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Ultrasensitive detection and rapid identification of multiple foodborne pathogens with the naked eyes

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ABSTRACT

In this study, a novel approach for ultrasensitive detection and rapid high-throughput identification of a panel of common foodborne pathogens with the naked eyes is presented. As a proof-of-concept application, a multiple pathogen analysis array is fabricated through immobilizing three specific polyT-capture probes which can respectively recognize *rfbE* gene (*Escherichia coli* O157:H7), *invA* gene *(Salmonella enterica)*, *inlA* gene *(Listeria monocytogenes)* on the plastic substrates. PCR has been developed for amplification and labeling target genes of *rfbE, invA, inlA* with biotin. The biotinated target DNA is then captured onto the surface of plastic strips through specific DNA hybridization. The succeeding staining of biotinated DNA duplexes with avidin-horseradish peroxidise (AV-HRP) and biotinated anti-HRP antibody greatly amplifies the detectable signal through the multiple cycle signal amplification strategy, and thus realizing ultrasensitive and specific detection of the above three pathogens in food samples with the naked eyes. Results showed approximately 5 copies target pathogenic DNA could be detected with the naked eyes. This simple but very efficient colorimetric assay also show excellent anti-interference capability and good stability, and can be readily applied to point-of-care diagnosis.

1. Introduction

The ultrasensitive analysis of pathogenic bacteria is of vital importance in food safety monitoring. A one-year active surveillance on 10,000,000 people for food safety risk assessment conducted by China National Centre for Food Safety Risk Assessment (CFSA) in 2011 revealed that one in every 6.5 people contracts foodborne disease. 31 major known pathogens acquired in the United States caused an estimated 9.4 million episodes of foodborne illness every year (Scallan et al., 2011). *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* are considered as the most prominent pathogens causing the most food-related human illnesses worldwide. For example, *E. coli* O157:H7 can produce toxins such as Shiga-like toxins or Vero cytotoxins, thus causing hemorrhagic diarrhea and abdominal cramps (Chen et al., 2015). *Salmonella* are the most commonly identified bacterial pathogens causing gastrointestinal infections in humans. *Salmonella and E. coli* O157:H7 have also been associated with several major food poisoning outbreaks in the United States (Suo et al., 2010). *L. monocytogenes* is one of the most virulent foodborne pathogens, with a mortality rate of 20–40% in humans despite early antibiotic treatment (Stylianou et al., 2008).

Currently, conventional culture methods remain the most reliable and accurate techniques for foodborne pathogen detection. Microbiological method is an extremely time-consuming process which requires intensive labor, as it always takes 2–3 days for initial results, and up to 7–10 days for confirmation (Velusamy et al., 2010). Different methods with improvements in terms of rapidity, sensitivity, specificity and suitability for *in situ* detection and identification of foodborne pathogens have been developed to overcome the limitations of conventional methods (Law et al., 2015; Mortari and Lorenzelli, 2014; Rohde et al., 2015). For example, many molecule-based methods have been developed for rapid detection of pathogenic bacteria, including real-time PCR (Ibekwe and Grieve, 2003; Mothershed and Whitney, 2006), enzyme- linked immunosorbent assay (ELISA) (Park et al., 2008; Sunwoo et al., 2006), fluorescent labeling (Carrillo-Carrión et al., 2011; Wu et al., 2013), nanoparticle based methods (Chen et al.,

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2015; Huang et al., 2015; Zhang et al., 2010). Considering the possibility of the coexistence of several pathogens in one sample, multiplex detection and rapid identification of pathogens in a single analysis is more practically important and highly desirable (Ohk and Bhunia, 2013). Recently, the array-based pathogen detection approaches are becoming attractive owing to their advantage in high-throughput (multiple and rapid) analysis and specific identification (Gehring and Tu, 2011). For example, nucleic acid-based microarray (Eom et al., 2007; Miller and Tang, 2009), protein microarray (Anjum et al., 2006; Cai et al., 2005; Charlermroj et al., 2014), carbohydrate-based microarray (Parthasarathy et al., 2006), and whole-cell microarrays (Thirumalapura et al., 2006) have been developed for multiplexed pathogen detection in a single-assay format (Suo and Wang, 2013). However, most of those array-based approaches generally uses fluoresce probes (Xu et al., 2014), electrochemical probes (Arora et al., 2011; Lam et al., 2013), or nanoparticle probe (Jung et al., 2010; Li et al., 2011), which always involve expensive fluorophores or sophisticated detectors.

Considering the key issues such as cost and simplicity in the development of rapid pathogen detection methods, especially in resource-constrained countries, we developed a novel approach using colorimetric detection arrays for simultaneous detection of multiple foodborne pathogenic DNA using the naked eyes. The overall diagram of this novel approach is presented in Fig 1. As a proof-of-concept application, a multiple pathogen analysis strip with a 3×3 array sets is fabricated to simultaneously detect *rfbE* gene (*E. coli* O157:H7), *invA* gene *(S. enterica)*, *inlA* gene *(L. monocytogenes)* through immobilizing three specific polyT-capture probes on the plastic substrates. Target genes of *rfbE, invA, inlA* has been amplified and labeled with biotin using a conventional PCR. The biotinated target DNA is then captured onto the surface of plastic strips through specific DNA hybridization. Consequently, a multiple cycle signal amplification strategy which is implemented by the repeated staining of biotinated DNA duplexes with avidin-horseradish peroxidise (AV-HRP) and biotinated anti-HRP antibody, is employed to amplify the detectable signals. At last, color development was produced using H_2O_2 and TMB to achieve visual detection with the naked eye or record with smartphone for storage, and transmission. The plastic array strips fabricated in this study show excellent anti-interference capability and good reproducibility, and can be readily applied to point-of-care diagnosis.

2. Materials and methods

2.1. Materials

All oligonucleotides were synthesized and then purified using HPLC by TaKaRa (Dalian, China). Taq DNA polymerase and other reagents required for PCR were purchased from TaKaRa. The biotinated anti-HRP antibody was purchased from Abcam (Hong Kong) Ltd., and the AV-HRP was purchased from Life Technologies Co. (U.S.A.). All other chemicals were of analytical grade and from Sigma. The Dr. Chip N3 plastic array and Dr. Chip DIY Kit™ including Dr. Hyb™ Buffer and HA buffer were obtained from Dr. Chip Biotech Inc.

2.2. Bacteria and culture plating methods

E. coli O157:H7 (ATCC 43888), *Salmonella* (ATCC 1111), *L. monocytogenes* (ATCC 35152) and *Campylobacter jejuni* (ATCC 43474) were purchased from American Type Culture Collection (ATCC, Rockville, MD). The lyophilized cells were rehydrated with tryticase soy broth (TSB, BD Diagnostics), plated on the tryticase soy agar (TSA, BD Diagnostics) and grown at 37 °C for 24 h prior to use. The viable cell number was determined by a microbial plate count method. 1 mL of dilutions were plated on the TSA and incubated at 37 °C for 24 h, and the resulting colonies were counted to determine the number of colony forming units per milliliter (CFU/mL).

2.3. Design of capture probes and PCR primers for E. coli O157:H7, Salmonella, Listeria

The specific gene targets used for the detection of *E. coli* O157:H7, *Salmonella*, *Listeria* were designed based on previous report (Bifulco et al., 2013; Mainar-Jaime et al., 2008; Mukhopadhyay and Mukhopadhyay, 2007). Briefly, the capture probes and primers specifically targeting genes of *rfbE* (for *E. coli* O157:H7 detection), *invA* (for *S. enterica* detection), *inlA* (for *L. monocytogenes* detection) were designed in the selected sequence regions using the Primer Premier 5.0 software, and BLAST search was used to verify that the primers and probes share low or no sequence similarity to any other microorganisms in the database of National Center for Biotechnology Information (NCBI). Each primer set was designed to ensure that amplicon would cover the entire length of its corresponding probe immersed on the plastic array. It is worth to mention that the designed capture probes contain a 20 T-base sequence in 5′ end which is used to facilitate their immobilization onto Dr. Chip N3 plastic array (Chang et al., 2012). Primers of *C. jejuni* as control were selected according to the previous report (Suo et al., 2010). The nucleotide sequences of these genes were retrieved from the GenBank under the accession numbers shown in Table 1.

2.4. Fabrication of colorimetric detection arrays

In the following experiments, Dr. Hyb™ Buffer was used as the spotting buffer. The three specific polyT-capture probes (Table 1) which can respectively recognize genes of *rfbE*, *invA*, *inlA* were adjusted to a certain concentration and then manually spotted with a 3×3 array set onto the Dr. N3 plastic array, on which each spot was fabricated with a 0.1-μL of the capture probe solution

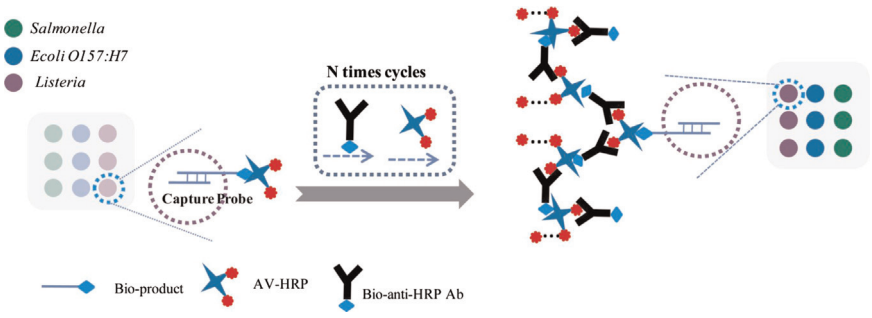

Fig. 1. Schematic diagram of a colorimetric array assay using multiple cycle signal amplification to simultaneously detect multiple foodborne pathogens with the naked eyes.