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Determination of foodborne pathogenic bacteria by multiplex PCR-microchip capillary electrophoresis with genetic algorithm-support vector regression optimization

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ABSTRACT

A rapid and sensitive method based on microchip capillary electrophoresis with condition optimization of genetic algorithm-support vector regression (GA-SVR) was developed and applied to simultaneous analysis of multiplex PCR products of four foodborne pathogenic bacteria. Four pairs of oligonucleotide primers were designed to exclusively amplify the targeted gene of *Vibrio parahemolyticus*, *Salmonella*, *Escherichia coli* (*E. coli*) O157:H7, *Shigella* and the quadruplex PCR parameters were optimized. At the same time, GA-SVR was employed to optimize the separation conditions of DNA fragments in microchip capillary electrophoresis. The proposed method was applied to simultaneously detect the multiplex PCR products of four foodborne pathogenic bacteria under the optimal conditions within 8 min. The levels of detection were as low as 1.2×10^2 CFU mL^{-1} of *Vibrio parahemolyticus*, 2.9×10^2 CFU mL^{-1} of *Salmonella*, 8.7×10^1 CFU mL^{-1} of *E. coli* O157:H7 and 5.2×10^1 CFU mL^{-1} of *Shigella*, respectively. The relative standard deviation of migration time was in the range of 0.74–2.09%. The results demonstrated that the good resolution and less analytical time were achieved due to the application of the multivariate strategy. This study offers an efficient alternative to routine foodborne pathogenic bacteria detection in a fast, reliable, and sensitive way.

1. Introduction

With the rapid expansion of food trade and highly increased mobility of today's populations, foodborne diseases resulting from consumption of food contaminated with pathogenic bacteria have been of vital concern to public health [1]. It is estimated that these foodborne microorganisms are responsible for approximately 76 million cases, 325,000 hospitalizations, and 5200 deaths in the United States annually, and the situations are much more serious in the developing countries [2]. Such challenges will continue and in some cases may be aggravated in the future. To reduce health hazards and economic losses due to foodborne diseases, rapid and specific methods to detect these pathogens are necessary for ensuring food safety.

Escherichia coli (*E. coli*) O157:H7, first identified as a human pathogen in 1982, is known as a leading cause of hemorrhagic colitis [3]. *Salmonella* and *Shigella* can lead to Salmonellosis and Shigellosis, respectively. *Vibrio parahaemolyticus* is a bacterium found in the sea foods and causes watery diarrhea often with abdominal cramping, nausea, vomiting, fever and chills. These four pathogens are the most prevalent foodborne pathogens, accounting for the largest number of outbreaks, cases, and deaths [4,5]. Classical methods of pathogen identification include inoculating into a medium, culturing, staining, followed by confirmation by biochemical and/or serological tests. It often takes up to 5–7 days to get a confirmed result for a particular pathogenic organism. Although having been accepted for decades, these methods are extremely labor intensive and time consuming, and cannot therefore meet the requirement of public health emergencies for a rapid and accurate response to the presence of foodborne pathogenic bacteria. The sequence of DNA provides highly specific biological information at every taxonomic level and is ideal for the specific detection of microorganisms [6]. Polymerase chain reaction (PCR) techniques that allow rapid amplification and specific detection of nucleic acid molecules have been fundamental in the microbiological research field during the last years. More recently, the use of the multiplex PCR has provided the rapid and inexpensive detection of several pathogens in a single tube. However, the currently used slab gel electrophoresis (AGE) to detect PCR products is time-consuming, insufficient in resolution and sensitivity, and frequently leading to false negative findings. Capillary electrophoresis (CE) is a powerful method with low sample consumption and short analysis time, and has sufficiently demonstrated its potentiality and versatility for solving analytical problems of chemical substances in

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food analysis [7–9]. It is noteworthy to mention that the application of CE to microbiological research is relatively rare. Alarcón et al. [10] exploited the novel concept of PCR in combination with CE to detect *Salmonella*, *Listeria monocytogenes*, and *Staphylococcus aureus* in artificially inoculated raw beef samples. Their work demonstrated that when compared with AGE, the use of CE to analyze PCR products achieved much better results in terms of resolution and sensitivity. As a new method combining CE technology and microfluidic platform, microchip electrophoresis has become a promising analytical technique. When coupled with laser induced fluorescence (LIF) detection, it is more applicable to routine food microbial analysis because of its higher sensitivity, resolution, reproducibility, and faster speed.

The optimization of the separation conditions in microchip capillary electrophoresis is a multivariate procedure in which many factors can affect separation efficiency, such as concentration of sieving matrix, running voltage, concentration of nucleic acid stain dye and running buffer. The traditional methods for determining the optimal separation conditions usually vary one variable while keep others at specified levels [11]. However, this one-dimensional approach disregards the interactions among the factors and often fails to obtain the optimal conditions. Recently, experimental design [12], overlapping resolution mapping [13], artificial neural network (ANN) [14,15] and response surface methodology (RSM) [16,17] have been utilized in CE and microchip capillary electrophoresis for the optimization of experimental conditions. Support vector regression (SVR), which is a novel learning machine algorithm based on statistical learning theory, has extraordinary generalization capability [18]. It has emerged as an alternative and powerful technique to solve the problems of nonlinear relationship, small sample and local minima. These advantages of SVR are just compatible with the features of the complicated nonlinear relationship between the separation conditions and the resolutions in microchip capillary electrophoresis. Like that of other algorithms, the generalization performance of the SVR model relies on the parameters that need to be defined by the user. However, there are no general guidelines to properly set these parameters. Genetic algorithm (GA) is a randomly optimized method simulating natural selection and genetic variation in the process of biological evolution. It can identify the optimal parameter settings in SVR model and perform a global search independent of specific solution models [19,20].

To our knowledge, it has not been found so far any work on the application of GA-SVR for the optimization of separation conditions in microchip capillary electrophoresis. Furthermore, most multiplex PCR assays for pathogen detection have focused on only two or three different types of organisms [10,21]. Neither has been reported the simultaneous detection of *Vibrio parahemolyticus*, *Salmonella*, *E. coli* O157:H7 and *Shigella* by a quadruplex PCR-based procedure followed by microchip capillary electrophoresis. In this study, we have developed a way for the determination of four foodborne pathogenic bacteria by using multiplex PCR-microchip capillary electrophoresis with LIF detection and using GA-SVR chemometric approach to optimize microchip capillary electrophoresis method parameters. It is a rapid, sensitive, and inexpensive method to bacterial identification, and can ultimately be used in routine food microbial analysis.

2. Experimental

2.1. Materials

Hydroxypropyl methylcellulose (HPMC) for microfluidic chip electrophoresis with a viscosity of 40–60 cP for a 2% aqueous solution at 20 °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). pUC Mix Marker,8 DNA Marker was purchased from Fermentas (MBI, Lithuania). Ethidium bromide (EB) was purchased from Sigma (St. Louis, MO, USA). Oligonucleotide primers were synthesized by SBS Biotech Company (Beijing, China). Deionized and purified water obtained from Milli-Q pure water system (Millipore, Bedford, MA, USA) was used throughout the experiments.

2.2. Bacterial strains

A total of 13 target bacterial strains from different sources were used in this study, including *Vibrio parahemolyticus* ATCC17802, *Salmonella enteritidis* 51041 and *Shigella sonnei* 51203 obtained from China Center of Common Culture Collection; two clinical isolates of *Vibrio parahemolyticus* obtained from Center for Disease Control and Prevention of Chengdu; *E. coli* O157:H7 882364 and *E. coli* O157:H7 EDL933 from Center for Disease Control and Prevention of Sichuan Province; *E. coli* O157:H7 from Center for Disease Control and Prevention of Henan Province; *Bacillus breslaviensis*, *Salmonella choleraesuis*, *Shigella flexneri* 2a, *Shigella boydii*, *Shigella dysenteriae* I strains provided by West China School of Public Health in Sichuan University.

Ten nontarget bacterial strains were used for specificity assays, which include *E. coli* 8099, *Bacillus cereus*, *Bacillus subtilis*, *Listeria monocytogenes*, *Bacillus proteus*, *Citric acid bacillus*, *Staphylococcus aureus* 6538, *Staphylococcus albus*, *Hemolytic streptococcus* and *Vibrio aquatilis* strains provided by West China School of Public Health in Sichuan University.

2.3. Instrumentation

The microfluidic chip system was assembled in-house equipped with a helium–neon greenie laser (1.5 mW, Edmund Scientific, Barrington, NJ, USA) which was used to generate an excitation beam at 543.5 nm. After having been reflected by a viewfinder, the laser beam was focused onto the chip channel through a 10× microscope objective. The resulting fluorescence emission passed through a 590 nm bandpass filter to isolate the ethidium bromide emission, and was focused by a focusing lens, then collected and transmitted by a photomultiplier tube. A work station was used to monitor the photocurrent of the PMT. The eletropherograms were recorded and processed by chemical workstation (ZB2020). The high voltage power supply of GY63 type was purchased from analytical research center in college of Science of Northeast University (Shenyang, China) and was utilized to apply electrophoretic fields.

The poly(methyl methacrylate) (PMMA) microchips used in this study were purchased from Dalian Institute of Chemical Physics, Chinese Academy of Sciences (Dalian, China). The effective length of the separation channel is 40 mm and the cross-sectional volume is calculated to be 177 pL [11]. The multiplex PCR was performed in a MasterCycler gradient thermocycler (Eppendorf, Madrid, Spain).

2.4. Bacterial culture conditions and DNA extraction

All strains of *Vibrio parahemolyticus*, *Salmonella*, *E. coli* O157:H7 and *Shigella* were cultured by using Luria–Bertani (LB) broth at 37 °C for 12 h in a air bath oscillating incubator. After enrichment, an aliquot of 1.0 mL of each individual culture was transferred separately into 1.5 mL Eppendorf tube and centrifuged at 12,000 rpm for 1 min. The pellet was resuspensed in 500 μL sterilizing deionized water and boiled for 10 min to lyse the bacterial cells. Before used in the quadruplex PCR, the lysate of four bacteria were mixed together with an equal volume. The representative strains of these four bacterial species were *Vibrio parahemolyticus* ATCC17802, *Salmonella enteritidis* 51041, *E. coli* O157:H7 from Center for Dis-