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High-throughput detection of food-borne pathogenic bacteria using oligonucleotide microarray with quantum dots as fluorescent labels

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ABSTRACT

Bacterial pathogens are mostly responsible for food-borne diseases, and there is still substantial room for improvement in the effective detection of these organisms. In the present study, we explored a new method to detect target pathogens easily and rapidly with high sensitivity and specificity. This method uses an oligonucleotide microarray combined with quantum dots as fluorescent labels. Oligonucleotide probes targeting the 16SrRNA gene were synthesized to create an oligonucleotide microarray. The PCR products labeled with biotin were subsequently hybridized using an oligonucleotide microarray. Following incubation with CdSe/ZnS quantum dots coated with streptavidin, fluorescent signals were detected with a PerkinElmer Gx Microarray Scanner. The results clearly showed specific hybridization profiles corresponding to the bacterial species assessed. Two hundred and sixteen strains of food-borne bacterial pathogens, including standard strains and isolated strains from food samples, were used to test the specificity, stability, and sensitivity of the microarray system. We found that the oligonucleotide microarray combined with quantum dots used as fluorescent labels can successfully discriminate the bacterial organisms at the genera or species level, with high specificity and stability as well as a sensitivity of 10 colony forming units (CFU)/mL of pure culture. We further tested 105 mock-contaminated food samples and achieved consistent results as those obtained from traditional biochemical methods. Together, these results indicate that the quantum dot-based oligonucleotide microarray has the potential to be a powerful tool in the detection and identification of pathogenic bacteria in foods.

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1. Introduction

Rapid detection of food-borne pathogenic bacteria is of great importance for public health. To minimize the prevalence of food-borne diseases and reduce microbial contaminations in food supplies, effective monitoring of the occurrence and distribution of bacterial pathogens in foods is essential. Currently, conventional methods commonly used in the field are based on cultivation of target pathogens or indicator microorganisms on specific media. However, these methods require several days for completion and are time-consuming. In addition, the culture-based methods sometimes lack specificity in selecting or identifying unknown pathogens in foods (Abubakar et al., 2007; Suo et al., 2010). More recently, several DNA-based methods have been developed to detect pathogenic bacteria. The polymerase chain reaction (PCR) is a very convenient and important technique for the detection of DNA of a specific microorganism (Horakova et al., 2008; Liu et al., 2008; Llop et al., 1999; Mao et al., 2007; Sawada et al., 1992; Takaishi et al., 2003; Versalovic et al., 1995). A number of methods based on probes, restriction fragment length polymorphisms, and multiplex PCR have been reported in previous studies to detect pathogenic bacteria (de Las Rivas et al., 2005; Kim et al., 2007; Naravaneni and Jamil, 2005; Priest et al., 1994). Real-time PCR systems for quantitative analyses of pathogenic bacteria have also been developed (Fricker et al., 2007; Fukushima et al., 2007; Lopez and Pardo, 2010). Although these DNA-based methods have been used to detect pathogenic bacteria, they share a common limitation when faced with the complicated distribution of various strains, species, and genera of pathogenic bacteria. Moreover, these approaches cannot simultaneously detect multiple pathogenic bacteria in parallel using a single experimental cycle. However, this high degree of parallelism can be achieved using microarray technology.

Microarray technology is based on the hybridization of oligonucleotide probes on a slide to another nucleotide population. In recent years, microarray technology has enabled high-throughput detection of multiple pathogens in a large number of samples and has been widely applied to DNA detection and genotyping because of its miniature arrangement, high performance, and ease of automation (Call et al., 2003; Cremonesi et al., 2009; Drost et al., 2009; Hacia et al., 2000; Huang et al., 2006; Jin et al., 2006; Kim et al., 2003; Park et al., 2004; Pasquini et al., 2008; Peplies et al., 2003; Tiberini et al., 2010). The labeled molecules used

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in DNA microarrays to detect binding events are usually fluorescent organic dyes. Although they provide a sensitive, safe, and low cost detection system, they suffer from several limitations. First, organic dyes are sensitive to photo bleaching and are often not bright enough for the quantification of a specific signal over background. Second, the fluorescence spectrums of organic dyes are not symmetric and each fluorophore is characterized by its specific optimal wavelength of excitation, which limits their multiplexing capabilities (Resch-Genger et al., 2008). Therefore, there is an urgent requirement to develop DNA microarrays that do not rely on organic dyes.

Current advances in nanotechnology have provided a novel and promising class of semiconductor nanocrystal quantum dots (QDs). QDs possess a number of advantages over traditional dyes, such as a high quantum yield, long photostability, and high extinction coefficient (Chan et al., 2002; Tan et al., 2002). Several groups have reported the application of QDs in nucleic acid diagnostic methods. For example, QDs have been used to detect complementary target nucleic acid sequences in fluorescent in situ hybridization (FISH) assays (Pathak et al., 2001). Single nucleotide polymorphism (SNP) discrimination and two-color, two-target detection systems have also been demonstrated with QD labels in a microarray format (Gerion et al., 2003). QDs have also been used with FISH to label the HER2gene locus in breast cancer cells (Xiao and Barker, 2004). QDs have been used successfully for the detection of Cryptosporidium parvum (Lee et al., 2004), Escherichia coli O157 (Hahn et al., 2005) and Mycobacterium spp. (Gazouli et al., 2010). Moreover, it has been shown that two food-borne strains Escherichia coli O157:H7 and Salmonella typhimurium labeled with QDs were identified simultaneously (Zhao et al., 2009). These experiments showed that QDs have superior sensitivity and photostability compared to conventional dyes. However, few studies have examined the detection of pathogenic bacteria from foods using an oligonucleotide microarray with QDs as fluorescent labels.

In the present study, we established a sensitive and specific oligonucleotide microarray using QDs as fluorescent labels to detect 11 common species of food-borne pathogenic bacteria that are the most common in food or most dangerous to human health (Gomes et al., 2013; Haagsma et al., 2013; Kozak et al., 2013). We also evaluated the assay by testing for specific bacterial strains within real samples.

2. Materials and methods

2.1. Strains

The standard bacteria strains, including food-borne and nosocomial pathogens, used in this study were obtained from the Chinese Medical Culture Collection Center (CMCC, Beijing China) and are listed in Table 1. The isolated strains used in this study were isolated from foods and are listed in Table 2. The negative control strains tested included Aspergillus fumigatus (CMCC A1), Fonsecaea pedrosoi (CMCC D6a), Sporothrix schenckii (CMCC D1), Mucor racemosus (CMCC 33440), Candida albicans (CMCC C1a), Microsporum canis (CMCC M3b), Citrobacter spp. (CMCC 48025), Clostridium perfringens (CMCC 64615), Aeromonas hydrophila (CMCC 12017), and Bacillus cereus (CMCC 63303). Bacteria cultures were serially diluted to the appropriate inoculum levels and confirmed by plating in standard plate count agar (PCA) in triplicate. Artificially inoculated samples were tested using conventional culture methods with serological confirmation and the VITEK test system (BioMerieux SA, France). All fungi were inoculated onto potato dextrose agar slants at 30 °C for 72–120 h and were stored at 4 °C until further use.

2.2. Preparation and cultivation of bacteria in food samples

Food samples (pork, chicken, fish, and milk) were purchased from local supermarkets. For bacterial detection from raw foods, cultures were pre-enriched by homogenizing 25 g of meat in 225 ml of nutrient broth (Becton Dickinson, Kansas, USA) or 25 ml of milk in 225 ml of universal pre-enrichment broth (Becton Dickinson, Difco). The preparations were then incubated at 37 °C overnight and DNA was extracted.

2.3. Preparation of mock-contaminated food samples

To test the effectiveness of this novel procedure in a real-world application, we introduced bacteria into the samples to represent mock-contaminated food samples. In these experiments, only those food samples that were confirmed to be negative for pathogens by both culture and PCR methods were used. A total of 105 food samples from different local manufacturers were used in this experiment, including 35 pork, 42 chicken, 23 fish, and 5 milk samples. Food samples were mock contaminated by inoculating each sample with 10^2–10^6 CFU of a strain prior to homogenization and enrichment. We found that only 2 h of incubation was required to enrich bacteria to detectable levels, without the need to isolate the organism.

2.4. Extraction of bacterial DNA from pure cultures and foods

For pure cultures, DNAs were extracted using the boiling method as previously described (Afghani and Stutman, 1996). DNAs from various bacteria species in food samples were extracted using the QIAamp DNA Mini Kit (Qiagen, GmbH, Germany) according to the manufacturer's instructions and were used as templates for amplification.

2.5. Sequencesof the primers and probes

The universal primers were based on the conserved region of the 16S rRNA gene. The forward primer sequence was 5′-aactggaggaaggtggggat-3′, and the reverse primer sequence was 5′-aggaggtgatccaaccgca-3′. Primers were synthesized by Invitrogen (USA) and the forward primer was labeled with biotin at the 5′ end. The oligonucleotide probes were targeted to the variability of the 16S rRNA gene regions (Wang et al., 2007). Probes (Table 3) were synthesized by Invitrogen (USA) and modified with NH_2 to increase binding to the glass slide as well as hybridization intensity.

2.6. PCR amplification of the target gene

PCR was performed in 50 μl containing 5 μl 10× buffer (Takara), 200 μM dNTP mixture (Takara), 0.1 U/μl Takara Taq (5 U/μl), 1 μM forward primer, 0.1 μM reverse primer, and 2 μl supernatant containing bacterial DNA. Sterile distilled water was added to a final volume of 50 μl. The PCR mixtures were denatured at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s. The PCR product was verified using 1% agarose gel electrophoresis and visualized with ethidium bromide.

2.7. Generation of oligonucleotide microarrays

In the present study, 14 oligonucleotide probes were used (Table 3) to design a microarray model for the detection of bacteria pathogens (Fig. 1A). Four categories of designed oligonucleotide probes were identified based on their efficacies. Category 1 included oligonucleotide probes 3–12, which is a cluster of genus- or species-specific probes used to identify bacteria at the genus or species level. Category 2, composed of oligonucleotide probe 1, is a G^+ probe shared by all G^+ bacteria and used to detect all types of G^+ bacteria. Category 3, which included oligonucleotide probe 2, is a G^- probe shared by all G^- bacteria and was used to detect all types of G^- bacteria. Category 4 included oligonucleotide probes 13 and 14, which were positive and negative control probes that were used to reflect the effectiveness of the hybridization system and to serve as reference coordinates for scanning.