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Development of a gold nanoparticle-based universal oligonucleotide microarray for multiplex and low-cost detection of foodborne pathogens

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ABSTRACT

Bacterial foodborne diseases remain major threats to food safety and public health, especially in developing countries. In this study a novel assay, combining gold nanoparticle (GNP)-based multiplex oligonucleotide ligation-PCR and universal oligonucleotide microarray technology, was developed for inexpensive, specific, sensitive, and multiplex detection of eight common foodborne pathogens, including *Shigella* spp., *Campylobacter jejuni*, *Bacillus cereus*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica*, *Staphylococcus aureus*, and *Vibrio parahaemolyticus*. The target fragments of the eight pathogens were enriched by multiplex PCR and subjected to multiplex ligase detection reaction. Ligation products were enriched and labeled with GNPs by universal asymmetric PCR, using excess GNP-conjugated primers. The labeled single-stranded amplicons containing complementary tag sequences were captured by the corresponding tag sequences immobilized on microarrays, followed by silver staining for signal enhancement. Black images of microarray spots were visualized by naked eyes or scanned on a simple flatbed scanner, and quantified. The results indicated that this assay could unambiguously discriminate all eight pathogens in single and multiple infections, with detection sensitivity of 3.3–85 CFU/mL for pure cultures. Microarray results of ninety-five artificially contaminated and retail food samples were consistent with traditional culture, biochemical and real-time PCR findings. Therefore, the novel assay has the potential to be used for routine detection due to rapidity, low cost, and high specificity and sensitivity.

1. Introduction

Food safety remains one of the most important global health issues; indeed, foodborne diseases currently constitute a major threat to public health, particularly in developing countries (Akhtar et al., 2014). Bacterial foodborne pathogens are a leading cause of foodborne diseases, with *Escherichia coli* O157:H7, *Salmonella enterica*, *Staphylococcus aureus*, *Shigella* spp., *Listeria monocytogenes*, *Campylobacter jejuni*, *Bacillus cereus* and *Vibrio* spp. responsible for the majority of foodborne disease outbreaks (Velusamy et al., 2010; Zhao et al., 2014). Suspected bacterial pathogens involved in foodborne diseases are numerous, and various pathogens often induce similar clinical manifestations; therefore, the development of a rapid, accurate, high-throughput and low-cost detection method is crucial for effective control and prevention of foodborne diseases in resource-limited areas.

Currently, routine detection of bacterial pathogens still relies on culture-based isolation combined with biochemical identification. However, this is time-consuming and labor intensive, and can only identify one pathogen per assay. Conventional individual or multiplex PCR and real-time PCR assays have been established as rapid, specific, and sensitive tools for detecting various foodborne pathogens. However, the detection capability of these methods is restricted to a few targets in a single reaction (Huang et al., 2007). To overcome this limitation, the oligonucleotide microarray technology has been applied for the simultaneous detection of a variety of pathogenic bacteria (Mitterer et al., 2004; Wang et al., 2007; You et al., 2008; Suo et al., 2010). This technique generally utilizes 16S rRNA, 23S rRNA, and virulence genes as target genes, and combined with multiplex PCR to achieve high detection sensitivity. Nevertheless, amplification disparity resulting from primer competition, and extensive optimization of multiplex amplification and microarray hybridization limit their multiplex detection capability (Kim et al., 2008). In addition, cross-hybridization is often obtained, especially for closely related species (Zhou et al., 2011). More recently, universal microarray assays coupled with

Abbreviations: GNP(s), gold nanoparticle(s); GUMA, gold nanoparticle-based universal oligonucleotide microarray assay; PCR, polymerase chain reaction; MOL-PCR, multiplex oligonucleotide ligation-PCR; PBS, phosphate buffered saline; LDR, ligase detection reaction; SSC, saline-sodium citrate; SDS, sodium dodecyl sulfate; PBN, 10 mM phosphate buffer containing 0.3 M $NaNO_3$; ddH_2O, double-distilled water; SD, standard deviation; CFU, colony forming unit

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multiplex oligonucleotide ligation-PCR (MOL-PCR) have been described for the detection of several bacterial pathogens (Stucki et al., 2012; Wuyts et al., 2015). This overcomes the drawbacks of bias amplification, operational complexity, and cross hybridization in conventional oligonucleotide microarray assays. However, these assays are usually based on the Luminex platform, and require expensive labeling reagents and sophisticated instruments. In recent years, gold nanoparticles (GNPs) instead of fluorophores as labels, have been increasingly used in microarray-based detection of foodborne pathogens due to large surface-to-volume ratio, high stability, easy preparation, and pollution-free and low-cost advantages (Yeh et al., 2012; Liu et al., 2015). However, the above assays are generally based on the conventional oligonucleotide microarray technology, and rely on time-consuming and complicated biotin-streptavidin based labeling.

In the present study, a GNP-based universal oligonucleotide microarray assay (GUMA) combining GNP-based MOL-PCR with silver enhancement detection, was developed to simultaneously detect the 8 most common pathogenic bacteria causing foodborne diseases (*Shigella* spp., *Campylobacter jejuni*, *Bacillus cereus*, *Escherichia coli O157:H7*, *Listeria monocytogenes*, *Salmonella enterica*, *Staphylococcus aureus* and *Vibrio parahaemolyticus*). The schematic overview of the GUMA is illustrated in Fig. 1. The novel assay was evaluated by testing multiple reference or isolated strains, spiked and retail food samples for specificity, multiplexing capability, sensitivity, and practical reliability.

2. Materials and methods

2.1. Bacterial strains

Twenty standard or isolated strains of target and non-target bacterial pathogens were used to optimize and evaluate the specificity and sensitivity of the developed assay. Some reference strains were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), including *Salmonella enterica serovar* Enteritidis (CMCC 50041), *Salmonella typhimurium* (CMCC 50115), *Shigella flexneri* (CMCC 51571), *Shigella sonnei* (CMCC 51592), *Staphylococcus aureus* (CMCC 26003), *Listeria monocytogenes* (CMCC 54001), *E. coli O157:H7* (CMCC 44828), and *Bacillus cereus* (CMCC 63301). The remaining reference strains were stored in our laboratory, including *Campylobacter jejuni* (ATCC 25923), *Vibrio parahaemolyticus* (ATCC 17802), *Enterobacter sakazakii* (ATCC 25944) and *Escherichia coli* (ATCC 25922). *Salmonella typhi*, *Salmonella choleraesuis* and *Salmonella paratyphi A*, *B*, *C*, and *Shigella dysenteriae* and *Shigella boydii* strains isolated from diarrheal patients were identified previously on a Vitek automated system (bioMerieux, Marcy l'Etoile, France) with serotyping confirmation. Bacterial strains were inoculated into 5 mL Luria-Bertani (LB) broth and cultured overnight at 37 °C with shaking at 200 rpm except for *Campylobacter jejuni*, which was cultured in 5 mL Preston broth at 42 °C under microaerophilic conditions, and *Vibrio parahaemolyticus* in 5 mL 3% sodium chloride alkaline peptone water (APW). Overnight cultures were adjusted to a McFarland unit of 0.5 (approximately 10^8 CFU/mL), and counted by plating on standard plate count agar (PCA) in triplicate. Each bacterial culture was 10-fold serially diluted in phosphate buffered saline (PBS, pH 7.4).

2.2. Preparation and culture of bacteria

Eighty retail food samples were purchased from local supermarkets and farmers markets, including 18 pork, 18 chicken, 18 fish, 6 egg, 5 milk and 15 breakfast samples. For the detection of target bacteria in actual food samples, 25 g (mL) of each sample was homogenized in 225 mL of appropriate enrichment broth and incubated under optimal growth conditions described above, according to the national food safety standards of China (GB 4789.4-2010, 4789.5-2012, 4789.7-2013, 4789.9-2014, 4789.10-2010, 4789.14-2014, 4789.30-2010, and GB/T 4789.6-2003). The overnight enriched cultures were grown on corresponding CHROMagar™ selective chromogenic media (CHROMagar, France), and suspected colonies were identified by the Vitek 2 compact system (bioMerieux). Food samples tested negative for the eight pathogens by traditional culture methods were used to prepare artificially contaminated samples. A total of 15 mock-contaminated food samples were prepared by inoculating each negative sample (25 g or 25 mL) with 10^5 CFU of single or mixed reference strains prior to homogenization. Each mock-contaminated sample was added to 225 mL of corresponding enrichment broth for target pathogens and homogenized. DNA was extracted from enrichment cultures after incubation for 2 h.

2.3. DNA extraction

Bacterial DNA extraction from pure cultures was performed by the boiling method. Bacterial cells were collected from 1 mL of culture broth by centrifugation at 11,000 × *g* for 5 min, and washed once with PBS. The pellets were resuspended in 100 μL of lysis buffer (1% Triton X-100, 10 mM Tris-HCl, 10 mM EDTA, pH 8.0) for *Listeria monocytogenes*, *Staphylococcus aureus* and *Bacillus cereus*, and sterilized water for the remaining bacterial species. The suspension was heated at 100 °C for 10 min, followed by centrifugation at 11,000 × *g* for 5 min. Two microliters of the resulting supernatant were used as DNA template for PCR. Bacterial genomic DNAs in artificially contaminated and actual food samples were extracted using QIAamp DNA Mini Kit (Qiagen, GmbH, Germany) according to the manufacturer's instructions. Each DNA extracted was finally eluted in 100 μL of sterile ddH_2O.

2.4. Primer and probe design

The target genes and specific primers of various pathogens were based on published literatures (Islam and Lindberg, 1992; Suo et al., 2010; Mäntynen and Lindström, 1998; Sharma, 2002; Burtscher et al., 1999; Croci et al., 2004; Blaiotta et al., 2004; Bej et al., 1999). Primer sequences were analyzed by BLAST search and the Oligo 6.0 software (Molecular Biology Insights, Cascade, USA) to ensure they had the highest specificities, with similar G-C contents and Tm values. A pair of adjacent upstream and downstream ligase detection reaction (LDR) probes for each pathogen was designed within the region amplified between the two primers using the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, USA). Non-homologous ten tag sequences and a pair of universal sequences with almost identical Tm values, and minimum hairpin structures and mismatches among them were selected as previously described (Hauser et al., 2006; Thierry et al., 2013). The 3′-ends of upstream LDR probes were linked to the upstream universal sequence, and phosphorylated at the 5′ ends; meanwhile, the 5′-ends of downstream LDR probes were linked orderly to tag sequences and the downstream universal sequence. The universal forward primer was identical to the downstream universal sequence; the universal reverse primer complementary to the upstream universal sequence, and contained a 5′-thiol modification to allow the covalent coupling of GNPs. The tag sequence for each pathogen was used as capture probe, and modified with a 5′-amino group (H_2N-$(CH_2)_6$) for immobilization on aldehyde-coated glass. The primers and probes specific for bacterial 16S rRNA genes (internal positive control) were included to monitor the reliability of PCR amplification and microarray hybridization (Jin et al., 2008). Primers and probes were synthesized by Sangon Biotech (Shanghai, China), and listed in Table 1.

2.5. GNP-conjugated primer preparation

Colloidal GNPs with a mean diameter of 13 nm were prepared by citrate reduction of $HAuCl_4$ as described previously (Fu et al., 2013). For the preparation of stable oligonucleotide-GNP conjugate, the lowest amount of 5′-thiolated universal reverse primer necessary to coat the exterior of GNPs was first determined as 0.4 μg per 1 mL of colloidal