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A sensitive assay based on specific aptamer binding for the detection of *Salmonella enterica* serovar Typhimurium in milk samples by microchip capillary electrophoresis

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ABSTRACT

The detection of *Salmonella enterica serovar* Typhimurium (*S. Typhimurium*) is very important for the prevention of food poisoning and other infectious diseases. Here we reported a simple and sensitive strategy to test *S. Typhimurium* by microchip capillary electrophoresis couple with laser-induced fluorescence (MCE-LIF) based on the specific reaction between the bacterium and corresponding aptamers. Based on the differences in charge to mass ratio between bacteria-aptamer complexes and free aptamers, a separation of the complexes and free aptamers could be obtained by MCE. The optimal parameters of the specific reaction including fluorescent dye concentration, Mg^{2+} concentration, incubation time, and pH of incubation solution were carefully investigated. Meanwhile, a non-specific DNA was exploited as a contrast for the detection of *S. Typhimurium*. Under the optimal conditions, a rapid separation of the bacteria-aptamer complex and free aptamers was achieved within 135 s with a limit of detection (S/N = 3) of 3.37×10^2 CFU mL^{-1}. This method was applied for the detection of *S. Typhimurium* in fresh milk samples and a recovery rate of 95.8% was obtained. The experimental results indicated that the specific aptamers are of enough biostability and the established method could be used to analyze *S. Typhimurium* in foods.

1. Introduction

Foodborne diseases, which result from consumption of food contaminated with pathogenic bacteria, are a vital public health concern [1,2]. *Salmonella enterica serovar Typhimurium* (*S. Typhimurium*), being one of the most common foodborne pathogens and being widespread in nature, is a major cause of illnesses and deaths every year [3]. Conventional detection methods rely on colony counting methods that require several days. Therefore, improving the means for detection of bacterial contamination is critical in food samples and environmental fields. Different methodologies have been used for the detection of *S. Typhimurium*, for example, electrochemical detection [4], PCR-based methods [5] and enzyme-linked immunosorbent assay (ELISA) [6]. However, one of the major challenges is that *Salmonella* and other pathogens have to be detected in real sample matrices such as foods, feces, and environmental samples. Given this, direct and specific identification of the pathogenic strain is very critical in basic research and clinical diagnosis.

PCR based molecular techniques are used as sensitive and rapid methods for detection of bacteria, but their reliability is limited by residual matrix associated inhibitors (e.g., food components, bile salts, urine, etc.), which often influence molecular detection. Removal of inhibitory substances is therefore the main step in the preparation of samples for PCR-based detection of food pathogens. The inherent instability of antibodies and their high preparation cost considerably restrict the application of ELISA in the detection of bacteria [7,8]. Building on these caveats and also the advantages of these techniques, a variety of specific aptamers instead of antibodies were selected for the detection of bacteria in analytical samples [9–17]. Aptamers are single-stranded DNA or RNA oligonucleotide molecules and are considered as nucleic acid versions of antibodies. Aptamers have a binding affinity towards specific targets that include small molecules, proteins, and even whole cells or bacteria. Aptamers were obtained by SELEX (systematic evolution of ligands by exponential enrichment) [18–20], and confer advantages such as small size and low cost. More importantly, unlike antibodies, aptamers can be easily labeled, synthesized, and are stable at a

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variety of temperatures as well as under a wide range of pH and salt concentrations.

Recently, Yuan et al. developed a simple and sensitive visual detection strategy for *S.* Typhimurium using aptamers as recognition and capture elements coupled with Au NPs labeling and silver enhancement signal amplification technology [10]; Wu et al. designed a comprehensive method for sensitive and specific quantitative detection of *S. Typhimurium* by gold nanoparticle-based enzyme-linked antibody-aptamer sandwich (nano-ELAAS) strategy [21]; Wang et al. developed a strategy in which a combination of carbon dots (CDs) and aptamers was used as a novel fluorescence probe for sensitive quantitative detection of *S. Typhimurium* [22]. With the development of microchip technology, microchip capillary electrophoresis (MCE) is currently of great interest owing to its rapid response rate, being highly informative and allowing the use of minuscule amounts of samples and reagents [23,24]. MCE has also been explored for the analysis of bacteria [25,26]. Recently, some researchers have explored to the detection of bacteria using MCE, a rapid analysis strategy that has low sample consumption and also allows automation of the analytical process. Law et al. described the determination of *Enteropathogenic E. coli* using MCE [27], while Shintani et al. showed the separation of lactic acid bacteria and *Saccharomyces cerevisiae* using quartz MCE [28]. Owing to the characteristically low sample volume, low concentration and short separation distance of MCE, an online sample stacking strategy was explored to improve the sensitivity of bacterial detection. Based on the sensitivity of bacterial detection, our group described an on-chip multiple-concentration method combining chitosan sweeping (CS), reversed-field stacking, and field-amplified sample stacking for highly efficient detection of *E. coli* [29]. To develop a viable new strategy for ensuring food safety, one possible approach for the detection of bacteria is using specific aptamers that bind to the bacterial cell.

In this study, the principal objective was to develop a rapid and sensitive strategy based on specific aptamers that can bind bacteria and its application to the separation and detection of *S. Typhimurium* by MCE. DNA aptamers against *S. Typhimurium* were isolated from random sequence libraries generated by Padma Sudha Rani Lavu and co-worker [19]. The aptamers, including a 76-nucleotide aptamer (ST1), with dissociation constants (K_d) approximately 123 ± 23 nM were selected for the analysis of *S. Typhimurium*. We hypothesized that aptamers could be used for detection of bacteria owing to the formation of aptamer-bacteria complexes, which could then be separated and detected by MCE for pathogen analysis. In this work, SYBR gold was used as chromosphere for labeling the aptamers and investigated the interaction between the aptamers and *S. Typhimurium* by MCE- LIF assay. Under optimal conditions, the rapid separation of the bacteria-aptamer complexes and free aptamers was achieved within 135 s by MCE-LIF with a relatively short detection length. A limit of detection (S/N = 3) of 3.37×10^2 CFU mL^{-1} and a recovery rate of 95.8% were achieved in this paper. Our assay thus facilitated the sensitive determination of pathogenic bacteria in artificially contaminated raw milk samples.

2. Materials and methods

2.1. Chemical reagents

All chemicals and reagents used were at least of analytical grade. Ethanol, hydrochloric acid, sodium chloride, potassium chloride, magnesium chloride, tris(hydroxymethyl)aminomethane (Tris), and ethylenediaminetetraacetic acid (EDTA) were provided by Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). SYBR gold solution was purchased from Invitrogen Corporation (Karlsruhe, Germany), and diluted with Tris-HCl buffer (TE buffer, 10.0 mM Tris-HCl, pH 8.0, 1 mM EDTA). The milk sample (fresh milk) was purchased from the local supermarket. All oligonucleotides were purchased from Sangon Biotech Co. (Shanghai, China) and purified by HPLC (Table 1). DNA-1000kit was purchased from Shanghai Genesci Medical technology Co., Ltd. *Escherichia coli* (*E. coli,* ATCC 25922), *Staphylococcus aureus* (*S. aureus,* ATCC 6538), *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*, ATCC 14028), *Listeria monocytogenes* (*L. monocytogenes,* ATCC 13932), and *Shigella flexneri* (*S. flexneri*, CMCC (B) 51572) were purchased from Shanghai Luwei Microbial Sci. & Tech. Co. Ltd. Yeast extract and peptone were purchased from Beijing Aoboxing Bio-tech Co. Ltd. All solutions were prepared using ultrapure water from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Bacterial strains used in bacteria culture and counting

E. coli, S. aureus, S. Typhimurium, L. monocytogenes and *S. flexneri* were cultured in Luria-Bertani (LB) broth at 37 °C for 20 h (THZ-103B, Shanghai Yiheng Scientific instruments Co., Ltd., Shanghai, China). Bacterial cell pellets were harvested during the exponential growth phase by centrifugation at 6000 rpm (TG1650-WS, Shang-Hai LuXiangYi Centrifuge Instrument Co., Ltd, Shanghai, China) for 5 min, and the pellets were washed five times with sterile water. The cell numbers in bacterial cultures were determined using a conventional plate counting method, and the results were converted to OD 600 values.

2.3. Sample preparation

Stock solutions of aptamers (100 μM) were prepared in 10 mM TE buffer (pH = 8.0). The stock solutions were diluted with 10 mM TE buffer to obtain the required concentrations. In order to obtain specific secondary structures, aptamers were incubated at 85 °C for 6 min and slowly cooled down to room temperature. Stock solutions of each bacterial strain were diluted in ultrapure water to the corresponding aptamer concentration in examination. Following this, the aptamers were incubated with the corresponding bacterial culture at 37 °C for 15 min. For quantitative assays, *S. Typhimurium* cultures at various concentrations were incubated with 1.2×10^{-7} M aptamers. The incubation buffer included 10 mM Tris-HCl with the corresponding concentrations of $MgCl_2$ at pH = 8.0. To detect the real sample matrix, the required concentration of the corresponding bacterium was spiked into raw milk, the aptamer (ST1) was added, and incubated at 37 °C for 15 min before MCE analysis.

2.4. Microchip capillary electrophoresis

The separation and detection of aptamers and bacteria-aptamer complexes was achieved using the MCE instrument (MCE-202 MultiNA, Shimadzu, Japan). The schematic illustration of this instrument is shown in Fig. 1. The quartz microchip was purchased from Shimadzu Company. A blue LED (470 nm; 20 mA) apparatus was installed in the MCE instrument along with four microchips that were applied for simultaneous detection of samples. The microchannel is 23 mm in the length for separation, 104 μm in width and 48 μm in depth. Briefly, the samples were introduced into the channel at different voltages: $V_1 = 280$ V, $V_2 = 510$ V, $V_3 = 320$ V, and $V_4 = 0$ V for 50 s and the samples were separated and detected for 135 s at $V_1 = 250$ V, $V_2 = 250$ V, $V_3 = 0$ V, and $V_4 = 1000$ V (Fig. S1). After MCE analysis, the microchannel was washed several times with ultrapure water in preparation for the subsequent experiment.

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