Establishment and Application of a Visual DNA Microarray for the Detection of Food-borne Pathogens

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The accurate detection and identification of food-borne pathogenic microorganisms is critical for food safety nowadays. In the present work, a visual DNA microarray was established and applied to detect pathogens commonly found in food, including *Salmonella enterica*, *Shigella flexneri*, *E. coli* O157:H7 and *Listeria monocytogenes* in food samples. Multiplex PCR (mPCR) was employed to simultaneously amplify specific gene fragments, *fim*Y for *Salmonella*, *ipa*H for *Shigella*, *iap* for *L. monocytogenes* and *ECs2841* for *E. coli* O157:H7, respectively. Biotinylated PCR amplicons annealed to the microarray probes were then reacted with a streptavidin-alkaline phosphatase conjugate and nitro blue tetrazolium/5-bromo-4-chloro-3′-indolylphosphate, *p*-toluidine salt (NBT/BCIP); the positive results were easily visualized as blue dots formatted on the microarray surface. The performance of a DNA microarray was tested against 14 representative collection strains and mock-contamination food samples. The combination of mPCR and a visual micro-plate chip specifically and sensitively detected *Salmonella enterica*, *Shigella flexneri*, *E. coli* O157:H7 and *Listeria monocytogenes* in standard strains and food matrices with a sensitivity of ~10^2 CFU/mL of bacterial culture. Thus, the developed method is advantageous because of its high throughput, cost-effectiveness and ease of use.

Keywords Micro-plate chip, food-borne pathogen, visual detection, multiplex PCR

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Introduction

Food-borne contamination and illness by food-borne pathogens have highlighted the importance of food safety. To date, more than 250 infectious diseases are known to be caused by food-borne pathogens.[1] Outbreaks of infectious diseases may lead to serious morbidity or mortality in children and the elderly. Researchers thus exert greater effort to develop rapid and sensitive methods to detect pathogens. Especially in such a situation of large-scale food poisoning, a rapid, high throughput and simple method is vital for testing a large number of samples.

At present, there are four categories of methods used for detecting food-borne pathogens, *i.e.* the conventional microbiological methods, the PCR based methods, the ELISA based methods and the microarray based methods.[2] However, each of these categories has its own advantages and limitations. The points we herein emphasized are: 1) facilitating the detection process by the visualization of results; 2) improving the detection throughput of existing microarray. For this end, mPCR and a micro-plate chip were employed in the present work. The former has been widely used to simultaneously detect multiple pathogens.[3-8] For the mPCR assay, the capacity of primers for specific amplification and amplicons with different size length fragments must be taken into consideration for subsequent detection by gel electrophoresis. However, gel

electrophoresis is not accurate for pathogens identification, other techniques have to be used, such as sequencing, microarray hybridization,[2] capillary electrophoresis,[9] *etc.* The DNA microarray technique is currently being widely used for gene expression profiling,[10,11] DNA sequencing,[12] disease diagnostics,[13] and genotyping.[14] The major advantages of this technology, including miniaturization, high performance, the ability to process samples in parallel, and ease of automation, have extended its application area in this decade. Thus combining mPCR and a DNA microarray is a powerful practical tool that is used widely for the detection and identification of different bacteria.[15-17] The obvious advantages of such a combined assay over traditional gel electrophoresis-based mPCR are that it does not depend on the size of the amplification products for product identification. However, the existing microarray has some limitations: 1) it is fragile for the cause of glass nature; 2) expensive equipment is needed because of using fluorescence detection; 3) a low detection throughput for a single slide microarray, which has a limitation for the testing of a large number of samples. Comparison to the existing DNA microarray, micro-plate chip was a novel type of microarray, in which the microarray was integrated into the individual wells of the micro-plate, *i.e.* one chip, one well. Such a design greatly improved the detection throughput comparison with the conventional slide-based microarray. Furthermore, different samples added into separated wells significantly reduced the mutual interference, while that in a slide substrate is a different situation due to carrying over among the different subarray.[18]

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Table 1   Probes and primers used in this study

Bacteria	Target gene	Sequence	Amplicon/ bp	Accession number	Ref.
Shigella	Invasion plasmid antigen (*ipa*H)	F: 5′CCTTTTCCGCGTTCCTTGA R: 5′biotin-CGGAATCCG GAGGTATTGC P: 5′poly(T)$_{15}$CGCCTTTCCGATACCGTCTCTGCA	64	M32063	19
Salmonella	Fimbriae Y protein (*fim*Y)	F: 5′GCGGCGTTGGAGAGTGATA R: 5′biotin-TACCACGCAGGGAAAGACACCGCCG P: 5′poly(T)$_{15}$CATTTCTTAAACGGCGGTGTCTTTCCCT	75	AE006468.1	This paper
L. monocytogenes	Invasion-associated protein (*iap*)	F: 5′CTGAATCTCAAGCAAAACCTGGT R: 5′biotin-CGCGACCGAAGCCAACTA P: 5′poly(T)$_{15}$ATACGATAACATCCACGGCTCTGGCTGG	174	DQ054587	20
E. coli O157:H7	Perosaminesynthetase (*ECs2841*)	F: 5′TCCTCAGCTATAGGGTGCTTTTGATA R: 5′biotin-CTCATCGAAACAAGGCCAGTTTTTTAC P: 5′poly(T)$_{15}$TATTTTTCCGAGTACATTGGCATCGTGTGG	86	BA000007	21
	Probe EV71	ATGAAGCATGTCAGGGCTTGGATACCTCG	—	—	22

At present, many companies provide such micro-plate chip-based products for the immunologic multiplex analysis of cytokines, but a DNA microarray constructed in a micro-plate was rarely reported. To facilitate practical use, a colorimetric reaction between alkaline phosphatase and its chromophoric substrate (NBT/BCIP), often used in ELISA experiment, was introduced in this method. In this system, biotin-labeled PCR amplicons are firstly hybridized with probes on the microarray. Then, the streptavidin conjugated alkaline phosphatase (Strep-AP) in blocking buffer binds to the biotinylated site. The BCIP in the detection buffer reacts to Strep-AP, and produces a blue precipitate at the site of enzymatic activity. NBT acts as a co-precipitant agent for the BCIP reaction, forming a dark-blue precipitate. Such an alteration makes the positive results to be visualized as a color dot formatted on the chip surface, which can be directly observed by unaided eyes for qualitative analysis.

In the present study, we developed a method for the simultaneous detection of four major food-borne pathogens: *Salmonella spp.*, *Shigella spp.*, *E. coli* O157:H7 and *Listeria monocytogenes*. By combining mPCR and a micro-plate chip, the developed method not only overcomes the shortcoming of m-PCR (dependence on the size of the amplification products for product identification), reducing the requirement of expensive equipment, but also greatly improves the throughput of detection. The performance parameters of the method were evaluated, including the specificity and the sensitivity. Generally, this method exhibits high through-put, ease of use and is quite cost-competitive with the existing technologies.

Experimental

Bacterial strains, cultural condition and DNA isolation

The bacterial strains used in this study included *Salmonella typhimurium* (10503), *Salmonella dublin* (10523), *Salmonella thompson* (10514), *L. monocytogenes* (22201), *Shigella flexneri* (11304), *Shigella bogdii* (11306), *E. coli* O157:H7 (10102), *Pseudomonas aeruginosa* (12625), *E. coli* O157:H7 ATCC 43894, *Campylobacter jejuni* ATCC33291, *L. monocytogenes* ATCC 19114, *L. monocytogenes* ATCC 19115, *Yersinia enterocolitica* ATCC 23715, *Staphylococcus aureus* ATCC33591. All strains were grown at 37°C for 18 – 24 h with constant shaking at 220 rpm in 5 mL of Luria–Bertani (LB) broth containing 3 g/L yeast extract. The total genomic DNA was isolated using a commercially available Wizard® Genomic DNA Purification Kit (Promega, Beijing, China).

Primer and probe design

The probes and primers used in this paper are listed in Table 1. The reverse primers for PCR were synthesized with a biotin modification at the 5′-end for detection. Each probe has a poly(T)$_{15}$ modification at their 5′-end for immobilization and as a spacer arm. Probe EV71 was used as a positive control for confirming the hybridization efficacy (biotin-labeled EV71 PCR amplicon was incorporated in the hybridization buffer supplied with the DR. Chip DIY Kit™).

Micro-plate chip preparation

DNA probes were spotted at the bottom of each well of a polystyrene strip plate (from NUNC) by a BIODOT machine (BD6000; CA, USA). The probes were cross-linked to the well surface by exposing to UV light using a UV cross-linker set at 254 nm, 3 min (Scientz03-II; Ningbo Xinzhi Biotechnology Co., Ltd., China), and then washed five times with 0.01 M phosphate-buffered saline (pH 7.4), 0.85% (w/v) NaCl (PBS) containing 0.05% Tween 20 (PBST), individually vacuum-packed in aluminums bags and stored at 4°C before use.

mPCR amplification

mPCR reactions were carried out in 50 μL reaction mixtures containing 1 × PCR buffer, 2 mmol/L MgCl$_2$, 0.1 mmol/L dNTP, 0.2 μmol/L of a primer for *Shigella* and *E. coli* O157:H7, 0.3 μmol/L of a primer for *Salmonella* and *L. monocytogenes*, 1 U of *TaKaRa Ex Taq* enzyme (Takara, Japan), and 2 ng/μL DNA templates. For a sensitivity assay, 5 μL of the supernatants extracted by boiling 200 mL of a serially diluted cell suspension of a fresh culture of four pathogens were used as templates. The PCR parameter was set as 95°C for 5 min, 35 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 30 s.

Standard assay protocol

A hybridization process was performed according to the instructions of a DR. Chip DIY Kit™ (DR. Chip Biotechnology, Inc.). All of the reagents, including DR. Hyb™ Buffer, Strep-AP, wash buffer, NBT/BCIP and the detection buffer, were supplied with the Kit. In brief, PCR products were denatured in boiling water for 5 min, and immediately chilled on ice for 5 min; 15 μL of ice-cold PCR products were mixed with 200 μL DR. Hyb™ Buffer (DR. Chip Biotechnology, Inc.) and transferred to the chip well, incubated at 63°C with vibration for 40 min, and washed twice with the wash buffer (DR. Chip Biotechnology, Inc.). The chip was then added to a mixture solution containing 0.2 μL of Strep-AP (DR. Chip
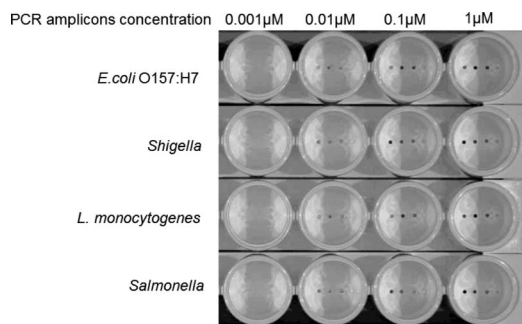
Fig. 1   Probes concentration optimization.   The concentration of probes in each well is 10, 20, 30 and 40 μM, respectively.

Fig. 2   (a) Pattern of immobilized probes.   A1, A4, D1, D4, Hybridization positive control; B1, B2, *E. coli* O157:H7; B3, B4, *Salmonella*; C1, C2, *Shigella*; C3, C4, *L. monocytogenes*.   (b) Specificity of the micro-plate chip assay for four pathogens.   PCR amplicons from one of four pathogens were hybridized with chip. From left to right: *Salmonella*, *Shigella*, *L. monocytogenes*, *E. coli* O157:H7 and simultaneous detection of four pathogens, respectively.

Biotechnology, Inc.; 0.5 μL/mL in blocking buffer) and 200 μL of the blocking reagent (DR. Chip Biotechnology, Inc.), and incubated at room temperature (25°C) for 30 min and washed twice again with the wash buffer.   Then, 4 μL of NBT/BCIP and 196 μL of the detection buffer (DR. Chip Biotechnology, Inc.) were added to the chip well and incubated for 7 min at room temperature in the dark, followed by washing twice with distilled water.   Positive hybridization results were indicated on the microarray as deep-blue spots that could be read directly by the naked eye for qualitative analysis.

Food sample detection

Food samples (beef, fish and milk) were purchased from a local market.   For the detection of bacteria from raw foods, a pre-enrichment was performed by homogenizing 25 g of meat in 225 mL of nutrient broth or 25 mL of milk in 225 mL of universal pre-enrichment broth, and then incubating the preparations at 37°C overnight; then, DNA samples were extracted.   In mock-contamination experiments, those pathogens in negative food samples were confirmed by both the culture and PCR methods.   They were artificially contaminated as follows: 25 g of food samples were inoculated with $10^2 – 10^6$ CFU of a strain before homogenization, and were then enriched directly without the need for any pre-enrichment or selective enrichment steps.   The isolates were confirmed by traditional methods.   The assay was carried out as described above.

Results and Discussion

Results

To establish the optimal capture probe concentration, 30 nL of synthetic probes with four different concentrations (10, 20, 30, and 40 μM) were spotted on the well surface.   The prepared chips were hybridized for 30 min at 45°C with PCR amplicons at concentrations of 0.001, 0.01, 0.1 and 1 μM in 200 μL of the reaction buffer.   The tested process was performed as the standard assay protocol described.   As shown in Fig. 1, the signal intensity decreased when the target concentration was lowered, while 10 μM probes for *Shigella* and *Salmonella*, 20 μM probes for *E. coli* O157:H7 and *L. monocytogenes* gave better results, which were adopted as the optimal concentration for subsequent spotting.

After establishing the optimal capture probes concentration, DNA probes, including pathogens specific probes (two repeats), hybridization control probes, were spotted on the well surface of the micro-plate as the predesigned pattern.   A series of experiments were carried out for testing the performance of the
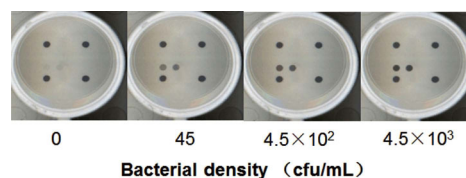

Fig. 3   Results of a *Shigella* sensitivity experiment.

micro-plate chip.   For specificity testing, the prepared chip was hybridized with PCR amplicons from single pathogen template amplification.   As expected, only those spots containing the specific probes were detected (as shown in Fig. 2(b), the first four), which indicates a remarkable specificity in this assay. Although it rarely occurred in practice that food contaminated by four pathogens occurred at the same time, when four pathogens were simultaneously amplified, positive results could also be simultaneously presented on the chip surface (Fig. 2(b), the last one).   To test the sensitivity, genomic DNA extracted from a cell culture suspension with different dilution was used as a template for PCR amplification.   The results showed that the change of the hybridization signal is consistent with the increase in the density of pathogen.   There was no visible signal when the density of pathogens was 0 CFU per milliliter, and a signal equal to that of positive control was detected when the density of *Salmonella* was 5.0×10^2 CFU, *Shigella* was 4.5×10^2 CFU, *E. coli* O157:H7 was 5.5×10^2 CFU and *L. monocytogenes* was 5.0×10^2 CFU, respectively.   As a model, the result of the sensitivity experiment for *Shigella* is shown in Fig. 3.   The results showed that 0 CFU per milliliter samples of *Shigella* gave no visible signal, 45 CFU per milliliter samples of *Shigella* gave weak signals, and 4.5×10^2 CFU per milliliter gave signals equal to that of the positive control, while samples of *Shigella* with densities of 4.5×10^3 CFU per milliliter gave strong signals.

To validate the practical performance of detection, different food samples including raw food samples (beef, milk and fish) and mock-contaminated food samples were used in this experiment.   For artificially contaminated food samples, the established micro-plate chip method successfully detected the added strains (data not shown).   Of the 20 food samples,
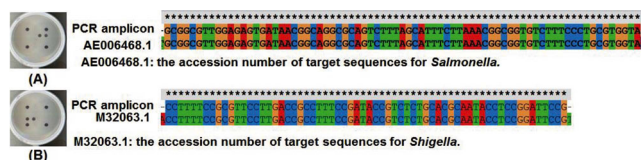
Fig. 4   Food sample detection by a micro-plate chip and PCR plus sequencing alignment (A) food sample contaminated by *Salmonella*; (B) food sample contaminated by *Shigella*.

four samples were identified as being positive by the micro-plate chip method; 2 were for *Salmonella*, and 2 were for *Shigella*, respectively. No isolated bacteria were discovered in the other samples. Contaminated food samples identified by micro-plate chip were further confirmed by PCR products sequencing alignment using Clustal X 2.0 software. All of the strains identified by the micro-plate chip were in agreement with the results of PCR products sequencing (Fig. 4).

Discussion

We developed an mPCR-based micro-plate chip assay for the detection of food-borne pathogens. Such a method integrated the advantages of mPCR and the micro-plate chip. In addition, for considering the ease of use, a colorimetric reaction by alkaline phosphatase and its substrate (NBT/BCIP) was introduced into this assay. Such a design not only facilitated the detection process (the positive results could be directly visualized by unaided eyes), but also reduced the requirement for expensive equipment. To validate the performance of this method, a series of experiments were carried out. The prepared micro-plate chip was used to detect four pathogens strains and authentic food samples. A positive signal only occurring at a spot containing the specific target probes, which demonstrates the high specificity of our assay. The detection level achieved in this study is comparable to, or better than, other similar detection techniques.[16,17] Moreover, our assays are cheaper, have high throughput and are more convenient than the existing DNA microarrays.

In conclusion, our mPCR-based visual micro-plate chip assay offers a reliable and specific method for the detection of four food-borne pathogens in a complex cell culture and food samples, and has the potential for use in the routine diagnosis of these pathogens in the food industry.

Acknowledgements

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