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SHORT COMMUNICATION

Simultaneous Detection of 10 Foodborne Pathogens using Capillary Electrophoresis-Based Single Strand Conformation Polymorphism

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Abstract

This report outlines the development of a rapid, simple, and sensitive detection system for pathogenic bacteria using a capillary electrophoresis-based, single strand conformation polymorphism (CE-SSCP) combined with PCR. We demonstrate that this method, used with primers targeting the V4 region of the16S rRNA gene, is capable of the simultaneous detection of 10 microbes that could be associated with foodborne illness, caused by animal-derived foods: *Salmonella enterica*, *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Campylobacter jejuni*, *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium perfringens*, *Yersinia enterocolitica*, *Vibrio parahaemolyticus*, and *Enterobacter sakazakii*. The traditional detection techniques are time-consuming and labor-intensive, due to the necessary task of separate cultivation of each target species. As such, the CE-SSCP-PCR method, that we have developed, has the potential to diagnose pathogens rapidly, unlike the traditional technique, in order to prevent foodborne illness in a much more efficient manner.

Key words: CE, SSCP, PCR, simultaneous detection, foodborne pathogens

Introduction

The rapid and sensitive diagnosis of foodborne pathogens is important for the prevention of foodborne illnesses (Sakamoto *et al*., 2001), particularly as the outbreaks resulting from increased institutional food services and westernized food practices have significant economic and social impact, and there is now increased public awareness of food safety. However, conventional methods for the detection of pathogens, such as plating and biochemical tests, are labor intensive and take several days to perform (Gracias and Mackillip, 2004). Immunoassays and molecular methods have therefore been developed to allow the rapid analysis of pathogens, and intensive study into the use of PCR-based methods has recently been carried out as it is both rapid and sensitive. Capillary electrophoresis-based single strand conformation polymorphism (CE-SSCP) may also be used to identify pathogens; it is a powerful system for the simultaneous detection of multiple microorganisms (Larsen *et al*., 2007; Oh *et al*., 2009; Shin *et al*., 2008, 2010; Zinger *et al*., 2007).

The "Standards for Processing and Ingredients of Specifications Livestock Product" (Animal Plant and Fisheries Quarantine and Inspection Agency, 2011) stipulate that the six foodborne pathogens, including *Salmonella* spp., *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Clostridium perfringens*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7, should not be detected in edible meat or heat-treated and ready-to-eat food products. Other pathogens such as *Bacillus cereus* (particularly the diarrheal toxin-producing strains), *Campylobacter jejuni*, and *Yersinia enterocolitica*, which are well known to contaminate raw and processed meat (Altekruse *et al*., 1999; McNallay *et al*., 2004; Oh and Cox, 2009) and *Enterobacter sakazakii*, which is frequently found in infant food such as powder milk (Iversen and Forsythe, 2003; Jos

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Van *et al*., 2001), should also be controlled. We therefore developed a CE-SSCP protocol capable of parallel detection of the foodborne pathogens including *Salmonella enterica*, *L. monocytogenes*, *E. coli* O157:H7, *C. jejuni*, *S. aureus*, *B. cereus*, *C. perfringens*, *Y. enterocolitica*, *V. parahaemolyticus*, and *E.sakazakii*. This method may have potential as a suitable early warning system for foodborne outbreaks.

Materials and Methods

Bacterial strain culture and genomic DNA isolation

Ten foodborne pathogenic bacteria were selected for this study as reference strains (Table 1). Aerobic or facultative anaerobic bacteria, including *Salmonella enterica*, *L. monocytogenes*, *E. sakazakii*, *E. coli* O157:H7, *V. parahaemolyticus*, *Y. enterocolitica*, *B. cereus*, and *S. aureus*, were grown in tryptone soya broth (TSB, Difco Laboratories, USA) for 24 h at 37°C. The pathogen *C. perfringens* was grown under anaerobic conditions in TSB for 24 h at 37°C, while *C. jejuni* was grown microaerobically in Hunt broth (MBcell, Korea) containing 5% horse blood (MBcell, Korea) for 48 h at 42°C. The pathogen cultures were grown to concentrations of approximately 10^8-10^9 CFU/mL, and mixed inoculum was prepared by combining equal amounts of all 10 pathogens. DNA was extracted from 1 mL of the pathogen mixture using the DNeasy kit (Qiagen, Inc., USA) according to the manufacturer's instructions.

Table 1. Bacterial strains used in this study

Bacteria	Serovar/strain	Source[1)]
Bacillus cereus		ATCC 14579
		ATCC 10876
		ATCC 11778
Campylobacter jejuni		KCCM 41773
Clostridium perfringens		ATCC 3624
		ATCC 13124
Enterobacter sakazakii		ATCC 12868
Escherichia coli	O157:H7	ATCC 43894
		ATCC 27325
Listeria monocytogenes	serotype 1	ATCC 19111
		ATCC 15313
Salmonella enterica	Typhimurium	ATCC 19585
	Typhimurium	ATCC 13311
	Typhi	ATCC 6539
	Typhi	ATCC 33459
	Paratyphi C	ATCC 13428
	Paratyphi B	ATCC 10719
Staphylococcus aureus		ATCC 25923
		KCCM 12214
Vibrio parahaemolyticus		ATCC 17802
		ATCC 27969
		ATCC 33844
Yersinia enterocolitica		ATCC 23715
		ATCC 27739

[1)]ATCC, American Type Culture Collection; KCCM, Korea Culture Center of Microorganisms

PCR amplification

The primer sets used to amplify DNA for separation analysis targeted the three different variable regions of the 16S rRNA gene (Table 2; Gillman *et al*., 2001). Sets of forward and reverse primers for each variable region were prepared, whereby 1 of each primer was fluorescently labeled at the 5 end with 6-carboxyfluorescein (6-FAM). *Pfu* polymerase PCR premix and primers were obtained from Bioneer, Inc. (Daejeon, Korea), and PCR was performed in a 20-μL reaction volume containing genomic DNA, 10 pmol of each primer (forward and reverse), 0.25 mM of each dNTP, and 1 U of *Pfu* DNA polymerase in reaction buffer. The PCR cycle was as follows: initial denaturation for 4 min at 95°C; 25 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C, and extension for 30 s at 72°C; followed by a final extension for 7 min at 72°C. PCR products were diluted as appropriate in nuclease-free water and used for CE-SSCP analysis.

Polymer matrix preparation and CE-SSCP analysis

Polymer matrix was prepared before CE analysis by dissolving various concentrations of Pluronic F108 PEO-PPO-PEO triblock copolymers purchased from Sigma-Aldrich Inc. (USA) in 0.7 × EDTA buffer (Applied Biosystems, USA). CE-SSCP analysis was performed as previously described (Oh *et al*., 2009). Briefly, a 1-μL sample of the amplified 16S rRNA gene from each species was mixed with 13.5 μL deionized formamide (Applied Biosystems, USA) and 0.5 μL ROX 500 size

Table 2. Primers used in this study

Target region	Forward seq.	Reverse seq.	Product Size (bp)
V2	5'-GGC GGA CGG GTG AGT AA-3'	5'-GGA CTG CTG CCT CCC GTA G-3'	255 bp
V4	5'-TGC CAG CAG CCG CGG TAA-3'	5'-GGA CTA CCA GGG TAT CTA AT-3'	290 bp
V8	5'-AAC TGG AGG AAG GTG GGG AT-3'	5'-AGG CCC GGG AAC GTA TTC AC-3'	220 bp

standards (Applied Biosystems, USA); the sample mixtures were denatured at 94°C for 4 min followed by immediate cooling on ice for 3 min. CE-SSCP analyses were performed using an ABI Prism 310 Genetic Analyzer using non-coated capillaries (47 cm×50 μm; Applied Biosystems, USA), configured in accordance with the manufacturer's instructions. Samples were electrophoresed using an injection voltage of 15.0 kV, an electrophoresis voltage of 15.0 kV, and a running temperature of 35°C. The syringe pumping time necessary to achieve complete polymer replacement in the capillary varied with polymer type and concentration. A CCD camera fitted to the ABI 310 Genetic Analyzer was used to detect fluorescence at wavelengths from 525 to 650 nm. Virtual filter sets were optimized for the ABI PRISM dye set (6-FAM), and dyes were excited with a 10 mW argon ion laser at 488 nm and 514 nm.

Results and Discussion

Development of the CE-SSCP-PCR method

Control of foodborne pathogens in livestock and in the environment is important because these pathogens could contaminate animal-derived food products (Altekruse *et al*., 1999; McNallay *et al*., 2004). Ten food-borne pathogens, *Salmonella enterica*, *S. aureus*, *V. parahaemolyticus*, *C. perfringens*, *L. monocytogenes*, *E. coli* O157:H7, *B. cereus*, *C. jejuni*, *Y. enterocolitica*, and *E. sakazakii*, were therefore selected as a model set for the development of a CE-SSCP method capable of the simultaneous detection of these 10 target microbes.

As pieces of single-stranded DNA from the same amplicon can have different sequences, resulting in different CE-SSCP elution times, CE-SSCP analysis can discriminate between differences in the conformations of single-stranded DNA molecules (Andersen *et al*., 2003; Shin *et al*., 2010; Wesche *et al*., 2005). In contrast, PCR can be used to identify pathogens based on sequence, leading us to perform PCR analysis on our DNA mixture of 10 pathogens prior to CE-SSCP. In total, six primer sets were tested for their ability to amplify the V2, V4, and V8 regions of 16S rRNA (Gillman *et al*., 2001), as each region was amplified using identical primer sets where either the forward or reverse primer was fluorescently labeled. Although the V2 region is reported to be the most variable region in the 16S rRNA gene (Andersen *et al*., 2003; Kullen *et al*., 2000), our results demonstrate that the primer set targeting the V4 region of the 16S rRNA with a fluorescently labeled forward primer produced the best resolution and allowed 10 peaks corresponding to our 10

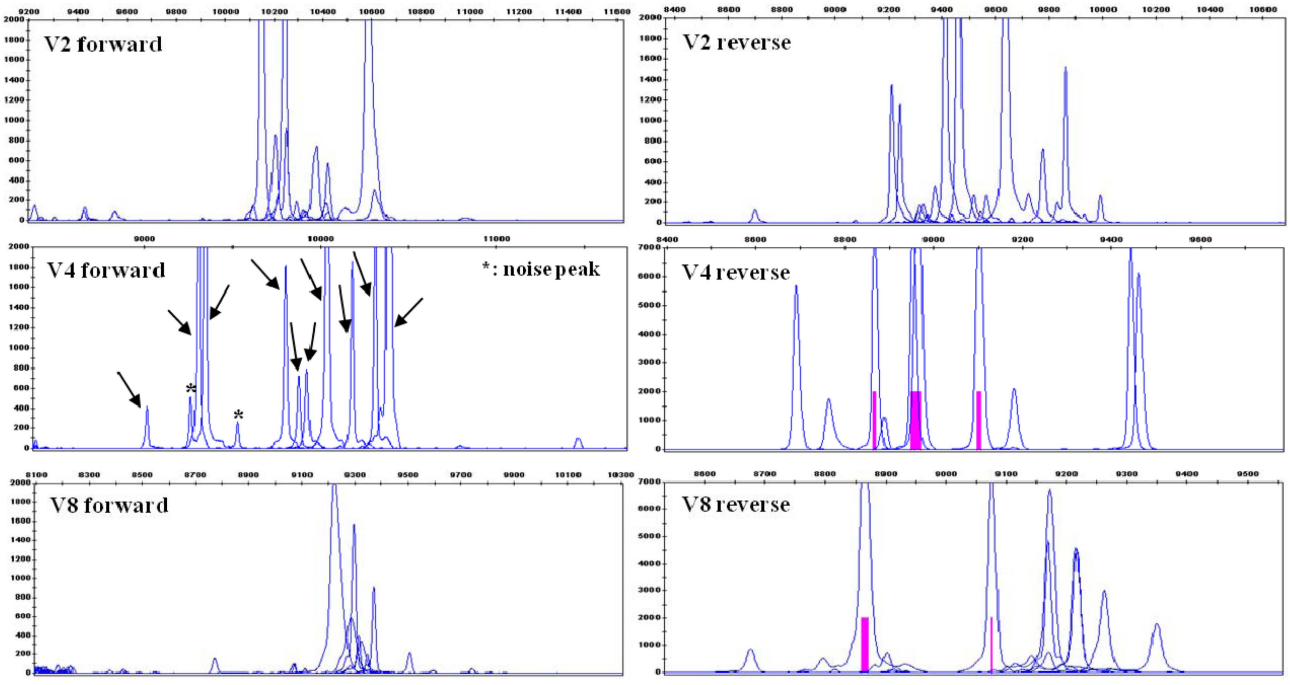

Fig. 1. Electropherograms showing the results of CE-SSCP following the PCR amplification of the V2, V4, and V8 regions of the 16S rRNA genes of 10 pathogenic species, including *Salmonella enterica*, *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Campylobacter jejuni*, *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium perfringens*, *Yersinia enterocolitica*, *Vibrio parahaemolyticus*, and *Enterobacter sakazakii*. The forward or reverse indicate fluorescently labeled primer.

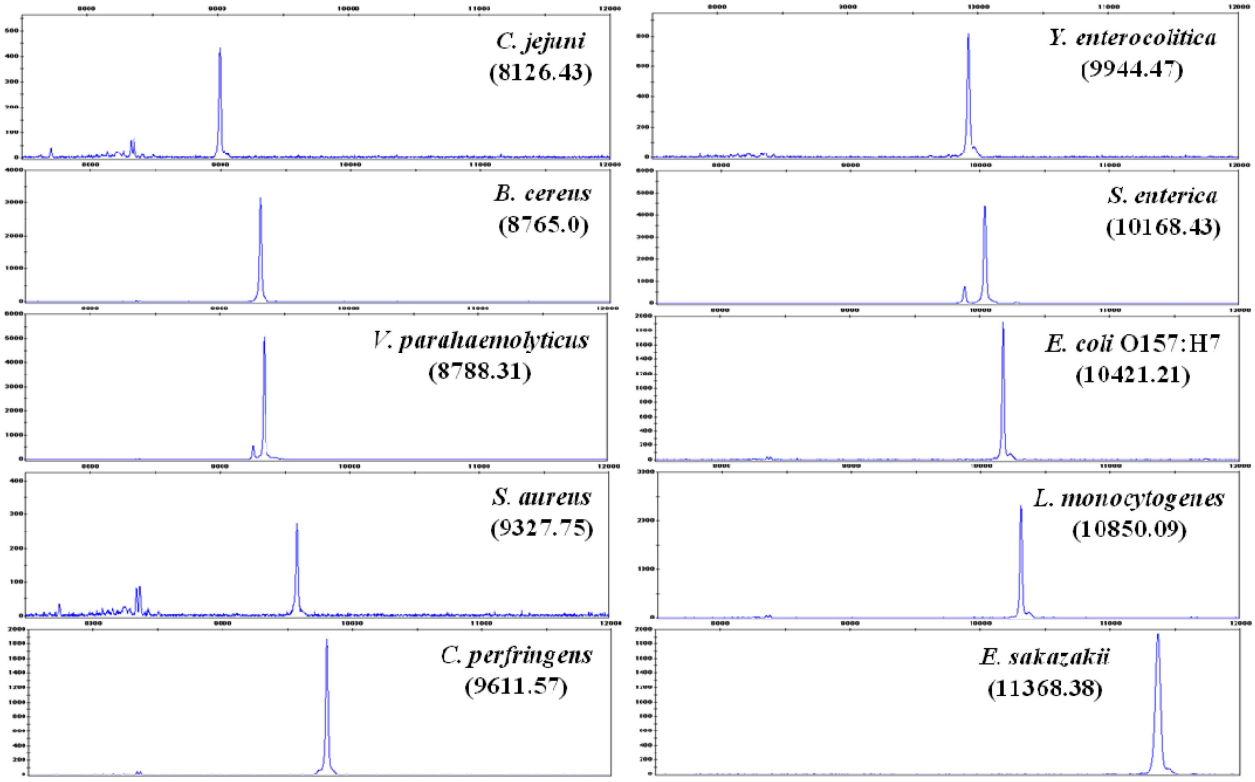

Fig 2. Electropherograms of CE-SSCP electrophoresis for the V4 region of the 16S rRNA genes of 10 pathogenic microbes.

target organisms to be separated by CE-SSCP (Fig. 1).

This finding is similar to that of a study (Claesson *et al*., 2010) that examined several variable regions, including V1 forward-V2 reverse, V2 forward-V3 reverse, V3 forward-V4 reverse, V4 forward-V5 reverse, V5 forward-V6 forward, and V7 forward-V8 reverse, and showed that the combination of V3 forward-V4 reverse, and V4 forward-V5 reverse primers had the best predictive power for strain identification. The V4 region of the 16S rRNA may therefore have more variable sequences than other regions for our target bacteria. However, the separation power of different primers might differ among target microbial strains. Therefore, all of the primer sets used in this study should be tested before choosing the most appropriate primer set for other target organisms.

Identification and separation of peaks in CE-SSCP

We analyzed the ability of our CE-SSCP method to identify pathogenic species from a mixed culture of 10 target microbes. All strains in Table 1 were analyzed individually, and the same species showed the same retention times in CE-SSCP (Fig. 2). Our results demonstrate that the peaks corresponding to each individual strain from our pathogen mixture have identical retention times to those observed when single strains were analyzed (Fig. 3). The accurate identification of identical peaks corresponding to each species was verified with CE-SSCP by systemically changing the concentrations of each strain, as this resulted in changes to the area of the peak corresponding to that target strain without affecting the other peaks (data not shown). Using this method, 1 to 100 pg of genomic DNA per μL was detected, which is equivalent to 10^1 to 10^3 CFU/mL (Oh *et al*., 2009; Zheng *et al*., 2004). Although the electropherograms showed smaller noise peaks in addition to the major peaks (Fig. 3), the retention times of these peaks differed from those of the peaks corresponding to the target organisms and did not interfere with the results. Further, the peaks corresponding to *B. cereus* and *V. parahaemolyticus* are not distinctly separated because of the similar three-dimensional conformations of these organisms. However, these peaks can be differentiated on the basis of the exact and reproducible retention time corresponding to each organism (8765.0 vs 8788.31, respectively) as shown in Figs. 2 and 3.

In conclusion, we demonstrated a diagnostic system that was developed using CE-SSCP and PCR. Traditional detection techniques are time consuming and labor intensive because each target species needs to be cultivated separately. In contrast, this method can simultaneously detect 10 foodborne pathogens within 5 h. The control of

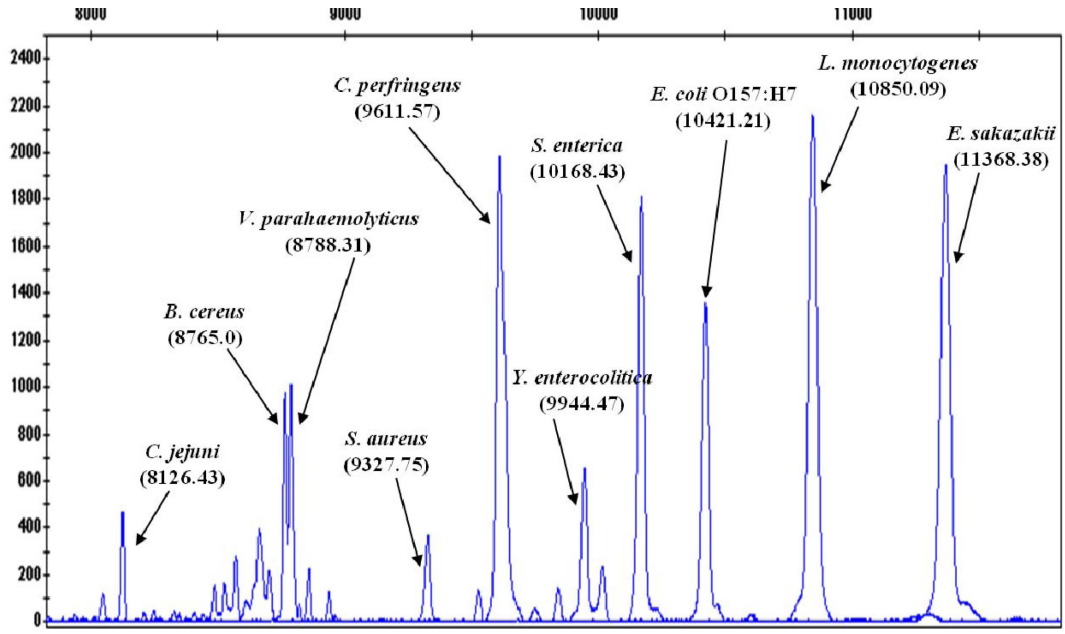

Fig. 3. Electropherograms showing the results of CE-SSCP of a mixture of 10 target microbes. The x and y axes represent data points and fluorescence intensity, respectively.

such pathogens is important for the animal-derived food industry, accordingly, this method can be used to provide an early warning of the outbreak of foodborne diseases. However, this method needs to be validated further, using actual food products, before it is used for the management of food safety in industry.

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