SHORT COMMUNICATION

Identification of thermotolerant *Cronobacter* strains using multiplex real-time polymerase chain reaction

EVA KACLÍKOVÁ – KATARÍNA ORAVCOVÁ

Summary
Cronobacter sp. is considered an opportunistic pathogen implicated in food-borne life-threatening infection, in particular in neonates and infants. Contaminated powdered infant formula was identified as the primary source of *Cronobacter* sp. at very low levels. Multiplication of survived cells of thermotolerant *Cronobacter* strains was often found a crucial factor for causing outbreaks. The aim of our work was to develop and evaluate a detection method for thermotolerant *Cronobacter* strains using triplex TaqMan real-time polymerase chain reaction (PCR) targeting a specific sequence for *Cronobacter* sp., a thermotolerance marker and an internal amplification control. The proposed method was evaluated on 48 strains, including 32 *Cronobacter* sp. and 16 related species of *Enterobacteriaceae* family. The results showed 100% inclusivity and exclusivity, and 100% correlation with the time required to kill 90 % of cells exposed at 58 °C (D_{58}-value). The developed real-time PCR method facilitated rapid and specific detection of thermotolerant *Cronobacter* strains isolated from powdered infant formula products or from the production chain.

Keywords
Cronobacter; thermotolerance; multiplex real-time polymerase chain reaction; powdered infant formula

Cronobacter sp. has been implicated in severe form of neonatal meningitis associated with powdered infant formula as the route of transmission [1], whereas the bacteria are widely distributed in environment and various foods. The increased stress tolerance, including thermotolerance of some *Cronobacter* strains [2, 3], can promote their survival in production facilities. Considering the mode of production and the practice in the preparation of reconstituted infant formula, termotolerant *Cronobacter* strains could be a risk factor related to their presence in powdered infant formula products, particularly in those intended for neonates.

The study on ability of twelve *Cronobacter* strains to survive heating in rehydrated infant formula at 58 °C [4] demonstrated D_{58}-values (time required to kill 90 % of cells exposed at 58 °C) ranging from 30.5 s to 591.9 s, with the strains appearing to fall into two distinct heat resistance phenotypes including ATCC 51329 (group of thermosensitive strains) and ATCC 29544 (group of thermotolerant strains). Thermal resistance among the studied *Cronobacter* strains varied up to 20-fold. These two groups of strains of markedly different thermotolerance were used by WILLIAMS et al. [5] to identify a biomarker specific for this phenotypic trait via protein expression profiles using liquid chromatography-mass spectrometry. From the individual expression profiles, unique proteins corresponding to thermoresistant strains were identified. One of these proteins was identified, sequenced and identified as homologous to a hypotethical protein found in a thermotolerant bacterium *Methylobacillus flagellatus* KT and then reverse-engineered into specific polymerase chain reaction (PCR) primers.

In this work, a method for the detection of thermotolerant *Cronobacter* strains was developed and evaluated. The method uses triplex TaqMan

Eva Kaclíková, Department of Microbiology, Molecular Biology and Biotechnology, Food Research Institute, National Agricultural and Food Centre, Priemyselná 4, 824 75 Bratislava 26, Slovakia.
Katarína Oravcová, Department of Microbiology, Molecular Biology and Biotechnology, Food Research Institute, National Agricultural and Food Centre, Priemyselná 4, 824 75 Bratislava 26, Slovakia. Current address: School of Medicine, University of St. Andrews, North Haugh, St. Andrews KY16 9TF, United Kingdom.

Correspondence author:
Eva Kaclíková, e-mail: kaclikova@vup.sk

Tab. 1. D_{58}-values and results of multiplex real-time PCR for 48 strains of *Enterobacteriaceae* family.

Strain	D_{58} [s]	PCR result		
		*dna*G	Mfla 1165	IAC
Cronobacter sakazakii ATCC 29004	188 ± 17	+	+	n/a
Cronobacter sakazakii ATCC 29544	122 ± 14	+	+	n/a
Cronobacter sakazakii LMG 2786	148 ± 19	+	+	n/a
Cronobacter sakazakii ILS 744/03	129 ± 11	+	+	n/a
Cronobacter sakazakii 051206/10	163 ± 10	+	+	n/a
Cronobacter sakazakii 201206/21	136 ± 18	+	+	n/a
Cronobacter sakazakii 210307/19	287 ± 29	+	+	n/a
Cronobacter sakazakii 091007/14	182 ± 9	+	+	n/a
Cronobacter sakazakii 061107/08	212 ± 24	+	+	n/a
Cronobacter sakazakii ATCC BAA 894	39 ± 17	+	–	n/a
Cronobacter sakazakii 220108/52	28 ± 8	+	–	n/a
Cronobacter sakazakii LMG 2759	34 ± 5	+	–	n/a
Cronobacter sakazakii LMG 2762	28 ± 5	+	–	n/a
Cronobacter sakazakii CCM 3460	26 ± 7	+	–	n/a
Cronobacter sakazakii CCM 3479	22 ± 6	+	–	n/a
Cronobacter sakazakii ILS 1155/04	32 ± 9	+	–	n/a
Cronobacter sakazakii 201206/19	30 ± 11	+	–	n/a
Cronobacter sakazakii 201206/23	39 ± 6	+	–	n/a
Cronobacter sakazakii 210307/06	36 ± 8	+	–	n/a
Cronobacter sakazakii 280108/01	22 ± 10	+	–	n/a
Cronobacter sakazakii 050208/13	32 ± 8	+	–	n/a
Cronobacter sakazakii 120808/27	19 ± 5	+	–	n/a
Cronobacter malonaticus LMG 23826	137 ± 15	+	+	n/a
Cronobacter malonaticus 120808/24	151 ± 12	+	+	n/a
Cronobacter malonaticus 161007/35	14 ± 5	+	–	n/a
Cronobacter malonaticus 260808/02	28 ± 11	+	–	n/a
Cronobacter dublinensis LMG 23823	38 ± 12	+	–	n/a
Cronobacter dublinensis 260808/01	29 ± 8	+	–	n/a
Cronobacter turicensis LMG 23827	20 ± 7	+	–	n/a
Cronobacter turicensis 290708/07	22 ± 9	+	–	n/a
Cronobacter muytjensii ATCC 51329	42 ± 11	+	–	n/a
Cronobacter condimenti LMG 26250	28 ± 7	+	–	n/a
Enterobacter aerogenes CCM 2531	33 ± 7	–	–	+
Enterobacter amnigenus CCM 3430	18 ± 6	–	–	+
Enterobacter cancerogenus CCM 2421	38 ± 10	–	–	+
Enterobacter cloacae CCM 1903	185 ± 20	–	+	n/a
Enterobacter cloacae CCM 2320	141 ± 15	–	+	n/a
Enterobacter gergoviae CCM 3459	28 ± 7	–	–	+
Citrobacter brakii CCM 3393	161 ± 17	–	+	n/a
Citrobacter freundii CCM 4475	176 ± 14	–	+	n/a
Citrobacter koseri CCM 4472	41 ± 10	–	–	+
Citrobacter koseri CCM 2537	29 ± 7	–	–	+
Escherichia coli CCM 4787	24 ± 6	–	–	+
Escherichia coli CCM 2024	21 ± 7	–	–	+
Escherichia vulneris CCM 3681	117 ± 12	–	+	n/a
Salmonella Enteritidis CCM 4420	22 ± 6	–	–	+
Salmonella Typhimurium CCM 4419	28 ± 9	–	–	+
Yersinia enterocolitica CCM 5671	37 ± 10	–	–	+

D_{58} – time required to kill 90 % of cells exposed at 58 °C, values represent mean ± standard deviation; *dna*G – *Cronobacter* genus-specific PCR (using FAM-labelled probe); Mfla 1165 – thermotolerance marker-specific PCR (using Cy5-labelled probe); IAC – internal amplification control (using HEX-labelled probe; should be positive in case of negative A and B).
n/a – not applicable (in case of positive FAM or Cy-5 signal).

real-time PCR targeting specific DNA sequences for *Cronobacter* sp., a thermotolerance marker and an internal amplification control.

Cronobacter strains were isolated and identified in our laboratory [6] or obtained from official culture collections: American Type Culture Collection (ATCC, Manassas, Virginia, USA), Belgian Co-ordinated Collections of Microorganisms (BCCM/LMG Bacteria Collection, Gent, Belgium), and from Czech Collection of Microorganisms (CCM, Brno, Czech Republic). Two strains were kindly provided by Prof. Roger Stephen from University of Zürich, Zürich, Switzerland). DNA from culture suspensions was extracted by cell lysis at 95 °C in buffered solution, as described previously [7].

Genus-specific TaqMan real-time PCR targeting *dna*G gene of macromolecular synthesis (MMS) operon [8] with 6-carboxyfluorescein (FAM)-labelled probe was used. The specificity of the system was previously evaluated in our laboratory using 97 *Cronobacter* sp. and 85 non-*Cronobacter* strains of *Enterobacteriaceae* family demonstrating 100% inclusivity and 100% exclusivity [9]. The TaqMan real-time PCR with cyanine 5 (Cy5)-labelled probe (ThrF: gcg caa att ctg ggt tat gg, ThrR: cag ccg act ttt gct caa tg, ThrP: Cy5-cca cgt tgg cgt gga tat cgt ggg-BHQ3) for the detection of thermotolerant strains, targeting a sequence of the gene encoding for Mfla 1165 homological protein [5], was originally developed in our laboratory. Real-time PCR for internal amplification control using pUC19 DNA as a target and 6-hexachlorofluorescein (HEX)-labelled probe were developed in our laboratory (pUC19F: cgc gtt ggc cga ttc a, pUC19R: tca ctg ccc gct ttc ca, pUC19P: HEX-taa tgc agc tgg cac gac agg ttt cc-BHQ1).

Real-time PCR was performed in a PTC-200 thermal cycler coupled to a Chromo 4 continuous fluorescence detector (MJ Research, Waltham, Massachusetts, USA) with increasing amplification curve taken as a positive result. Two positive and three negative controls were included in each run.

The reference method for the determination of thermotolerance was based on colony forming units enumeration of survived *Cronobacter* cells after their incubation at 58 °C, with the D_{58}-values being calculated and expressed as previously described [10]. Despite the difficulties to compare *D*-values for individual strains obtained by different researchers, as they are influenced by many factors including growth conditions or heat-cold shock treatment, *D*-values measured in the same experimental conditions showed very good reproducibility.

The developed method was evaluated with 32 *Cronobacter* strains and 16 related species of *Enterobacteriaceae* family (from genera *Enterobacter*, *Citrobacter*, *Escherichia*, *Salmonella* and *Yersinia*), including 11 potentially more thermotolerant *Cronobacter* strains and 5 potentially more thermotolerant non-*Cronobacter* strains. As more thermotolerant, strains with D_{58} value higher than 100 s (ranging from 117 s to 287 s) were considered. Strains considered thermosensitive showed D_{58} values lower than 50 s (ranging from 42 s to 14 s). All 16 thermotolerant strains provided positive results and all 31 thermosensitive strains negative results of PCR for thermotolerance marker (Tab. 1), thus indicating 100% inclusivity and 100% exclusivity. The multiplex approach facilitated, in single analysis, as an added value to European Comission requirements [11], identification of thermotolerant *Cronobacter* strains, which were positive by both *Cronobacter* genus-specific PCR and by thermotolerance marker-specific PCR.

Because the method is rapid and specific, it is suitable for microbiological analysis of strains isolated from powdered infant formula products. The approach developed in this study can be utilized also to design analogical methods for identification of thermotolerant strains of other food-borne pathogens. The new methods can also use internal amplification control (IAC) system developed in this study to eliminate false negative results.

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