Original Research Article

Isolation and Characterization of *Cronobacter* spp. from Environmental and Food Resources

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Abstract

Cronobacter spp. (formerly *Enterobacter sakazakii*) has been isolated from a wide range of environmental and several food sources. *Cronobacter* spp. is an opportunistic pathogen causing serious infection in infants, particularly neonates. The aim of this study was to isolate and characterize *Cronobacter* spp. from food sources (infant food, herbs and spices and vegetables) and from environmental sources as dust from vacuum cleaners. Isolation of *Cronobacter* spp. was performed on selective chromogenic agars, firstly using commercial ESIA agar and thereafter on Kim and Rhee-KR agar described in the literature. Phenotypic characteristics were obtained by commercial miniaturized biochemical ENTEROTEST 24 kits and the final confirmation of isolated strains was performed by molecular techniques (PCR, PCR - DGGE analysis, and 16S rDNA sequencing). Altogether, 99 samples were analyzed (47 samples of foods and 52 samples of dust). In total, 43 isolates of presumptive *Cronobacter* spp. were initially identified, however, only 22 isolates (51%) were identified as *Cronobacter* spp. with high identity scores (75-99%). The occurrence of presumptive cronobacters in environmental samples was significantly higher than in samples of food (18 out of 52 vs. 4 out of 47; P = 0.003). No cronobacters were found in 17 samples of infant food, 3 isolates originated from herbs and spices, 1 isolate from spinach and 18 isolates from samples of dust (households, restaurants, dormitory rooms). It can be concluded that *Cronobacter* spp. is a ubiquitous pathogen contaminating food and environment. *Cronobacter* spp. could be well identified by means of ENTERO24 test kits with high probability. Both phenotypic and genotypic methodology could be used for identification of *Cronobacter* spp. and they can be combined for reliable identification.

Keywords: Infant food; environment; dust; *Cronobacter* spp.

INTRODUCTION

Cronobacter spp. (formerly *Enterobacter sakazakii*) was defined by Farmer et al. (1980). It has been recognized as an emerging opportunistic pathogen. The first cases of illness attributed to this organism occurred in 1958 in England (Urmenyi and Franklin, 1961). *Cronobacter* spp. occasionally causes foodborne illness in premature bodies and neonates. The pathogen is occasionally present in infant milk formula. It has been associated with outbreak of a rare form of infant meningitis, necrotizing enterocolitis (NEC), bacteraemia and neonate deaths (Simmons et al., 1989; Lai, 2001; Kucerova et al., 2010).

Cronobacter spp. is a gram-negative, facultatively aerobic, rod-shaped bacterium. This organism is a microbiological hazard occurring in the infant food chain with historic high morbidity and mortality in neonates (Lai, 2001). The ubiquitous *Cronobacter* spp. is considered as remarkably resistant to dryness, osmotic stress and moderately thermotolerant, accompanying contamination in a wide range of sources such as clinical specimens (Urmenyi and Franklin, 1961), foods (Iversen and Forsythe, 2003; Kandhai et al., 2004), and environmental sources (Friedemann, 2007; Jaradat et al., 2009).

Isolation of *Cronobacter* spp. has been based on the report by Muytjens et al. (1984) that cronobacters have a unique α-glucosidase reaction. The DFI medium (Iversen et al., 2004), OK medium (Oh and Kung, 2004), *Enterobacter sakazakii* chromogenic plating medium (ESPM) (Restaino et al., 2006), and *Enterobacter sakazakii* isolation agar (ESIA) have been recommended by the International Organization for Standardization-International Dairy Federation (Besse et al., 2006). Meanwhile, Kim and Rhee (2011) have used Kim and Rhee-KR medium as a selective, differential and cost-effective medium for isolating *Cronobacters* spp.

Cronobacter spp. produces basal yellow-pigmented colonies on TSA agar (Tryptone Soy Agar, Iversen et al., 2007) and it has been proposed recently to be reclassified as novel five species and one genomospecies

in a new genus named *Cronobacter* within the family *Enterobacteriaceae* in 2008, based on partial 16S rRNA sequence analysis (Iversen et al., 2008). Biochemical, chromogenic assays, PCR and 16S rRNA sequencing should be utilized for reliable identification. The aim of this study was to isolate *Cronobacter* spp. from the wide range of food and environment sources in an attempt to find the reservoir of this pathogen, and characterize the presence of *Cronobacter* spp. by means of a biochemical test kit. Finally, we also confirmed the identity of presumptive *Cronobacter* spp. isolates by molecular techniques such as PCR method, PCR - DGGE analysis, and 16S rDNA sequencing.

MATERIALS AND METHODS

Sample collection

In the first stage, samples of foods including infant foods, herbs and spices, and vegetables were purchased from local markets. Thereafter, environmental samples focused on dusts were obtained from vacuum cleaners in households, restaurants, and dormitory rooms, and were analyzed. Samples were collected in Prague, Czech Republic.

Bacteria enrichment

Ten grams of samples were weighed and put into sterile bottles containing 90 ml of peptone water phosphate buffer (pH = 7). After shaking on stirrer, bottles with samples were incubated at 37 °C overnight. Sub-samples (0.1 ml) were taken and resuspened into tubes containing 10 ml selective mLST media (modified Lauryl Sulphate Tryptose medium with vancomycin at 10 mg/10 ml) and then incubated at 44 °C for 24 hours (CSN P ISO/TS 22964). Lauryl sulphate broth was obtained from Merck KGaA (Darmstadt, Germany).

Isolation of *Cronobacter* spp.

In this study, two methods of *Cronobacter* spp. isolation were used. The ESIA method (Besse et al., 2006) was used at the beginning of the project for isolation of *Cronobacter* spp. from food and environmental source. Thereafter, new Kim and Rhee method (Kim and Rhee, 2011) was used for *Cronobacter* spp. isolation. Briefly, 10 µl from mLST incubate were taken and streaked on Petri dishes with selective chromogenic Enterobacter Sakazakii Isolation Agar (ESIA) using bacteriological needle. ESIA was obtained from AES Laboratories (Bruz, France). Agar plates were then incubated at 44 °C overnight. Typical colonies of small to medium size (1 mm to 3 mm), green and blue-green emerged which were assumed as positive screening for the *Cronobacter* spp. Using the same procedure 10 µl of the mLST media incubate were plated on the Kim and Rhee-KR agar. Plates were incubated at 44 °C for 24 hours. Bacteria created specific colonies with violet-colored centers surrounded by a transparent to opalescent border that were assumed as *Cronobacter* spp. colonies.

Pro-specific propagation

Positive *Cronobacter* spp. colonies both from ESIA agar and Kim and Rhee-KR agar were picked up and plated on Petri dishes with TSA agar. Plates were incubated at 25 °C for 48 hours. Typical yellow colonies emerged and cell morphology was examined microscopically after Gram staining. Thereafter, isolates showing good growth were picked up and subjected to further characterization using biochemical test kit. Confirmed cultures were preserved in broth containing 50% glycerol and stored at -20 °C for further confirmation.

Cronobacter spp. characterization

Typical yellow colonies were picked up from TSA Petri dishes agar, resuspended in physiological saline and subjected to subsequent biochemical characterization (initial presumptive identification of cronobacters). Procedures and interpretation of identification were performed following the ENTEROTEST 24 test kit manufacturer instruction (PLIVA-Lachema Diagnostic CZ). The Fisher´s Exact Test was used to evaluate difference in occurrence of cronobacters in food and environmental samples.

Molecular confirmation

After phenotypic characterization, presumptive identification of isolated strains was confirmed by molecular techniques such as *rpoB* based PCR method, PCR-DGGE analysis (denaturing gradient polyacrylamide gel electrophoresis), and 16S rDNA sequencing.

Isolation of bacterial DNA

Molecular characterization of isolates was performed as follows: Bacterial cells from overnight grown cultures were washed with 1× TE buffer, checked for purity, and centrifuged (0.5 ml of growth culture at 2897.856 g for 5 minutes at room temperature). Decant the supernatant, 1 ml of 1 × TE buffer was added to the cells, mixed by vortex

mixer and centrifuged again. Supernatant was removed and washed once more with 1xTE buffer. After that, cells were used for DNA isolation according to the kit protocol for gram negative bacteria of Qiagen – DNeasy Tissue Kit and at the end of isolation process, 200 µL of bacterial DNA solution were used for next amplification.

Amplification of bacterial DNA

RedTaq Ready Mix (Sigma) was used for preparation of PCR templates which was done in a box sterilized by UV light (15 minutes). PCR reaction was performed in 200 µL microtubes in Biometra thermocycler, containing 15 µl Red Taq Ready Mix (Sigma, R2648-20RXN) containing 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dNTPs mix, universal primers sequence (338GC and 534R) at 10 pmol each (0.5 µl forward and 0.5 µl reverse primers), 1 µl PCR product and 13 µl dH_2O. As PCR conditions, 1 min at 94 °C followed by 55 °C for 2 min and 72 °C for 3 min were applied. Cycling was completed by a final elongation step at 72 °C for 5 min. The amplification was performed in a thermocycler (Biometra, Germany). After PCR the amplicons were separated on a 1% agarose gel, stained with ethidium bromide and visualized under UV light. The correct size products were excised from the gel and purified using the MinElute™ gel extraction kit (Qiagen, Switzerland).

Purification of DNA Amplicon

It was done by PCR purification kit of QIAGEN according to manufacturer's protocol. Elution was done into 200 µL. Thereafter, those amplicons were utilized and/or kept in -40 °C refrigerator for sequence confirmation.

PCR technique confirmation

For amplification, mixtures (total volume 30 µl) containing 15 µl Red Taq Ready Mix (Sigma, R2648-20RXN) with a concentration of 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dNTPs mix, three sets of primers sequence (Stoop *et al*., 2009) at 10 pmol each (0.5 µl forward and 0.5 µl reverse primers), 1 µl PCR product and 13 µl dH_2O were prepared. Thermal cycling was carried out using an initial denaturation step of 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min and 30s of annealing. Annealing temperatures as well as elongation time were chosen dependent on expected fragment size and primer constitution. Elongation was performed at 72 °C. Amplification conditions were optimized by gradually increasing the annealing temperature in the assay. The reaction products were resolved on a 1.5% agarose gel followed by ethidium bromide staining and examination under UV light (Stoop et al., 2009).

PCR - DGGE analysis confirmation

DGGE is an electrophoretic method with constant 60 ºC heat and it is used to identify single base changes in a segment of DNA. The universal bacterial primers 534R and 338GC (annealing temperature at 55 °C) were used to amplify 1500 bp of the 16S rDNA gene. The amplicons obtained from the first PCR were then subjected to DGGE analysis. DGGE was performed using about 5 µl per lane of PCR products. These were electrophoresed in 1.5 mm

Table 1: Environmental and food sample tested for the presence of *Cronobacter* spp.

Samples	No. of samples	Positive isolates	Percentages of isolates	Presumptive *Cronobacter* spp.
Food source	**47**	**8**	**18.18**	**4**
Infant food	**17**	**0**	**0**	**0**
Milk formulas	*7*	*0*	*0*	*0*
Chocolate*s*	*6*	*0*	*0*	*0*
Jams	4	*0*	*0*	*0*
Herbs and spices	**15**	**7**	**15.90**	**3**
Herbs	*4*	*0*		*0*
Spices	*6*	*4*	*9.08*	*0*
Instant teas	*5*	*3*	*6.81*	*3*
Vegetables	**15**	**1**	**2.27**	**1**
Environmental source	**52**	**36**	**81.82**	**19**
Households	**18**	**14**	**31.81**	**7**
Restaurants	**17**	**13**	**29.54**	**6**
Dormitory rooms	**17**	**9**	**20.45**	**6**
Total	**99**	**44**	**100**	**23**

[1]ESIA agar, [2]ENTEROTEST 24

thick polyacrylamide gels, submerged in 7L of 1xTAE buffer. The linear denaturing gradient ranged from 35% to 60%. Electrophoresis was performed for 18h at 60V. Gels were stained with ethidium bromide, unstained with deionised water, visualized on an UV transilluminator and photographed. The EC3 gel (UVP Co.) documentation system was used (Muyzer et al., 1993).

16S rDNA sequencing confirmation

After sequencing PCR product by BigDye Termination kit and purifying the sequencing product by QIAGEN according to manufacturer's protocol the amplified fragments were sequenced (Iversen *et* al., 2008) using the universal primers as fD1 and RP2, and again BigDye Terminator kit (Applied Biosystems). Full-length 16S rDNA sequences were manually checked for errors and edited in ChromasLite program. Edited sequences were exported into FASTA format and identification of sequences was done on web databases, like BLASTn (for bacteria select and other database), multiple sequences can be aligned and compared in BioEdit program.

RESULTS AND DISCUSSION

In this study, isolation of *Cronobacter* spp. is worth mention in an attempt to pinpoint their source from a range of food and environmental sources (Iversen and Forsythe, 2003; Friedemann, 2007). In total 99 samples were analyzed (47 samples of food and 52 samples obtained from the environment) and investigated for the presence of *Cronobacter* spp. However, it was noteworthy to state that occurrence of presumptive cronobacters in environmental samples was significantly higher than in samples of food (18 out of 52 vs. 4 out of 47; P= 0.003). Thus, the highest positive result of *Cronobacter* spp. isolation was observed in dust (Table 1). No sample of infant milk formula contained *Cronobacter* spp. This positive result agreed with report of Iversen and Forsythe (2004) that final product after pasteurization would be free pathogenic bacteria.

Thereafter, 20 presumptive isolates of shape-rod were subjected to molecular technique as a step of confirmation for evaluation of genetic traits using based on amplification products. Initial PCR-DGGE analysis

Table 2: Characterization and confirmation *Cronobacter* spp. isolates by biochemical test kit and molecular methods as PCR analysis and 16S rDNA sequencing

Samples analyzed	Source	ENTERO24 test kit		PCR test			16S rDNA sequencing	
		result	%	[c]Csakf/Csakr	[d]Cmalf/Cmalr	[c]Cturf/Cturr	result	%
S1-3	Tea	**Cronob.*	77.78	+	-	-	*C. sakazakii*	100
S4-2	Dust	*Cronob.*	87.36	+	-	-	*C. sakazakii*	99
S3-1	Spices	*Cronob.*	94.90	+	-	-	*C. sakazakii*	99
S3-2	Spinach	*Cronob.*	95.64	+	-	-	*C. sakazakii*	99
S5-1	Dust	*Cronob.*	97.95	+	-	-	*C. sakazakii*	99
S5-2	Dust	*Cronob.*	97.74	+	-	-	*C. sakazakii*	99
S4-5	Dust	*Cronob.*	77.68	+	-	-	*C. malonaticus*	99
S7-1	Dust	*Cronob.*	77.70	+	-	-	*C. sakazakii*	100
S7-2	Dust	*Cronob.*	95.64	+	-	-	*C. sakazakii*	99
S8-2	Dust	*Cronob.*	99.00	+	-	-	*C. sakazakii*	98
S8-3	Dust	*Cronob.*	95.60	+	-	-	*C. sakazakii*	100
S8-4	Dust	*Cronob.*	89.00	+	+	-	*C. sakazakii*	100
S10-1	Dust	*Cronob.*	95.40	+	+	-		
S10-2	Dust	*Cronob.*	95.50	+	-	-		
S11-3	Dust	*Cronob.*	75.00	+	+	-		
S11-5	Dust	*Cronob.*	77.70	+	+	-		
S11-6	Dust	*Cronob.*	93.00	+	+	-		
S11-7	Dust	*Cronob.*	94.90	+	-	-		
S11-8	Dust	*Cronob.*	95.64	+	+	-		
S2-7	Tea	*Cronob.*	97.94	+	-	-	*C. sakazakii*	99
Control	[#]ICT	[a]*C. malo.*	99.85	-	+	-	*C. malonaticus*	100
Control	ICT	[b]*C. saka.*	97.94	+	-	-	*C. sakazakii*	100

**Cronob.*= *Cronobacter* spp., [#]ICT = Institute of Chemical Technology of Prague, [a]C. malo. = *Cronobacter malonaticus*, [b]*C. saka.* = *Cronobacter sakazakii*, [c]Csakf/Csakr = Csakf 5′-ACGCCAAGC CTATCTCCGCG-3′/ Csakr 5′-ACGGTTGGC GTCATCGTG-3′, [d]Cmalf/Cmalr = Cmalf 5′-CGTCGTATC TCTGCTCTC-3′/ Cmalr 5′-AGGTTGGTG TTCGCCTGA-3′, [c]Cturf/Cturr = Cturf 5′-CGGTAAAAG AGTTCTTCGGC-3′/ Cturr 5′-GTACCGCCA CGTTTCGCC-3′

showed different 12 types of band lane from isolates (Figure 1). Two control strains of *Cronobacter* spp. (*C. sakazakii* and *C. malonaticus*) had unfortunately a similar DAN lane band. Therefore, it was not possible to confirm any clear identity of isolates. The division between *C. sakazakii* and *C. malonaticus* seems to be problematic and these organisms are so closely related that differentiation was not always feasible, even using 16S rDNA sequencing (Forsythe, 2010). There have been several comparative studies performed to determine the usefulness of biochemical test kits and chromogenic diagnostic tools for identification of *Cronobacter* spp. (Iversen et al., 2007; Jaradat et al., 2009). However, these studies have not given consistent results highlighting the need for other methods of confirmation such as PCR analysis and 16S rDNA sequencing (Lehner et al., 2006; Hassan et al., 2007).

PCR analysis was used as the second confirmation to help ascertain the identity of all presumptive isolates according to primers sequence and methods of Stoop et al. (2009). In this confirmation, it is worth mentioning that all 20 presumptive isolates gave positive response for primers of *Cronobacter sakazakii* and at least 6 isolates were *Cronobacter malonaticus* (Table 2). Moreover, 13 presumptive isolates were subjected to final 16S rDNA sequencing confirmation. It is noteworthy to mention that all 13 isolates were confirmed as *Cronobacter* spp. and there were herein differentiations in genomospecies between 12 isolates which were confirmed as *Cronobacter sakazakii* and only one isolate was *Cronobacter malonaticus.* In general, in *Cronobacter* spp. isolates is important to use both genetic and phenotypic characteristics for classification (Iversen et al., 2007; Barron et al., 2007).

CONCLUSIONS

Cronobacter spp. is a ubiquitous pathogen present in nature, often contaminating food. Environmental sources represent its natural reservoir. *Cronobacter* spp. endangers human health, especially health of neonates and immuno-compromised persons. *Cronobacter* spp. could be well identified by means of ENTEROTEST 24 kits. Both phenotypic and genotypic methodology could be used for putative identification of *Cronobacter* spp. and the combination of methods is crucial for reliable confirmation of identity.

ACKNOWLEDGEMENTS

This study was supported by the Czech Science Foundation (project No. P503/10/0664). The authors thank Oxana Savka for her valuable assistance.

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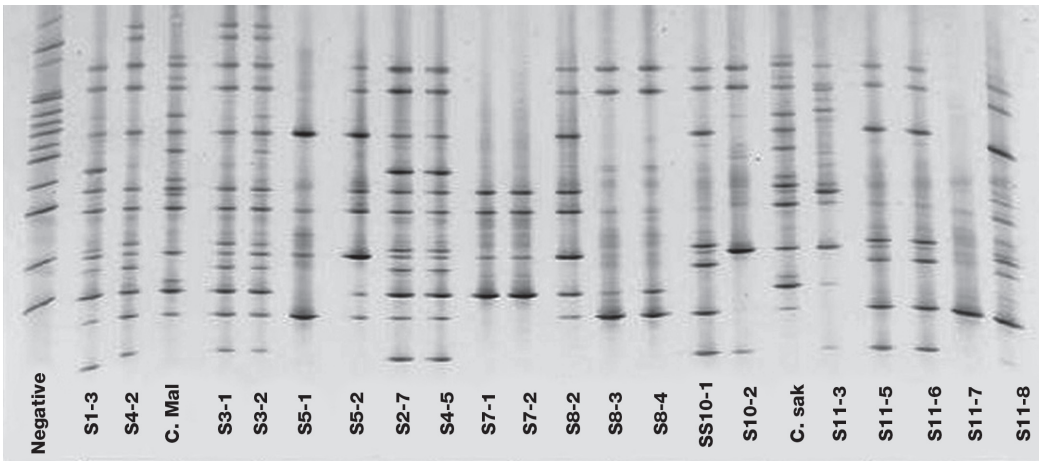

Type1	Type2	Type3	Type4	Type5	Type6	Type7	Type8	Type9	Type10	Type11	Type12
S1-3 S2-7 S4-5	S4-2 S3-1 S3-2	C. Mal C. Sak	S5-1	S5-2 S8-2	S7-1 S7-2	S8-3 S8-4	S10-1 S10-5 S10-6	S10-2	S11-3	S11-7	S11-8

Figure 1: DGGE patterns for confirmation presumptive isolates

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Received for publication: December 21, 2011
Accepted for publication: February 23, 2012

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