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Rapid and sensitive detection of *Cronobacter* spp. (previously *Enterobacter sakazakii*) in food by duplex PCR combined with capillary electrophoresis–laser-induced fluorescence detector

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ABSTRACT

Cronobacter spp. (*Enterobacter sakazakii*) is an emerging opportunistic pathogen with a 40–80% mortality rate in infants and immunocompromised crowd resulting from the consumption of contaminated food. A novel method for detecting *Cronobacter* spp. in food samples by duplex polymerase chain reaction (PCR) in combination with capillary electrophoresis–laser induced fluorescence (CE–LIF) detector has been developed. The specific gene sequences of 16S–23S rDNA internal transcribed spacer (ITS) and the outer membrane protein A (OmpA) of *Cronobacter* spp. were amplified by duplex PCR. The PCR products were separated and determined sensitively by CE–LIF within 12 min. The relative standard deviations of migration time for the detected DNA fragments were 2.01–2.91%. The detection limit was as low as 1.6×10^1 cfu/mL of *Cronobacter* spp. Besides, the specificity of the method was verified by 24 non-*Cronobacter* bacterial strains. A total of 120 commercial infant food formula were tested for the presence of *Cronobacter* spp. by using the proposed method. This current study demonstrates that the combination of CE–LIF method with duplex PCR is rapid, sensitive and environmental friendly, and has the potential to be adapted for the routine detection of *Cronobacter* spp. in food samples. To the best of our knowledge, this is the first use of CE–LIF for the detection of *Cronobacter* spp.

1. Introduction

Cronobacter spp., which used to be called *Enterobacter sakazakii* is a Gram-negative bacillus and belongs to Enterobacteriaceae family [1–3]. This bacterium is a ubiquitous microorganism and has been detected in nearly all environmental samples, including soil, water and rivers, the guts of human and animals, and a wide spectrum of food and food ingredients [4–7]. In 1961, Urmenyi and Franklin reported the first two known cases of neonatal meningitis caused by *Cronobacter* spp. [2]. Since then, this microorganism has gradually attracted international concern. *Cronobacter* spp. is an opportunistic pathogen. Although the incidence of *Cronobacter* spp. infections is low, its mortality rates are 40–80% in infected infants [8]. A number of neonatal life-threatening infections, including meningitis, sepsis, brain abscesses and necrotizing enterocolitis are implicated with *Cronobacter* spp. [6,9]. Some surviving patients may develop serious mental impairment and neurological sequelae after recovery. Besides, infections of *Cronobacter* spp. in adults have been reported, especially among the vulnerable individuals such as the elderly and the immunocompromised [10,11]. Within just the past decade, an increasing number of infection cases caused by *Cronobacter* spp. have been reported worldwide [4,6,12]. Moreover, *Cronobacter* spp. exhibits extraordinary proliferation force in food due to its high tolerance to desiccation and osmotic pressure [13]. Even very low numbers of *Cronobacter* spp. can cause the patients to suffer from serious injury and death. Therefore, it is particularly imperative to establish a sensitive, rapid, and reliable method to identify this emerging microorganism of concern in foods.

However, the procedure recommended by US Food and Drug Administration (FDA) for isolation and identification of *Cronobacter* spp. is labor intensive and time-consuming, involving several steps of enrichment and isolation, followed by biochemical analysis that may take 5 days or longer to complete [14]. Additionally, the morphological characteristic of *Cronobacter* spp. is similar to that of other *Enterobacter* species grown on Violet Red Bile Glucose Agar (VRBGA), which would lead to inaccurate results. Molecular assays have proven useful as they offer an alternative means to identify organisms rapidly and specifically from a wide variety of sources

Abbreviations: VRBGA, Violet Red Bile Glucose Agar; PCR, polymerase chain reaction; LAMP, loop-mediated isothermal amplification; PFGE, pulsed-field gel electrophoresis; CPA, cross-priming amplification; MC, methylcellulose; OmpA, outer membrane protein A; ITS, 16S–23S rDNA internal transcribed spacer.

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[15]. However, agarose gel electrophoresis and imaging observations are required after conventional polymerase chain reaction (PCR), which would take a long time, and require a large volume of samples and naked eyes to observe the results with poor sensitivity. The use of real-time quantitative PCR makes it possible to obtain a fast, robust and sensitive analysis of *Cronobacter* spp. However, the high cost of the exclusive equipment and Taqman probes, together with significant technical demands, has limited its applications [16]. Recently, there have reported several protocols, including loop-mediated isothermal amplification (LAMP), pulsed-field gel electrophoresis (PFGE), cross-priming amplification (CPA) combined with immune-blotting analysis for *Cronobacter* spp. detection [17–19]. But these methods have some drawbacks, such as complexity of primer design, lack of simultaneous analysis of several target genes, and requirement of expensive kits.

A recent study by Yang et al. [20] reported the use of a PCR–denaturing high pressure liquid chromatography (DHPLC) assay to identify *Cronobacter* spp. in milk and dairy product. In this work, DHPLC was employed to detect the PCR products after the 16S–23S rRNA gene of *Cronobacter* spp. was amplified. Compared to DHPLC, capillary electrophoresis (CE) has several advantages such as low sample consumption, high sensitivity and resolution. The combination of CE and highly sensitive laser induced fluorescence (LIF) detection has showed the unique feature in modern applications of biological molecules analysis [21,22]. The objective of this work was to develop a new method combing the selectivity and the sensitivity provided by PCR with the speed of analysis, resolving power and low sample requirements of CE–LIF to improve the detection of *Cronobacter* spp. in food, and to investigate the prevalence of this pathogen in commercial infant food formula using the established method. To the best of our knowledge, there has been no report on the detection of *Cronobacter* spp. in food by a PCR-based procedure followed by CE–LIF.

2. Experimental

2.1. Materials

Methylcellulose (MC) for capillary electrophoresis with a viscosity of ~4000 cP for a 2% aqueous solution at 25 °C was purchased from Sigma (St. Louis, MO, USA). Tris-(hydroxymethyl) aminomethane (Tris), boric acid and disodium-EDTA were purchased from Shanghai Analytical Reagent Factory (Shanghai, China). Gene Ruler™ Low Range DNA Ladder was purchased from Fermentas (MBI, Lithuania). SYBR Green I used as nucleic acid stain for CE was purchased from Bosai Gene Diagnostics Technology Co. Ltd. (Hangzhou, China). Oligonucleotide primers were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China). Deionized and purified water obtained from Milliq pure water system (Millipore, Bedford, MA, USA) was used throughout the experiments.

2.2. Bacterial strains

Cronobacter spp. ATCC 51329 used for the target bacterial strains was provided by Center for Disease Control and Prevention of Chengdu.

Twenty-four non-target bacterial strains were utilized for specificity assays, including *Vibrio parahaemolyticus* ATCC17802, *Salmonella enteritidis* 51041 and *Shigella sonnei* 51203, *Staphylococcus aureus* ATCC25923, *S. aureus* ATCC23213 (China Center of Common Culture Collection), *Escherichia coli* O157:H7 EDL933 and *E. coli* O157:H7 882364 from Center for Disease Control and Prevention of Sichuan Province, *Salmonella typhimurium*, *Salmonella choleraesuis*, *Shigella flexneri 2a*, *Shigella boydii*, *Shigella dysenteriae* I, *Listeria monocytogenes*, *E. coli* 8099, *Bacillus vularis* and *S. aureus* 6538, *E. coli* ATCC 25922, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus proteus*, *Citric acid bacillus*, *Staphylococcus albus*, *Hemolytic streptococcus* and *Vibrio aquatilis* strains provided by West China School of Public Health in Sichuan University.

2.3. Instrumentation

The capillary electrophoresis system equipped with laser-induced fluorescence detector was assembled in-house as described previously [23]. A high voltage power supply (5–30 kV, Cailu scientific instruments company, Beijing) was used to provide electric fields. In this system, a solid state laser (Beijing Viasho Technology Co. Ltd., China) was utilized to generate an excitation wavelength of 473 nm. The laser beam was focused onto the window of the capillary by a 10 cm focal length and 25 mm diameter quartz lens. The resulting fluorescence emission at 520 nm was collected by a photomultiplier tube (PMT) and a 520 nm cut-off filter which was placed in front of the PMT to isolate the laser emission. Fluorescence signals were recorded and processed by chemical workstation (ZB2020).

2.4. Bacterial culture conditions and DNA extraction

Cronobacter spp. was cultured in LB broth (10 g bacto tryptone, 5 g yeast extract and 5 g NaCl in 1 L water) at 37 °C in an air bath oscillating incubator. The genomic DNA was extracted after overnight incubation. Cells were harvested from 1.0 mL cultures by centrifugation at 12,000 rpm for 30 s. Cell pellets were re-suspended in 100 μL sterile water and then heated in a boiling water bath for 10 min to lyse the bacteria cells. After centrifugation for 30 s at 12,000 rpm, the supernatant was stored at −20 °C and was used as template DNA.

2.5. Duplex PCR conditions

Duplex PCR assay, which was designed to amplify two DNA targets simultaneously in a single reaction tube, was employed in this work. The reaction mixture (50 μL total volume) contained: 1.2× PCR buffer, 1.5 mmol L^{-1} $MgCl_2$, 0.30 mmol L^{-1} dNTPs, primers (0.2 μmmol L^{-1} ITS-F/R, 0.5 μmmol L^{-1} OmpA-F/R), 2.5 U of *Taq* DNA polymerase, and 2.0 μL DNA template. Negative control of amplification was performed with 2.0 μL of sterile water instead of DNA template. Thermal cycling parameters were as following: initial denaturation at 94 °C for 5 min; 35 cycles of denaturing at 94 °C for 45 s, annealing at 58 °C for 90 s, elongation at 72 °C for 60 s and final elongation at 72 °C for 7 min.

2.6. Detection of duplex PCR products by capillary electrophoresis–laser induced fluorescence detector

The PCR products were analyzed in the CE–LIF system assembled in-house equipped with a solid state laser of 473 nm excitation wavelength. Bare fused-silica capillary with 75 μm i.d. was purchased from Yongnian Optical Fiber Factory (Hebei, China). A 50-cm section of capillary column with the effective length of 42 cm was prepared. Subsequently, the capillary walls were coated with linear polyacrylamide in order to diminish the electroosmotic flow and surface adsorption [24,25]. The running buffer used in this work was 60 mM TBE (Tris, boric acid and disodium-EDTA, pH 8.3). SYBR Green I nucleic acid stain was added to the separation buffer containing 0.9% MC (w/v).

The PCR products were injected into the capillary at 10 kV for 10 s and separated at 12.0 kV with running temperature of 20 °C. The laser induced fluorescence detection equipment was utilized to detect the fluorescence of 520 nm wavelength generated by combination of SYBR Green I and DNA fragments. After each run, the

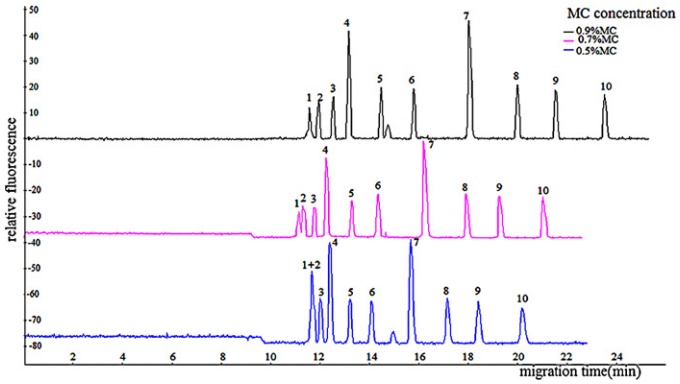

Fig. 1. The separation effects of Gene Ruler™ Low Range DNA Ladder under different concentrations of MC. Running buffer: 60 mmol L^{-1} TBE; capillary: 50 cm (42 cm effective length) × 75 μm i.d.; injection: 10 s at 10 kV; separation voltage: 8 kV; temperature: 20 °C. Peaks: (1) 25 bp; (2) 50 bp; (3) 75 bp; (4) 100 bp; (5) 150 bp; (6) 200 bp; (7) 300 bp; (8) 400 bp; (9) 500 bp; and (10) 700 bp.

capillary column was rinsed for 2 min with 0.01 mol L^{-1} H_3PO_4. Gene Ruler™ Low Range DNA Ladder was used as DNA size markers to assess the system.

3. Results and discussion

3.1. Optimization of separation conditions in capillary electrophoresis

3.1.1. Optimization of methyl cellulose (MC) concentration

MC, as the dynamic coating material and sieving matrix, is widely employed in capillary electrophoresis for fast separation of DNA fragments with shorter migration time [26]. However, the separation performance of DNA fragments strongly depends on MC concentration. In this study, the separation characteristics of Gene Ruler™ Low Range DNA Ladder were investigated under various concentrations of MC ranging from 0.5% to 0.9% (w/v). Fig. 1 shows that the higher the MC concentration was, the better the resolutions of DNA fragments would be achieved. All other DNA fragments could be achieved the baseline separation except 25 bp and 50 bp with no resolution when the sieving matrix concentration was 0.5%. The resolution between 25 bp and 50 bp DNA fragments (R_S = 0.68) was achieved, by increasing MC concentration to 0.7%. The only unresolved fragments could be separated with the appropriate resolution of 1.69 by increasing the MC concentration to 0.9%. Therefore, 0.9% MC was selected as the sieving matrix in this study.

3.1.2. Optimization of electric field strength

The electric field strength on the resolutions and the migration time of Gene Ruler™ Low Range DNA Ladder were investigated at levels ranging from 120 V cm^{-1} to 240 V cm^{-1}. In general, the resolutions of DNA fragments were decreased while the running time was reduced significantly by applying higher electric fields. All the DNA fragments were separated with adequate resolutions by applying low electric field strength of 120 V cm^{-1}. However, the total run time was as long as 46 min. Therefore, novel electric field strength was needed to reduce the run time without excessive loss of resolution. The run time was reduced to 18 min, by altering the electric field strength to 240 V cm^{-1}. At the same time, the baseline resolutions of $R_S \geq 1.50$ were obtained between all the adjacent DNA fragments (see Fig. 2). Thus the electric field strength of 240 V cm^{-1} was more suitable for the rapid separation of DNA fragments in terms of the migration time and resolutions.

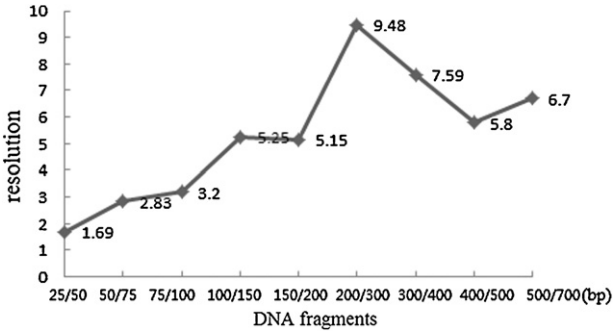

Fig. 2. The resolutions of Gene Ruler™ Low Range DNA Ladder under the electric field strength of 240 V cm^{-1}.

3.2. The selection of PCR primers

In PCR assay, primers which work in pairs (including forward primer and reverse primer) will bind to two separated strands on either side of the target DNA sequence so that the region between them is amplified. Therefore, the suitable target sequences are of critical importance for the specificity of PCR assay. At present, the methods for PCR identification of *Cronobacter* spp. mostly focus on these target sequences of α-6-glucosidase activity gene [15,27], zpx gene [28], rpsU-dnaG gene [29], outer membrane protein A (OmpA) gene [30], and 16S–23S of ribosomal DNA (rDNA) [31]. The 16S–23S rDNA internal transcribed spacer (ITS) sequences are extraordinarily suitable to provide highly specific biological information at bacteria taxonomic level. The lengths and sequences of ITS regions vary greatly with different bacterial species. Accordingly, ITS sequence can be utilized to discriminate *Cronobacter* spp. from all other *Enterobacter* and non-*Cronobacter* strains. The outer membrane protein A (OmpA) plays a critical role in the pathogenicity of *Cronobacter* spp. and its ability to adhere to and invade the host cells. Singamsetty [32] reported that the OmpA-deletion mutant was sevenfold less invasive than the wild type of *Cronobacter* spp. and the bacterium did not multiply inside human brain microvascular endothelial cells (HBMEC). In order to identify *Cronobacter* spp. and to judge its pathogenicity simultaneously, two primer pairs based on ITS gene and OmpA gene of *Cronobacter* spp. were synthesized for PCR amplification in this study [33]. The primer sequences are shown in Table 1.

3.3. Optimization of duplex PCR conditions by capillary electrophoresis

Several factors including the proportion and concentration of different primers, Mg^{2+} concentration and annealing temperature are the substantial contributors to analytical sensitivity and specificity of duplex PCR. Thus the three parameters were optimized in this study.

3.3.1. Optimization of primers proportion and concentrations

In duplex PCR, the proportion and concentrations of primers have a great influence on the amplification efficiency, leading to unbalanced products yields and low sensitivity of the method. Thus, it is necessary to optimize the concentration of different primer pairs. The effects of seven concentration ratios of ITS-F/R and OmpA-F/R (1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1: 3.5 and 1:4) on the amplification were investigated in this study (Fig. 3). When the concentration of ITS-F/R and OmpA-F/R was 0.40 μmol L^{-1} and 0.60 μmol L^{-1} respectively (the ratio was 1:1.5), the yields of ITS gene were much higher than that of OmpA gene and there were obvious primer dimers in the amplification products. Therefore, the amounts of two pairs of primers should be further reduced,

Table 1
Primer sequences for the detection of *Cronobacter* spp.

Primer name	Primer sequence (5′-3′)	Target	T_m (°C)	Amplicon size (bp)
OmpA-F	5′-CACTGTAAACGGCGCAAACAA-3′	OmpA gene	58.01	140
OmpA-R	5′-CGCCAGCGATGTTAGAAGAGG-3′	OmpA gene	61.92	
ITS-F	5′-CCGGAACAAGCTGAAAATTGA-3′	ITS gene	56.06	
ITS-R	5′-TCTTCGTGCTGCGAGTTTG-3′	ITS gene	57.56	98

while the relative proportion of OmpA-F/R should be increased in the amplification system. When the concentrations of ITS-F/R and OmpA-F/R were 0.20 μmol L^{-1} and 0.50 μmol L^{-1} respectively, the yields of these two target genes were almost equal to each other and there were less primer dimmers. Keeping the concentration of ITS-F/R and increasing the amount of OmpA-F/R, it was found that the yields of OmpA gene were not increased significantly while the amount of primer dimers in the system was increased. Therefore, the optimal concentrations of ITS-F/R and OmpA-F/R were 0.20 μmol L^{-1} and 0.50 μmol L^{-1} (the corresponding ratio was 1:2.5).

3.3.2. Optimization of annealing temperature

The annealing temperature for a PCR depends on the length and composition of the primers [34]. Optimization of the annealing temperature becomes increasingly important, since the yields of PCR amplification products and the reaction specificity can be affected directly by the annealing temperature in duplex PCR. To select the optimal annealing temperatures, 56 °C, 58 °C and 60 °C were tested according to T_m values of the primers. Both target genes, OmpA gene and ITS gene, can be amplified under the three annealing temperatures. However, the annealing temperature of 56 °C resulted in poor specificity, as evidenced by an increase in non-specific products. The annealing temperature of 58 °C provided the maximal amplification efficiency of ITS-F/R and OmpA-F/R and no non-specific products. With a further increase in annealing temperature, the product yields were reduced significantly. Therefore, the optimal annealing temperature was 58 °C.

3.3.3. Optimization of Mg^{2+} concentration

High Mg^{2+} concentration may lead to poor specificity of PCR assay in that the stringency of primer binding decreased with the increase of Mg^{2+} concentration. On the contrary, low Mg^{2+} concentration may have negative effect on the yields of PCR products [34]. Optimal Mg^{2+} concentration should be determined experimentally. The effects of Mg^{2+} concentrations in the range of 0.5–3.5 mmol L^{-1} in the duplex PCR system were investigated (shown in Fig. 4).

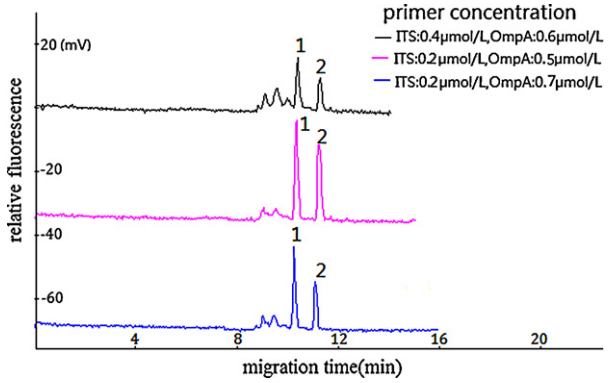

Fig. 3. Electropherograms of the duplex PCR products of *Cronobacter* spp. for optimization of the concentrations of primer pairs by CE. Mg^{2+}concentration: 1.5 mM; annealing temperatures: 58 °C. The figures indicate the PCR products: (1) the PCR product of ITS gene, 98 bp; and (2) the PCR product of OmpA gene, 140 bp.

When Mg^{2+} concentration of 0.5 mmol L^{-1} was used, the two target genes cannot be amplified effectively. Subsequently, Mg^{2+} concentration was increased to 1.5 mmol L^{-1}. In this condition, OmpA gene and ITS gene were amplified with high efficiency and non-specific amplification products were not found. Nevertheless, excessive Mg^{2+} concentration resulted in the nonspecific amplification and decreased efficiency. Consequently, Mg^{2+} concentration of 1.5 mmol L^{-1} was the optimum for the duplex PCR.

Under the optimal conditions, the electropherograms of Gene Ruler™ Low Range DNA Ladder and duplex PCR products of *Cronobacter* spp. by CE are shown in Fig. 5.

3.4. Analysis of the method performance

3.4.1. Specificity of primers

In order to assess the specificity of duplex PCR, the DNA extracted from 24 strains of non-target bacteria was separately added to two primer pairs to perform PCR under the optimal conditions. Experimental results indicated that no target gene was amplified, which demonstrated that the method established in this study was specific enough to discriminate *Cronobacter* spp. from all other bacteria tested.

3.4.2. Sensitivity of determination

Tenfold serial dilutions of *Cronobacter* spp. broth culture were prepared for the evaluation of the method sensitivity. The DNA extracted from each dilution was used as the template of duplex PCR and the amplified products were analyzed by CE–LIF. An criterion was developed to confirm the positive samples that could provide the signal-to-noise ratios of both the peaks of ITS and OmpA higher than 3. At the same time, a 1-mL portion from each dilution was plated onto LB agar and incubated for 24 h at 37 °C for enumeration of cultures. The final plate in these series having 30–300

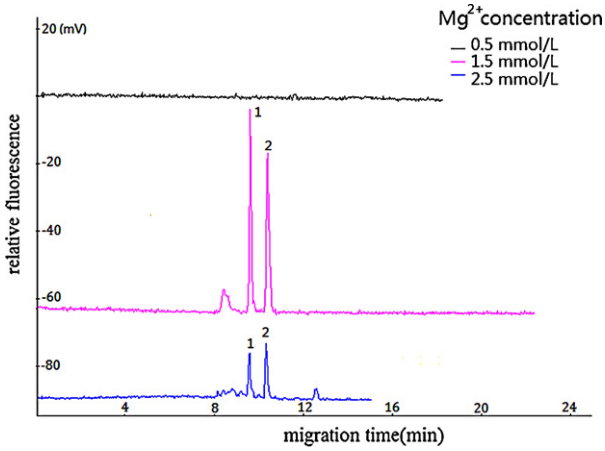

Fig. 4. Electropherograms of the duplex PCR products of *Cronobacter* spp. for optimization of Mg^{2+} concentration by CE. The concentrations of primer pairs of ITS-F/R and OmpA-F/R: 0.2 μmol L^{-1} and 0.5 μmol L^{-1}; annealing temperature: 58 °C. The figures indicate the PCR products: (1) the PCR product of ITS gene, 98 bp; and (2) the PCR product of OmpA gene, 140 bp.

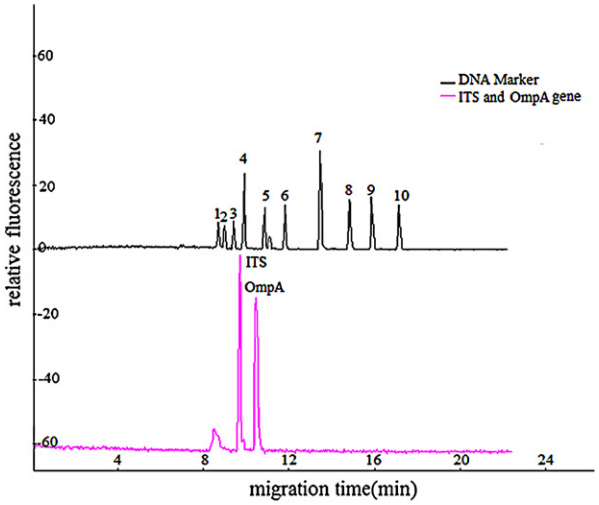

Fig. 5. Electropherogram of Gene Ruler™ Low Range DNA Ladder and duplex PCR products of *Cronobacter* spp. by CE. Peaks: (1) 25 bp; (2) 50 bp; (3) 75 bp; (4) 100 bp; (5) 150 bp; (6) 200 bp; (7) 300 bp; (8) 400 bp; (9) 500 bp; and (10) 700 bp. PCR conditions: the concentrations of primer pairs of ITS-F/R and OmpA-F/R were 0.20 μmol L^{-1} and 0.50 μmol L^{-1}, respectively; 1.5 mmol L^{-1} Mg^{2+}; annealing temperature: 58 °C.

colonies was used to calculate the number of bacteria (the colonies numbers on other plates were too many or too few for accurately counting). The results showed that the detection limit of the proposed method was the dilution of 10^{-8}, which was equivalent to 1.6×10^1 cfu/mL of *Cronobacter* spp.

3.4.3. Precision of the method

Under the optimized experiment conditions, the precision of the method was determined by injecting 5 replicate samples of both DNA makers and duplex PCR products of *Cronobacter* spp. The relative standard deviations for migration times and the area of peaks of the target DNA fragments were in the range of 2.01–2.91% and 1.51–5.30%, respectively.

3.5. Determination of food samples

3.5.1. Artificially inoculated food samples

The food samples including different brands of milk powder, instant noodles, fermented bread, beef and egg cakes were purchased from supermarkets. Each sample of 5 g was taken and added to 45 g sterile nutrient broth respectively, artificially inoculated with a small amount of *Cronobacter* spp. The broth was cultured at 37 °C for 5 h on a shaker. One milliliter aliquot of each inoculated food sample was centrifuged at 12,000 rpm for 2 min. After DNA extraction, the extract was used for duplex PCR amplification, and the amplification products were analyzed by CE–LIF under the optimal conditions described above. The results demonstrated that the proposed method could detect *Cronobacter* spp. specifically and accurately in artificially inoculated food samples.

3.5.2. Real samples

Although the reservoir of *Cronobacter* spp. infections is unknown, powdered infant formula milk is recognized as a primary source and vehicle of the microorganism [8]. In order to investigate the prevalence of *Cronobacter* spp. in commercial infant food and milk formula, a total of 120 different samples from 20 districts (counties) of Chengdu city were collected and tested from March 2009 to June 2012. These samples were manufactured or packaged in 13 different food factories from nine provinces of China. After enrichment and procession as already described, the samples were detected by using PCR with CE–LIF. The results showed that *Cronobacter* spp. was detected from nine of these samples. That is, 7.5% of the samples were contaminated with *Cronobacter* spp.

Analyses of commercial powdered infant formula products have revealed that small amounts of domestic commercial baby formula milk powder were contaminated by *Cronobacter* spp., which urged the competent departments related to food safety to establish the limited standard and testing guidelines of *Cronobacter* spp. in baby formula milk powder and to warn the manufacturers of powdered infant formula and milk powder to strengthen the quality control against contamination in the manufacturing procedures so as to reduce the health risks of PIF products contaminated by *Cronobacter* spp.

4. Conclusion

In this study, a new method incorporating duplex PCR and CE–LIF for specificity and sensitivity was proposed for the first time for the detection of *Cronobacter* spp. in food samples. The study can make the whole detection procedure of *Cronobacter* spp. faster, cheaper, more sensitive and environmental friendly. The suggested method is suitable not only for the detection of low amount of *Cronobacter* spp. in food, but also for the identification of other foodborne pathogenic bacteria of a large number of samples. Moreover, it can provide a reliable approach for locating points of contamination and identifying possible dissemination routes of *Cronobacter* spp.

Acknowledgements

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