A method for the detection of *Cronobacter* strains in powdered milk-based foods using enrichment and real-time PCR

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Summary

Cronobacter spp. are emerging food-borne pathogens implicated in fatal infections in neonates and infants. As the contaminated powdered infant formula (PIF) has been identified as the major source of infection, a zero tolerance of *Cronobacter* spp. is required for these products. The aim of our study was to evaluate real-time PCR-based method for *Cronobacter* detection in milk powder-based products. TaqMan real-time PCR targeting the *dnaG* gene of MMS-operon was used. The system was evaluated as 100% specific for *Cronobacter* spp., which was determined using 97 *Cronobacter* strains and 85 non-*Cronobacter* strains with a PCR detection limit of 1×10^2 CFU·ml^{-1}. Shortened two-step enrichment was used. Out of 50 milk powder-based samples analysed, six were positive by the PCR-based method and five by the standard method. Out of 15 artificially contaminated PIF with *Cronobacter* at 10^0 CFU per 10 g, 15 and 13 were positive by respective methods. Low detection limit of the complete method was not compromised either by *Enterobacteriaceae* background up to 10^7 CFU per 10 g, or by dryness and cold stress of *Cronobacter* cells used for artificial contamination. This method facilitated a reliable detection of *Cronobacter* spp. alternative to the currently available method, providing a considerable time reduction in comparison to the microbiological standard detection method.

Keywords

pathogenic *Cronobacter* spp.; real-time PCR; detection; powdered milk food

Cronobacter spp. (formerly *Enterobacter sakazakii*) is a bacterial genus within the *Enterobacteriaceae* family. The bacterium was called „yellow pigmented *Enterobacter cloacae*“ until 1980 [1], when it was renamed to *Enterobacter sakazakii*. The World Health Organization and the Food and Agriculture Organization categorized *E. sakazakii* together with *Salmonella enterica* as potencially dangerous contaminants in powdered infant formula in 2004 [2]. Recently, *E. sakazakii* has been re-classified to six species within the *Cronobacter* genus containing *C. sakazakii*, *C. malonaticus*, *C. muytjensii*, *C. dublinensis*, *C. turicensis* and *Cronobacter* genomospecies I [3]. All species have been linked retrospectively to clinical cases of infection in either infants or adults and therefore all *Cronobacter* spp. should be considered pathogenic [4].

Cronobacter spp. cause a rare but fatal infection manifested as meningitis, meningoencephalitis, sepsis and necrotizing enterocolitis primarily in low birth weight and immunocompromised neonates with high mortality rate of 50–80% [5, 6].

Whereas *Cronobacter* spp. is widespread in the environment and has been isolated from various food and environmental samples [7, 8], the primary source of the organism and the main vehicle for its transmission in neonatal infections was found to be the rehydrated powdered infant formula [9]. *Cronobacter* spp. have been found in samples of infant formula powders at very low levels and it was demonstrated that these low numbers (less than 1 CFU per 100 g of the infant formula) may be responsible for infection [10]. Post-process contamination of powdered infant formula is considered a likely source of *Cronobacter* spp., since the usual milk pasteurization treatment in the production process is adequate to destroy the bacterium. However, some infant products with added ingredients may be produced via dry blending, without heat treatment, while *Cronobacter* spp. has been isolated from wide variety of production facilities

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[11]. Moreover, the presence in dried foods and powdered ingredients may be due to the increased desiccation resistance of *Cronobacter* strains [12].

According to the currently valid European Commission Regulation No. 2073 [13], a zero tolerance of *Cronobacter* presence is required for powdered infant formula and powdered dietary foods for infants up to 6 months of age. Therefore, the reliable detection method is necessary to control the microbiological quality of final products and ingredients used in powdered infant formula (PIF) production as well. Currently available standard method ISO/TS 22964 [14] for the detection of *Cronobacter* spp. (*E. sakazakii*) is based on two-step enrichment including non-selective enrichment for 18 h ± 2 h followed by selective enrichment for 24 h, isolation of presumptive colonies on chromogenic medium and several confirmation tests. The procedure is time-consuming and labour-intensive, and the biochemical confirmation need not always provide reliable results. The confirmation of presumptive *Cronobacter* colonies selected from chromogenic medium utilizes the detection of the production of a yellow pigment and several biochemical tests. This identification may not be sufficiently reliable as not all *Cronobacter* strains produce the yellow pigment [15]. This procedure is considerably labourious and moreover typical colonies on the chromogenic media are formed also by other bacterial species in addition to *Cronobacter* spp. [16]. This procedure requires up to six days to confirm positive results.

Therefore, there is a strong need for more rapid and specific methods for detection and identification of *Cronobacter* spp. During the last few years, several PCR-based methods have been developed that enable identification of *Cronobacter* spp. to the genus and species level. Real-time PCR-based methods provide the powerful tool for highly specific and sensitive identification and are considered reliable alternatives to conventional methods for food control purposes. Real-time PCR methods for *Cronobacter* spp. (*E. sakazakii*) detection utilizing various targets have been developed recently [17–20] and subsequently evaluated as having various level of specificity [7]. PCR-based detection of *Cronobacter* strains targeting *dnaG* gene of MMS-operon developed by Seo and Brackett [17] has been widely evaluated and confirmed to be efficient, the molecular target being found to be reliable [21, 22].

In this study, we describe the rapid and sensitive *Cronobater* spp. detection method using TaqMan real-time PCR targeted to *dnaG* gene, suitable for routine application. The developed method has been applied to naturally and artificially contaminated powdered milk-based infant foods and evaluated by comparing real-time PCR-based detection with the standard one.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Cronobacter spp. (*Enterobacter sakazakii*) strains used in this study were obtained from American Type Culture Collection (ATCC, Manassas, Virginia, USA), Belgian Co-ordinated Collections of Microorganisms in Gent (BCCM/LMG Bacteria Collection) and from Czech Collection of Microorganisms (CCM, Brno, Czech Republic). Five strains isolated from fruit powder were kindly provided by Prof. Stephan from the Institute of Food Safety and Hygiene (ILS), Vetsuisse Faculty University of Zurich, Switzerland. Most of used *Cronobacter* strains were isolated from various food samples from the market in Slovakia and identified in our laboratory [23]. Numbers of strains belonging to different *Cronobacter* species used in this study are summarized in Tab. 1. Bacterial strains other than *Cronobacter* were obtained from culture collections or from

Tab. 1. Results of *dnaG*-targeted real-time PCR inclusivity on a panel of 97 *Cronobacter* strains.

Cronobacter spp.	Number of strains	PCR result	Strain used for artificial contamination
C. sakazakii	73	+	ATCC 29544
C. malonaticus	10	+	LMG 23826
C. dublinensis	6	+	LMG 23823
C. muytjensii	5	+	ATCC 51329
C. turicensis	3	+	LMG 23827

Tab. 2. Results of *dnaG* real-time PCR of 85 non-*Cronobacter* strains for exclusivity testing.

Genus	Number of strains	PCR result
Enterobacter	13	–
Campylobacter	5	–
Citrobacter	16	–
Edwardsiella	1	–
Escherichia	10	–
Klebsiella	1	–
Proteus	1	–
Salmonella	27	–
Serratia	1	–
Yersinia	10	–

reference laboratories (Tab. 2). Five selected strains, one of each species (Tab. 1), were used for artificial contamination of food samples. All strains were maintained at –18 °C in 20% glycerol solution or freeze-dried for long-period storage. Working cultures were prepared by inoculation in Brain Heart (BH) broth (Merck, Darmstadt, Germany) and incubation for 20 h ± 1 h at 37 °C. Decimal dilutions of the culture were prepared in 0.85% NaCl and the cell concentrations were determined by the plate-count technique on BH agar (Merck) incubated overnight at 37 °C.

Preparation of DNA samples and 5'-nuclease real-time PCR

DNA samples were prepared from overnight cultures in BH broth (Merck) using lysis by boiling, as described previously [24]. DNA from *Cronobacter sakazakii* type strain ATCC 29544 to be used as PCR positive control was extracted using QiaAmp DNA Mini Kit (Qiagen, Hilden, Germany). Each PCR reaction contained 200 nmol·l^{-1} of primers, 200 nmol·l^{-1} of the TaqMan probe (all from Qiagen Operon), 500 mmol·l^{-1} of each dNTP (Applied Biosystems, Foster City, California, USA), 1.5 U of HotStar Taq DNA polymerase (Qiagen), 1 × concentrated PCR buffer, 4.5 mmol·l^{-1} magnesium chloride, TaqMan Exogenous Internal Positive control VIC (Applied Biosystems), 2.5 ml of the DNA sample and water to make the total volume up to 25 ml. Real-time PCR was performed in a PTC-200 thermal cycler coupled to a Chromo 4 continuous fluorescence detector (MJ Research, Waltham, Massachusetts, USA) using a thermal programme consisting of the initial denaturation of 15 min at 95 °C, and 45 cycles of 15 s at 95 °C and 60 s at 60 °C. Two positive and three negative controls were included in each experiment.

Determination of PCR detection limit and detection probability

In order to determine the DNA-based PCR detection limit, DNA from the overnight culture of type strain *C. sakazakii* ATCC 29544 was isolated using QiaAmp DNA Mini Kit. Concentration of total extracted DNA was determined by using Quant-iT PicoGreen Assay (Invitrogen, Carlsbad, California, USA) with fluorescence measurement in a Tecan Saphire2 plate reader (Salzburg, Austria). Two parallel PCR analyses of each diluted sample DNA as a template were performed.

For the determination of the practical PCR detection limit and PCR detection probability, two independent sets of parallel PCR analyses of suitably diluted (ranging from 10^4 to 10^0 CFU·ml^{-1}) of *C. sakazakii* ATCC 29544 and *C. muytjensii* ATCC 51329 overnight cultures were performed, respectively.

Enrichment

Two-step enrichment of 10 g sample consisting of the pre-enrichment in 90 ml of Buffered Peptone Water (Merck) at 37 °C for 18 h ± 1 h and selective enrichment (1/100) at 45 °C in modified Lauryl Sulfate Tryptose (mLST) broth (Merck) with additional NaCl (Merck) to the final concentration of 0.5 mol·l^{-1} and vancomycin 10 mg·l^{-1} (Fluka, Buchs, Switzerland) was used. The selective culture was followed by the PCR and the standard *Cronobacter* detection after 5 h and 24 h of enrichment.

Food samples and artificial contamination

Samples of milk powder-based foods and infant formula used in this study were obtained from retail markets and drug stores in Slovakia. All samples were analysed for the presence of *Cronobacter* spp. after enrichment using microbiological detection according to ISO/TS 22964 and the real-time PCR-based detection method. For artificial contamination, five different *Cronobacter* species listed in Tab. 1 were inoculated at two initial concentration levels of 10^0 and 10^1 CFU per 10 g sample and 90 ml of pre-enrichment medium, respectively. Simultaneously, stressed cells at the same concentration levels were inoculated. The stressed cells were prepared by dessication of culture dilutions applied on the filter paper in air-ventilated oven at 45 °C followed by 6 h freezing at –18 °C under aseptic conditions. Model *Enterobacteriaceae* microflora consisting of *Enterobacter cloacae* CCM 1903, *Citrobacter braakii* CCM 3393, *Escherichia vulneris* CCM 3681, *Salmonella* Enteritidis CCM 4420 strains at levels of 10^8, 10^7 and 10^6 CFU per 100 ml of medium was used as the background microflora. It was prepared from decimally diluted mixture of overnight cultures of individual strains at proportional concentrations.

Reference method

The method according to ISO/TS 22964 Milk and milk products – Detection of *Enterobacter sakazakii* [14] was used as the reference one. *Cronobacter* strains were detected in natural and artificialy contaminated food samples using the two-step enrichment procedure described above. Presumptive *Cronobacter* strains were selected on Chromogenic *Enterobacter sakazakii* agar (DFI formulation; Oxoid, Basingstoke, United Kingdom) as green-blue colonies. Selected colonies were confirmed on the basis of the yellow pigment

production when exposed to light during incubation on Tryptone Soya Agar (Oxoid) with bile salts (Fluka). Identification was confirmed by profile determination using API 20E system (bioMérieux, Marcy l'Etoile, France).

RESULTS AND DISCUSSION

Evaluation of *dnaG real*-time PCR specificity

The specificity of the real-time PCR detection system targeting *dnaG* gene of MMS operon developed by SEO and BRACKETT [17] was evaluated using 97 *Cronobacter* spp. including 70 well-identified isolates from our laboratory [23] resulting in the determination of the value of 100% inclusivity, and 85 non-*Cronobacter* strains of *Enterobacteriaceae* family resulting in in the determination of the value of 100% exclusivity. The results of *dnaG* real-time PCR for all strains are summarized in Tab. 1 and Tab. 2. The oligonucleotide sequences of the primers and probe used in the study are presented in Tab. 3.

Several PCR methods based on different target sequences were developed and subsequently validated for their specificity by other researchers. CAWTHORN et al. [25] valuated the PCR amplifications using six different genus-specific primer pairs targeting 16S rRNA gene, gene responsible for α-glucosidase activity and ITS sequence between 16S and 23S rDNA published previously. The specificity was determined to range from 8% to 92%. JARADAT et al. [8] used eight sets of previously published *Cronobacter* spp.-specific primers targeting ITS sequences, 16S rRNA, *ompA, zpx, gluA* and *gluB* genes and none of the methods proved to be a reliable method for the identification of *Cronobacter* isolates being free of false positives or false negatives.

On the other hand, the sequence of *dnaG* gene of MMS operon used in our study was considered 100% specific for *Cronobacter* strains by other researchers as well [21, 22], without obtaining any false-positive or false-negative results. The described PCR method provided reliable *Cronobacter* identification.

PCR detection limit

DNA-based detection limit was determined using DNA isolated from *C. sakazakii* ATCC 29544 diluted to 100 fg, 50 fg, 10 fg, 7.5 fg, 5 fg, 2,5 fg, 1 fg, 0.5 fg and 0.1 fg. The DNA amount of ≥ 5 fg in PCR reaction was detected in 10 parallel analyses with 100% detection probability (data not shown). This is equivalent to approximately one genome copy calculated based on *C. sakazakii* genome size of 4.37 Mbp ≈ 4.8 fg, (database GenBank, accession No. NC 009778, National Center for Biotechnology Information, Bethesda, Maryland, USA).

Practical PCR detection limit was estimated using lysed cells from decimally diluted overnight cultures of two *Cronobacter* strains (*C. sakazakii* ATCC 29544 and *C. muytjensii* ATCC 51329). The results of PCR analyses showed that the detection probability of the cell suspension was 100% at a concentration of $(1.0 \pm 0.2) \times 10^2$ CFU·ml^{-1} (equal to 2.5 CFU per reaction) and at higher concentrations for both strains, and 80% for *C. sakazakii* and 70% for *C. mutjensii* at a concentration of $(0.5 \pm 0.1) \times 10^2$ CFU·ml^{-1} (equal to 1.25 CFU per reaction; data not shown).

Comparable or higher detection limits using the same PCR system were obtained by other research groups. Detection limit from 10^2 CFU·ml^{-1} to 10^3 CFU·ml^{-1} was determined using real-time PCR and different target sequences for *Cronobacter* detection [18, 20]. Conventional PCR provided the detection at a higher level of more than 10^3 CFU·ml^{-1} [26].

For simultaneous detection of *Cronobacter* using PCR according to SEO and BRACKETT [17] and *Salmonella* spp., a detection limit of 10^3 CFU·ml^{-1} was determined for both targets without enrichment [27].

Based on the results obtained in this study, the *Cronobacter* concentration of at least 10^2 CFU·ml^{-1} should be reached by the enrichment procedure to ensure the reliable detection by downstream real-time PCR.

Evaluation of the enrichment procedure with a shortened second step

The time reduction of the enrichment procedure was evaluated in an attempt to obtain results on the next day after the sample reception. Five different *Cronobacter* species (Tab. 1) at initial concentrations of 10^1 and 10^0 CFU were inoculated to two-step enrichment described above. Selective enrichment of 5 h and 24 h, respectively, was followed by real-time PCR detection and standard detection according to ISO/TS 22964 was used as the reference method. For both levels of contamination, the selective enrichment of 5 h was found to be sufficient for *Cronobacter* detection using real-time PCR (data not shown).

Similar evaluation was performed with added artificial background microflora of *Enterobacteriaceae* strains. PCR provided more sensitive detection in the case of model *Enterobacteriaceae* background microflora up to 10^7 CFU added

Tab. 3. Oligonucleotides used in the study (5'-3').

Designation	Sequence and labelling
EsMMSf	GGGATATTGTCCCCTGAAACAG
EsMMSr	CGAGAATAAGCCGCGCATT
EsMMSp	FAM-AGAGTAGTTGTAGAGGCCGTGCTTCCGAAAG-TAMRA

Tab. 4. Results of real-time PCR detection of *C. sakazakii* ATCC 29544 at two contamination levels in combination with three concentration levels of background microflora.

Enterobacteriaceae background microflora (CFU per 100 ml)	*C. sakazakii* ATCC 29544 (CFU per 100 ml)			
	Result of PCR detection		Result of standard detection	
	10^1	10^0	10^1	10^0
–	+	+	+	+
10^6	+	+	+	+
10^7	+	+	+	–
10^8	+	–	+	–

to the sample, in comparison to 10^6 CFU using standard method. However, at the extreme load of 10^8 CFU, the detection of 10^0 CFU of *C. sakazakii* per sample completely failed using both methods. The results for the detection of *C. sakazakii* ATCC 25944 at contamination levels of 10^0 CFU and 10^1 CFU per 100 ml culture, in combination with the background microflora of 10^6, 10^7 and 10^8 CFU of *Enterobacteriaceae* mixture, are presented in Tab. 4.

Although new alternative selective media were developed for better recovery of *Cronobacter* strains isolated from foods, we used the two-step enrichment according to the currently valid reference method in order to compare the efficiency of microbiological and real-time PCR-based detection of *Cronobacter* spp. in milk powder-based infant foods.

Detection of *Cronobacter* spp. in food samples

The two step enrichment with reduced selective enrichment of 5 h-duration and real-time PCR detection was compared with the reference detection method. Fifty samples of milk powder-based foods, including 20 samples of PIF, were analysed. Six samples were found positive for *Cronobacter* spp. by the real-time PCR-based method and five samples by the standard detection method (Tab. 5). Compared to real-time PCR-based detection, one sample of the infant food was false-negative using the standard detection method.

The efficiency of the developed real-time PCR-based detection method was further evaluated using 15 samples artificially contaminated with *C. sakazakii* ATCC 25944 at two concentration levels. From 15 samples of PIF artificially contaminated at a level of 10^1 CFU per 10 g, all

Tab. 5. Results of *Cronobacter* spp. detection in milk powder-based foods.

Food sample	Number of samples	Number of positive samples	
		PCR detection	Reference method
Powdered infant formula (up to 6 months)	20	0	0
Infant food with rice	5	0	0
Infant food with fruit	5	0	0
Infant food with cocoa	5	1	0
Hot chocolate	5	2	2
Milk shake	5	2	2
Milk powder	5	1	1

Tab. 6. Results of *Cronobacter* spp. detection in powdered infant formulae artificially contaminated at two levels of 10^1 and 10^0 CFU per 10 g.

Artificial contamination	Number of samples	Number of positive samples			
		by PCR		by standard method	
		10^1	10^0	10^1	10^0
Unstresed cells	15	15	15	15	13
Stressed cells	15	15	15	15	11

samples were detected by both methods. From 15 samples contaminated at a level of 10^0 CFU per 10 g, 15 were detected by real time PCR and 13 by standard method. When stressed *Cronobacter* cells were used for artificial contamination of 15 PIF samples, 15 were detected by real-time PCR-based method and 10 by the standard one (Tab. 6).

Insufficient detection ability using the standard method was observed also by other investigators. DERZELLE and DILASSER [20] analysed 41 samples of infant formulae and samples from production environment for the presence of *E. sakazakii*. Using the standard enrichment procedure, 23 samples were positive by real-time PCR and 22 by the standard method, giving the concordance of 97.5%.

CONCLUSION

Currently, the standard method available for *Cronobacter* spp. (*Enterobacter sakazakii*) detection in milk and milk products according to ISO/IDF 22964 is based on two-step enrichment followed by microbiological and biochemical identification. This method is laborious as well as time-consuming, it takes up to six days to confirm positive results. Generally, molecular-based methods have a well-grounded potential to overcome the insufficiences of identification procedures associated with the results based on the biochemical characteristics. The aim of our study was to develop and optimize sensitive and reliable method for the rapid and definite detection of *Cronobacter* spp. in foods, particularly milk-powder based infant foods. The developed real-time PCR-based method, which involves an optimized shortened selective enrichment, facilitated the detection of *Cronobacter* spp. on the next day after the sample reception. The method is fully effective also in the presence of background *Enterobacteriaceae* strains in concentrations up to 10^7 CFU per sample. Moreover, our results suggest that, besides being significantly faster, the molecular-based method may be more sensitive than the conventional culture method. The developed method can be considered as a faster and reliable alternative for *Cronobacter* detection.

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