27	27/27	27/27	27/27	26/27	26/27	27/27	27/27	26/27	27/27	27/27	27/27	27/27
Other *Enterobacteriaceae* (positive results/ tested strains)	6/10	1/10	1/10	1/10	NA[c]	8/10	0/10	4/10	0/10	0/10	0/28	0/28	0/28
Non-*Enterobacteriaceae* strains (positive results/ tested strains)	NT[d]	NT	NT	NT	NT	NT	NT	NT	NT	NT	0/1	0/1	0/1
Sensitivity (%)	93.1	100	100	100	96.4	96.4	100	100	96.4	100	100	100	100
Specificity (%)	62.5	90.9	90.9	90.9	NA	55.6	100	71.4	100	100	100	100	100

a - modified Lauryl Sulfate Tryptose broth/vancomycin (mLST/v); b - Triptone Soy Agar; c - Not Applicable; d - Not Tested.

showed positive results for melibiose in this medium. The strain *C. sakazakii* HPB 2891 showed a negative result for acid production from sucrose even after the additional 18-hour incubation period (Table 4).

The isolation media used in this study, DFI, ESIA and ESPM, were 100% sensitive (Table 4), while ESSM was 96.4% sensitive. The specificities of the DFI, ESIA and ESPM agars were all 90.9% (Table 4).

Production of yellow pigment was detected in all but one strain of *Cronobacter* spp. (*C. sakazakii* CDC 996-77), which developed a cream-coloured colony (Table 4). All the non-*Cronobacter* isolates, with the exception of isolates 57L and 5A, showed yellow-pigmented colonies on TSA (Table 4), indicating the low specificity of this test.

Phenotypic characterization

All the isolates and reference strains were phenotypically assessed using conventional biochemical assays recommended by the ISO methodology and the commercial kits API 20E, ID 32E and the Vitek 32 system. The identification schemes proposed by Farmer et al.[27] and Iversen et al.[28] were also applied to the isolates, which were characterized into biogroups (Tables 1 and 2). Presumptive *Cronobacter* speciation was determined by correlating biogroups with species, according to Iversen et al.[1]. The results given by the commercial tests (API 20E, Vitek 32 system and ID 32E) are presented using the former taxonomy (*E. sakazakii*) because the database for these kits had not been updated when this paper was prepared.

Using ID 32E, all *Cronobacter* strains were identified as *E. sakazakii* with percentage matches of 99.9% (Table 1), although strain HPB 2891 was designated as "doubtful profile". The Vitek 32 system confirmed the identity of all *Cronobacter* cultures except for the type strain *C. dublinensis* (CFS 237), which was misidentified as *Pantoea agglomerans,* with a percentage match of 95% and with only a 3% likelihood of being *E. sakazakii*. Most of the *Cronobacter* strains were correctly identified by the Vitek system, with values above 95%, except for the type strain of *C. malonaticus* (CDC 1058-77) and the *C. sakazakii* strain CFS 101, which showed percentage matches of 61% and 74% respectively (Table 1).

The results obtained with API 20E were, in some instances, in disagreement with the results of the conventional biochemical assays and with those of the other commercial kits. Strain HPB 2891 was considered by this kit's database as an "unacceptable profile". Two strains of *C. malonaticus* (type strain CDC 1058-77 and 2A) and six cultures of *C. sakazakii* (E922, CDC 3128-77, HPB 2875, HPB 2876, CFS 101 and ATCC BAA 894) presented very low percentage matches (51.1%) for *E. sakazakii* (Table 1). In addition, API 20E identified four non-*Cronobacter* strains (A1, D2, 501M e 57L) as *E. sakazakii,* most of them with high percentage values (Table 2).

Those strains confirmed as *Cronobacter* were subjected to enzymatic activity evaluation using the API ZYM (bioMérieux) kit. Twelve different enzymatic profiles were observed, and they were designated as "ZYM-types" 1 through 12 (Table 1). All strains showed α-glucosidase activity, characteristic for the genus. ZYM type 9 was the most prevalent, with 11 isolates (ten *C. sakazakii* and one *C. malonaticus*) showing this enzymatic profile. *C. turicensis* 3032, *C. dublinensis* CFS 237 and *C.* genomospecies 1 NCTC 9529 were the only strains classified as ZYM types 2, 11 and 12 respectively. *C. malonaticus* type strain (CDC 1058-77) was classified as the unique ZYM type 1 strain (Table 1), while the

other *C. malonaticus* strain (2A) was designated as ZYM type 9, similar to most of the strains evaluated in this study. *C. sakazakii* strains E922, HPB 2856 and HPB 2871 also presented unique profiles, designated as ZYM types 3, 7 and 8, respectively. The strain ATCC 51329 (*C. muytjensii*) showed the same enzymatic profile as two *C. sakazakii* strains (ATCC 29004 and isolate 1390).

The specificity and the sensitivity of the conventional biochemical assays and ID 32E were 100% (Table 4). The API 20E kit, frequently used by researchers and by food quality control/assurance laboratories for the identification of microorganisms present in PIF, showed 100% sensitivity but 71.4% specificity. On the other hand, the Vitek 32 system showed 100% specificity and 96.4% sensitivity (Table 4).

DISCUSSION

Cronobacter is a microbiological hazard associated with infant formula consumption and recognized as a cause of high case morbidity and mortality in neonates. The conventional methods for the detection of *Cronobacter* spp. are time-consuming and labor-intensive. There has also been a lack of consistency between results obtained with different detection and identification methods[14,24]. Despite all of the disadvantages, these conventional methods have been widely used for the detection of this genus in PIF at many countries' entry-exit inspection ports and quarantine bureaus[31].

This study used 37 strains, comprising isolates previously identified as *E. sakazakii*/*Cronobacter* spp. plus reference strains of *Cronobacter* to compare the results of the molecular assays (PCR detection of *dna*G, *glu*A and V1/V3 hypervariable region of the 16S rRNA gene region) to phenotypic profiles obtained by ISO methodology, conventional biochemical assays and miniaturized kits (API 20E, ID 32E, the Vitek system and API ZYM).

During the PCR assays, 19 reference strains (Table 3) of species commonly misidentified as *Cronobacter* spp. (negative controls) were included to evaluate a conventional PCR protocol for *dna*G gene detection using the primers designed by Seo and Brackett[22]. The original protocol has been used by several authors with consistent results[18,19, 23,32-34].

The proposed PCR protocol (*dna*G) and the other PCR protocols (*glu*A and V1/V3) identified 27 cultures as *Cronobacter* spp. (Table 1), and these genes were not detected in the strains used as negative controls. Results obtained using the new method, targeting *dna*G, showed equivalence to the protocol targeting *glu*A (Tables 1, 2 and 4). The results indicated that this protocol was 100% sensitive and specific. The *glu*A protocol was chosen as the gold standard for this study because it was considered 100% sensitive and specific by Iversen et al.[18].

Most of the strains isolated in Brazil were not confirmed as *Cronobacter* when the PCR protocols were used. According to the biochemical identification, they were classified among species commonly misidentified as *Cronobacter* (Table 2). One of these strains (8F) showed very low metabolic activity and could not be classified. Another strain (N1) was identified as a member of the recently described species *E. helveticus*[35]. This species can be found in the same ecological niches as *Cronobacter*, such as dried food products and factory environments, and it yields presumptively positive colonies on chromogenic agars designed for *Cronobacter* detection. These results show that molecular methods are faster and more reliable than some phenotypic assays for *Cronobacter* identification.

In this essay, it was verified that the selective enrichment step of the ISO/TS 22964:2006 method can be inhibitory to some *Cronobacter* strains (*C. sakazakii* HPB 2856 and *C.* genomospecies I type strain NCTC 9529) (Table 4). Limitations of the mLST/v enrichment broth have been reported elsewhere[17,36-38]. Further tests using regular LST formulation with and without vancomycin followed by incubation at 35 °C and 44 °C indicated that the inhibitory factor was the 44 °C temperature (data not shown). However, it is interesting to note that these strains were able to grow on ESIA at 44 °C. Thus, it may be a synergistic effect between the 44 °C temperature and the selective agent (sodium lauryl sulphate) in the culture medium. Proposals of new methodologies for *Cronobacter* spp. detection must consider the possible inhibitory effect of this temperature.

The chromogenic agars tested (ESIA, DFI and ESPM) showed equivalent performances, detecting all the *Cronobacter* isolates (Table 4). However, all three chromogenic agars supported the growth of the isolate N1 (*E. helveticus*), which also produced colonies typical of *Cronobacter* spp. on these media (Table 4). This isolate showed typical features of *Cronobacter* spp. in the selective enrichment and therefore could be presumptively identified as *Cronobacter*. However, the isolate did not produce acid from sucrose on ESSM agar and was not misidentified as *Cronobacter* by any of the identification

schemes used (Tables 2 and 4). DFI and ESPM agars were included in this study for performance evaluation because the new FDA protocol detection method, which is in process of validation[23], incorporates these media for the isolation of *Cronobacter* spp. colonies.

The use of ESSM as another tool for screening has to be evaluated with care. The 6-hour incubation period suggested by Restaino et al.[30] did not prove reliable in this study, with eleven (40.7%) *Cronobacter* isolates generating negative or doubtful results; a 24-hour incubation was necessary for consistent results. Iversen et al.[18] had the opposite problem, as they found transient melibiose-positive results at 6 hours. Additionally, sucrose-negative isolates, such as HPB 2891 (Table 4), may not be identified. For this reason, ESSM agar was considered 96.4% sensitive. This possibility has to be taken into account when using a culture media that utilizes this carbohydrate as a marker for *Cronobacter* spp.

Cronobacter spp. was previously designated as "yellow-pigmented *E. cloacae*". Based on this feature, the USFDA[15] and the ISO method[16] cite the use of yellow pigmentation as an indication of positive *E. sakazakii* (*Cronobacter* spp.) isolates. In this study, detection of a non-yellow pigmented *Cronobacter* isolate (*C. sakazakii* CDC 996-77) and yellow-pigmented non-*Cronobacter* isolates (A1, D2, 501M, 8F, M1, M3, M12 and N1) shows that this criterion is unreliable (Table 4). Moreover, it is possible that the ability of the Brazilian isolates to produce yellow pigment may have contributed to the misidentification of these strains during presumptive identification.

As previously described[18], the type strain of *C. muytjensii* (ATCC 51329) was the only *Cronobacter* culture that did not produce acid from methyl-α-D-glucopyranoside (data not shown). This strain developed typical colonies on the chromogenic agars, showed α-glucosidase activity detected by the commercial kits (ID 32E and API ZYM) and was positive for *glu*A by PCR assay. This result can be attributed to a possible difference between *Cronobacter* spp. in either specificity or inductility of the α-glucosidase enzyme and/or transport mechanism for the different glucopyranosides[18].

In this study, no correlation between enzymatic profiles and *Cronobacter* species, biogroups or origin of the isolates could be determined. Except for *C. sakazakii* and *C. malonaticus*, only one isolate from each species was tested, jeopardizing the comparison. Additional studies using a higher number of strains from each species and/or biogroups would be required to evaluate a possible relationship.

A comparison of the results for the identification of *Cronobacter* spp. obtained with the commercial kits showed that ID 32E was 100% sensitive and specific. These results are similar to those reported elsewhere[39,40] using the database version 3.0, available since the end of 2006. Reports of false-positive and false-negative results by ID 32E[32,38] were obtained with the previous database.

In this study, the Vitek 32 system showed a specificity of 100% but a sensitivity of 96.4%, which is in contrast to Fanjat et al.[40], who reported a 100% sensitivity for the identification of *E. sakazakii* (*Cronobacter*) using the system. Low sensitivity represents false-negative results and may lead to problems in food safety (production/quality assurance) or in public health (pathogen identification) laboratories.

In this study, API 20E showed a sensitivity of 100%, but its specificity was only 71.4%, the lowest specificity among the tested kits. Although this is the most widely used miniaturized kit for confirmation of presumptive *Cronobacter* colonies, several reports of false-positive and false-negative results can be found[18,30,32,38]. Therefore, the authors believe that the use of API 20E for the identification of *Cronobacter* should be evaluated with care, and ID 32E should be used preferentially.

Recently, Lampel and Chen[23] reported a new method devised by the FDA for the detection of *Cronobacter* spp. in PIF. According to these authors, the protocol is currently undergoing a collaborative validation study for final adoption into the FDA's *Bacteriological Analytical Manual*. This new method incorporates a bi-functional real-time PCR-based assay utilized as a screening tool and also for culture confirmation. FDA methodologies have been commonly adopted as official methods by many countries. However, the qPCR protocol may represent a technical barrier to those laboratories that do not have the financial, technical or physical resources to host the necessary equipment. Therefore, alternative molecular methods need to be considered for those laboratories, such as the use of the conventional PCR protocol targeting *dna*G proposed in this study. Thus, the developed conventional PCR protocol has proven its usefulness and effectiveness and it can be a good alternative for those laboratories not able to perform qPCR assays.

CONCLUSION

Most of the Brazilian isolates previously identified as *Cronobacter* spp by phenotypic characterization were not confirmed by the PCR protocols used in this study. Thus, we propose the use of PCR protocols targeting *glu*A and *dna*G to complement the detection or identification of *Cronobacter* spp. after traditional isolation and identification methods.

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