

Rapid and reliable detection of 11 food-borne pathogens using thin-film biosensor chips

Sulan Bai · Jinyi Zhao · Yaochuan Zhang ·
Wensheng Huang · Shi Xu · Haodong Chen ·
Liu-Min Fan · Ying Chen · Xing Wang Deng

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Abstract Traditional methods for identifying food-borne pathogens are time-consuming and laborious, so it is necessary to develop innovative methods for the rapid identification of food-borne pathogens. Here, we report the development of silicon-based optical thin-film biosensor chips for sensitive detection of 11 food-borne pathogens. Briefly, aldehyde-labeled probes were arrayed and covalently attached to a hydrazine-derivatized chip surface, and then, biotinylated

polymerase chain reaction (PCR) amplicons were hybridized with the probes. After washing and brief incubation with an antibiotin immunoglobulin G–horseradish peroxidase conjugate and a precipitable horseradish peroxidase substrate, biotinylated chains bound to the probes were visualized as a color change on the chip surface (gold to blue/purple). Highly sensitive and accurate examination of PCR fragment targets can be completed within 30 min. This assay is extremely robust, sensitive, specific, and economical and can be adapted to different throughputs. Thus, a rapid, sensitive, and reliable technique for detecting 11 food-borne pathogens was successfully developed.

Sulan Bai and Jinyi Zhao had equal contribution to this work.

S. Bai · S. Xu
Key Laboratory of Genetics and Biotechnology,
College of Life Sciences, Capital Normal University,
Beijing 100048, People's Republic of China

J. Zhao · W. Huang · Y. Chen (✉)
Institute of Food Safety,
Chinese Academy of Inspection and Quarantine,
Beijing 100123, People's Republic of China
e-mail: yqychen@yahoo.com.cn

J. Zhao
College of Food Science and Engineering,
Shandong Agricultural University,
Shandong 271018, People's Republic of China

Y. Zhang
Beijing Vocational College of Agriculture,
Beijing 102442, People's Republic of China

H. Chen · L.-M. Fan · X. W. Deng
College of Life Sciences, Peking University,
Beijing 100871, People's Republic of China

X. W. Deng (✉)
Department of Molecular, Cellular and Developmental Biology,
Yale University,
New Haven, CT 06520-8104, USA
e-mail: xingwang.deng@yale.edu

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Introduction

Food-borne pathogens are very diverse in their nature and keep causing major public health problems worldwide. Many high-risk pathogens that cause diseases in humans are transmitted through various food items. Therefore, the microbiological safety of food has become an important concern of consumers, industry, and regulatory agencies. Researchers are continuously searching for sensitive tools for detecting pathogenic microorganisms.

At present, there are four major categories of methods for detecting food-borne pathogens. The first group is the conventional microbiological methods. Conventional methods include blending the food product with a selective enrichment medium to increase the population of the target organisms, plating onto selective or differential agar plates to isolate pure cultures, and examining the cultures by phenotypic analysis or metabolic fingerprinting (monitoring the carbon or nitrogen

utilization). Conventional microbiological methods, considered to be the golden standard methods, are reliable and accurate, but time-consuming and labor intensive. It takes 2–3 days for results and up to 7–10 days for confirmation (Swaminathan and Feng 1994).

The second group is methods-based on the polymerase chain reaction (PCR). Since the mid-1980s, PCR technology has been proved to be an invaluable method for the detection of pathogens in food. Numerous papers have been published on PCR detection of different food-borne pathogens including *Escherichia coli* (Tsai et al. 1993; Naravaneni and Jamil 2005); *Salmonella* (Rahn et al. 1992); *Shigella* (Frankel et al. 1990); *Yersinia* (Ibrahim et al. 1992); *Vibrio cholerae* (Shangkuan et al. 1995); *Vibrio parahaemolyticus* (Tada et al. 1992); *Vibrio vulnificus* (Brauns et al. 1991); *Listeria monocytogenes* (Simon et al. 1996), and *Staphylococcus aureus* (Wilson et al. 1991). Real-time PCR is the most commonly used technology for quantification of specific DNA fragments. The amount of product synthesized during the PCR is measured in real time by detection of the fluorescent signal produced as a result of specific amplification. Real-time PCR requires special thermal cyclers and usually specific fluorescent probes. PCR method is rapid and sensitive, but sometimes shows false-positive and false-negative results and need further confirmation such as probe hybridization and restriction fragment length polymorphism (Baric and Dalla-via 2004).

The third group is methods-based on enzyme-linked immunosorbent assay (ELISA). The basic principle of antibody-based detection (immunoassay) is the binding of antibodies to target antigens, followed by the detection of the antigen–antibody complex. ELISA was widely used to develop methods for detecting pathogenic bacteria and bacterial toxins in foods (Notermans and Wernars 1991). This method is accurate and precise, ideal for qualitative and quantitative detection of many types of proteins in complex matrices when the targets are known, but is also laborious and time-consuming to develop and validate compared with nucleic acid tests. The sensitivities are low (in the range of 10^4 – 10^7 bacterial cells, which is achieved only after selective enrichment), and it takes about 3–4 h to complete (Swaminathan and Feng 1994). In addition, the successful use of antibodies to detect pathogens depends on the stable expression of target antigens in a pathogen, which are often affected by temperature, preservatives, acids, salts, or other chemicals in foods (Mendes-Ledesma et al. 2008).

The fourth and most recent group is the microarray-based techniques. These methods allow for the simultaneous identification of the increasing number of food-borne pathogens worldwide in a single reaction (Sergeev et al. 2004). The basic idea is that many selected probes are attached spotwise in an array format to a solid surface, and each spot contains

numerous copies of a probe. The array is subsequently hybridized with DNA isolated from the sample of interest labeled with fluorescence. During the hybridization phase, the labeled fragments will bind to the spotted probes on the basis of DNA complementarity. The larger the stretch of sequences complementarity is, the stronger the bond will be (Sergeev et al. 2004). As a high-throughput method, microarray-based techniques have some advantages, such as informative, highly repeatable, and potential to combine detection, identification, and quantification effectively of unlimited number of food-borne pathogens in a single experiment. However, the regular microarray methods need expensive equipments for array scanning and data collection, which may be beyond the budget of many laboratories, particularly those in the public sector such as trading standards laboratories and public analysts.

For a rapid, efficient, accurate, and inexpensive detection assay, we developed a method to detect food-borne pathogens on the surface of optical thin-film biosensor chips. Our preliminary result about detection and identification of four pathogens using the optical chips has been published in a journal only in Chinese (Zhao et al. 2008). Here, we expanded the work to detect 11 common food-borne pathogens in one chip and further optimized the assay in regarding to specificity and sensitivity. One of the advantages of this technology is that, due to the optical characteristics of the thin-film biosensor chip surface, experimental results can be visualized by the unaided human eye without any specific instruments. Therefore, this technology eliminates a large initial investment of purchasing expensive instruments and can be distributed to any individual research laboratory with basic molecular biology facilities. Generally, this method is rapid and robust, can be extensively multiplexed, which has excellent sensitivity and specificity, and is quite cost-competitive with the existing technologies.

Materials and methods

Strains of microorganisms used for the preparation of DNA templates

The bacterial strains used in this study were obtained from the Inspection and Quarantine Culture Collection (Beijing, China): *Salmonella typhimurium* (10503), *Salmonella dublin* (10523), *Salmonella thompson* (10514), *S. aureus* (22002), *Yersinia enterocolitica* (10901), *L. monocytogenes* (22201), *Shigella flexneri* (11304), *Shigella boydii* (11306), *Campylobacter jejuni* (13603), *E. coli* O157:H7 (10102), *V. parahaemolyticus* (12310), *V. cholerae* (12321), *Enterobacter sakazakii* (10403.17), *Pseudomonas aeruginosa* (12625). Microorganisms were grown at 37°C for 18–24 h,

with constant shaking at 220 rpm in 5 ml Luria–Bertani (LB) broth containing 3 g/L yeast extract except for that *C. jejuni* was cultivated in 5 ml Brucella broth under a microaerophilic condition, *V. parahaemolyticus* in 5 ml 3% sodium chloride alkaline peptone water, and *V. cholerae* in 5 ml alkaline peptone water.

DNA template preparation

Easy-DNA kit (Invitrogen Co., San Diego, CA, USA) was used for isolation of genomic DNA from all 11 bacterial species. The procedure was modified according to the instructions of the kit and Wang and Wang (1997). One milliliter of log-phase culture of the bacterial cells was centrifuged at 13,000 rpm for 5 min at 4°C and washed once with PBS. Of solution A (supplied by the kit), 350 µl was added to the pellet and mixed well; the mixture was heated at 65°C for 60 min for *L. monocytogenes*, *S. aureus*, and *Bacillus cereus* and 10 min for other bacterial species. A 2 µl aliquot of RNase (2 mg/ml) was added to the tube and heated at 65°C for 5 min, and then, 150 µl of solution B was added and mixed well. Chloroform (500 µl) was added and mixed well, then centrifuged at 14,000×g for 10 min. The upper phase was transferred to 1 ml 100% ethanol and mixed well, then cooled at –20°C for 20 min, and centrifuged for 10 min at 14,000 rpm. The pellet was washed with 75% ethanol and dried. The genomic DNA in the pellet was resuspended in 100 µl of TE buffer (10 mM Tris, 1 mM EDTA), and the DNA was quantified with a NanoDrop Instrument (NanoDrop Technologies, Wilmington, DE, USA) and diluted to 100 ng/µl.

PCR amplification

All PCR reactions were carried out in 50-µl reaction mixtures containing 1× PCR buffer, 2 mmol/l MgCl₂, 0.1 mmol/l dNTP, 0.2 µmol/l of each primer, 1 U of *rTaq* enzyme (TaKaRa, Japan), and 100 ng DNA templates. Reactions were initialized for 5 min at 94°C and then, 40 cycles of 94°C for 15 s, 55°C for 30 s and 72°C for 30 s were performed in a PTC-225 Peltier Thermal Cycler (MJ Research, USA), followed by a final 5 min incubation at 72°C. The PCR products were then quantified and sized by electrophoresis on agarose gels (2%, w/v). Gels were photographed with a UVIpro system (Uvitec, England).

Primer and probe design and synthesis

Oligonucleotides were synthesized by Invitrogen Co. (San Diego, CA, USA). PCR primers were designed against specific genes in 11 food-borne pathogens; these genes and the corresponding primer and probe sequences 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 an aldehyde group modification at their 5' ends followed by ten deoxyadenosine residues that constitute a “spacer,” then 40 nucleotides complementary to the corresponding target sequence.

Preparation of the optical thin-film biosensor chips

Thin-film biosensors are capable of transducing specific molecular interactions into signals visualized by the naked eye because the mass deposited on the thin-film surface by enzymatic catalysis alters the wavelength of light reflected by the optical layer and results in a perceived color change on the surface (Zhong et al. 2003; Bai et al. 2007). The biosensors were prepared following the procedure described by Zhong et al. (2003).

Standard assay protocol

Aldehyde-labeled oligonucleotides (probes) were spotted by manual pipette or a robotic Hamilton pipetting device using 200 or 40 nl per spot, respectively, onto biosensor chips from 1.0 µM stocks dissolved in 0.1 M sodium phosphate buffer, pH 7.8. PCR amplicon targets from 65 to 254 bp in length, at a concentration of 0.1 µM per 100-µl reaction, were denatured and hybridized on the chips for 10 min at 45°C in the hybridization buffer [5× saline–sodium citrate (SSC) and 5 mg/ml acid-treated casein (ATC)]. After washing three times in 0.1× SSC, the chips were incubated with an anti-biotin immunoglobulin G (IgG)–horseradish peroxidase (HRP) conjugate (Jackson ImmunoResearch; 1:1,000 dilution from a 1 mg/ml stock in a buffer containing 5× SSC, 5 mg/ml ATC, and 10% glycerol) for 5 min in hybridization buffer. The chips were rinsed with 0.1× SSC three times and then, 100 µl of tetramethylbenzidine (TMB) formulation from BioFxx Laboratories (Owings Mills, MD) was added onto the chips and incubated for 5 min at room temperature. The chips were then rinsed in ddH₂O, air-dried, and scored by eye. Digital images were taken with a simple dissection microscope (Olympus, SZX12) fitted with an inexpensive digital camera.

Sensitivity detection assay

To determine the sensitivity of the biosensor chips for detecting bacteria, *S. thompson* were incubated at 37°C for 18–24 h, and a dilution series was prepared. The bacterial DNA was extracted from the 1 ml dilution, followed by PCR and thin-film biosensor chips assay. At the same time, enumeration was done by spreading 200 µl dilutions onto LB agar plates.

Table 1 Sequences of PCR primers and capture probes for detecting food-borne pathogens

Species	Target gene	Sequences	Fragments size (bp)	Accession number	Reference
<i>Salmonella</i>	<i>Fimbriae Y protein</i>	F : 5'-GCGGCGTTGGAGAGTGATA R: 5'-biotin-AGCAATGGAAAAAGCAGGATG	103	AE008721	Yeh et al. 2002
<i>Shigella</i>	<i>Invasion plasmid antigen (ipaH)</i>	P: 5'-ALD -aaaaaaaaaCATTCTTAAACGGCGGTGCTTTCCCT F: 5'-CGCAATACCTCCGATTCC R: 5'-biotin-TCCGCAGAGGCACTGAGTT	65	CP000266	Liu et al. 2007
<i>S. aureus</i>	<i>Glutamate synthase-related protein (gsp)</i>	P: 5'-ALD-aaaaaaaaaACAGGTCGCTGCATGGCTGGAA F: 5'-GGTACTACTAAAGATTATCAAGACGGCT R: 5'-biotin-TTCTTACGACTAAATAAACGCTCA	147	CP001781.1	This paper
<i>Y. enterocolitica</i>	<i>16S rRNA</i>	P: 5'-ALD-aaaaaaaaaCAGAACACAAATGTTCCGATGCAACGT F: 5'-TTCTGCGAGTAACGTCAATCACA R: 5'-biotin-AAGAAGGCCTTCGGGTTGTAA	86	Z49830	This paper
<i>L. monocytogenes</i>	<i>Invasion-associated protein</i>	P: 5'-ALD-aaaaaaaaaATTAACTTTATGCCTTCCTCCTCGCTG F: 5'-CTGAATCTCAAGCAAAACCTGGT R: 5'-biotin-CGCGACCGAAGCCAACTA	174	DQ054587	Ha et al. 2002
<i>C. jejuni</i>	<i>Cytochrome c oxidase</i>	P: 5'-ALD-aaaaaaaaaATCGATAACATCCACGGCTCTGGCTGG F: 5'-TTGGTATGGCTATAGGAACCTTATAGCT R: 5'-biotin-CACACCTGAAGTATGAAGTGGTCTAAGT	116	CP000538	This paper
<i>E. coli O157:H7</i>	<i>Perosamine synthetase</i>	P: 5'-ALD-aaaaaaaaaATGGCATATCCTAAATTA F: 5'-TCCTCAGCTATAGGTGCTTTTG R: 5'-biotin-ATCGAAACAAGGCCAGTTTITTTAC	83	BA000007	Zhao et al. 2007
<i>V. parahaemolyticus</i>	<i>Membrane protein ToxS</i>	P: 5'-ALD-aaaaaaaaaTATTTTCCGAGTACATTGGCATCGTGTGG F: 5'-CATTCGCGTGGCAAAACATC R: 5'-biotin-GCGACCTTCTCTGAAATATTAATTGT	79	BA000031	Takahashia et al. 2005
<i>V. cholerae</i>	<i>Hemolysin</i>	P: 5'-ALD-aaaaaaaaaCGCACAAAGGCTCGACGGCTGA F: 5'-GCTTTATTTGTCGATGCGTTAAAC R: 5'-biotin-GATGCCAAAATTTGTGCGTATCA	188	AF003853	Williams and Manning 1991
<i>E. sakazakii</i>	<i>Macromolecular synthesis (MMS) operon</i>	P: 5'-ALD-aaaaaaaaaTCTTGGGCAATCGCATCGGTTGA F: 5'-CCGTTGACGTAGCACTGC R: 5'-biotin-CATAGAATTACGACGACGAACTTC	85	L01755	Seo and Brackett 2005
<i>P. aeruginosa</i>	<i>Gyrase B</i>	P: 5'-ALD-aaaaaaaaaTTCAAACGTTCTCGAGAGAAAGCGG F: 5'-CCTGACCATCCGTCGCCACAAC R: 5'-biotin-CGCAGCAGGATGCCGACGCC	254	CP000438	Motoshima et al. 2007

F forward PCR primer, R reverse PCR primer, P probe, ALD aldehyde modification

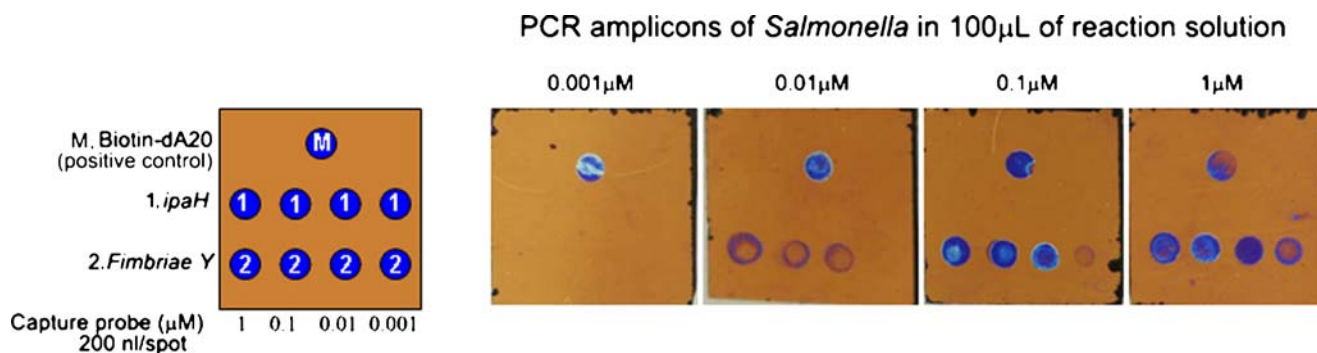


Fig. 1 Specificity and sensitivity of food-borne pathogen detection on a chip with capture probes spotted by hand at various concentrations. Capture probes for the *ipaH* gene from *Shigella* and *Fimbriae Y* gene from *Salmonella* were spotted by hand at a volume of 200 nl and

concentrations of 1, 0.1, 0.01, and 0.001 μM, respectively (left panel). M Biotin-dA20 at 1 μM, positive control. PCR amplicons of *Fimbriae Y* at concentrations of 0.001, 0.01, 0.1, and 1 μM, respectively, in 100 μl reaction solution were hybridized to four identical chips (right panels)

Practical detection sensitivity

Pork samples confirmed as negative for *S. thompson* were used as the matrix for this experiment. Six meat samples of 25 g were added to 225 ml of modified LB, respectively, and homogenized. *S. thompson* cultures grown to log phase were diluted, and 0, 10, 65, 6.75×10^2 , and 4.32×10^3 colony-forming unit (cfu) were added to 25 g pork samples, respectively. The contaminated homogeneous suspensions were incubated at 37°C for 18–24 h, and then, DNA was extracted from 1 ml of each contaminated suspension and analyzed by PCR and the thin-film biosensor chips assay.

Results

Defining sensitivity and specificity parameters of biosensor chips

In order to establish the suitable probe density for spotting on a biosensor chip, we chose probes for the *invasion plasmid antigen (ipaH)* gene from *Shigella* and *Fimbriae Y*

gene from *Salmonella* and diluted them to various concentrations. As shown in Fig. 1 (left panel), 200 nl of each probe at concentrations of 1, 0.1, 0.01, and 0.001 μM were manually spotted on the chips' surfaces. Identical chips were hybridized for 10 min at 45°C with *Fimbriae Y* PCR products from *Salmonella* at concentrations of 0.001, 0.01, 0.1, and 1 μM in 100 μl total reaction volume. The chips were washed once with $0.1 \times$ SSC at room temperature, followed by a 5 min incubation with anti-biotin IgG–HRP conjugate and a 5 min incubation with TMB substrate. The chips were then washed, dried, and visualized (Fig. 1, right panels). As expected, only the spots containing the *Fimbriae Y* probe, but not the *ipaH* probe, were detected, indicating a remarkable specificity in this assay. Although the signal intensity decreased as the target sequence concentration was lowered, as little as 0.01 μM of target was detected by the probe spotted at 0.01 μM, while the 1 μM probe gave better results. Probes spotted at concentrations higher than 1 μM did not increase detection sensitivity in the range of target concentrations tested, leading us to adopt the 1 μM probe concentration for subsequent spotting.

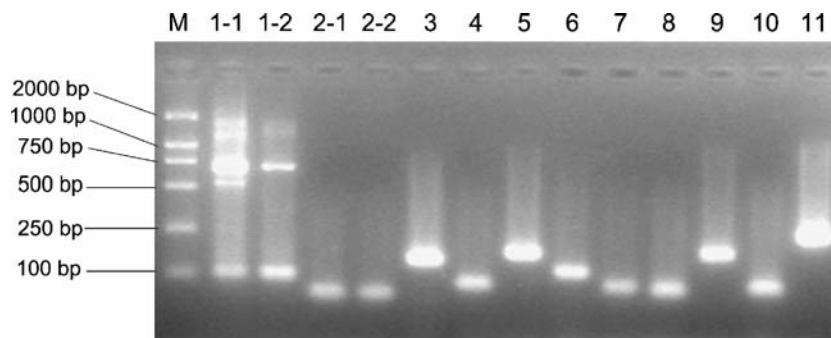


Fig. 2 Amplification of target DNA fragments for 11 specific genes from individual food-borne pathogen strains by PCR. PCR products were examined by agarose gel electrophoresis. M DNA maker DL2000; 1-1 *fimbriae Y* (from *S. typhimurium*); 1-2 *fimbriae Y* (from *S. dublin*); 2-1 *ipaH* (from *S. flexneri*); 2-2 *ipaH* (from *S. bogdii*); 3

gsp (*Staphylococcus*); 4 16 S rRNA (*Y. enterocolitica*); 5 *Iap* (*L. monocytogenes*); 6 cytochrome C oxidase (*C. jejuni*); 7 perosamine synthetase (*E. coli* O157:H7); 8 *ToxS* (*V. parahaemolyticus*); 9 hemolysin (*V. cholerae*); 10 *MMS* (*E. sakazakii*); 11 gyrase B (*P. aeruginosa*)

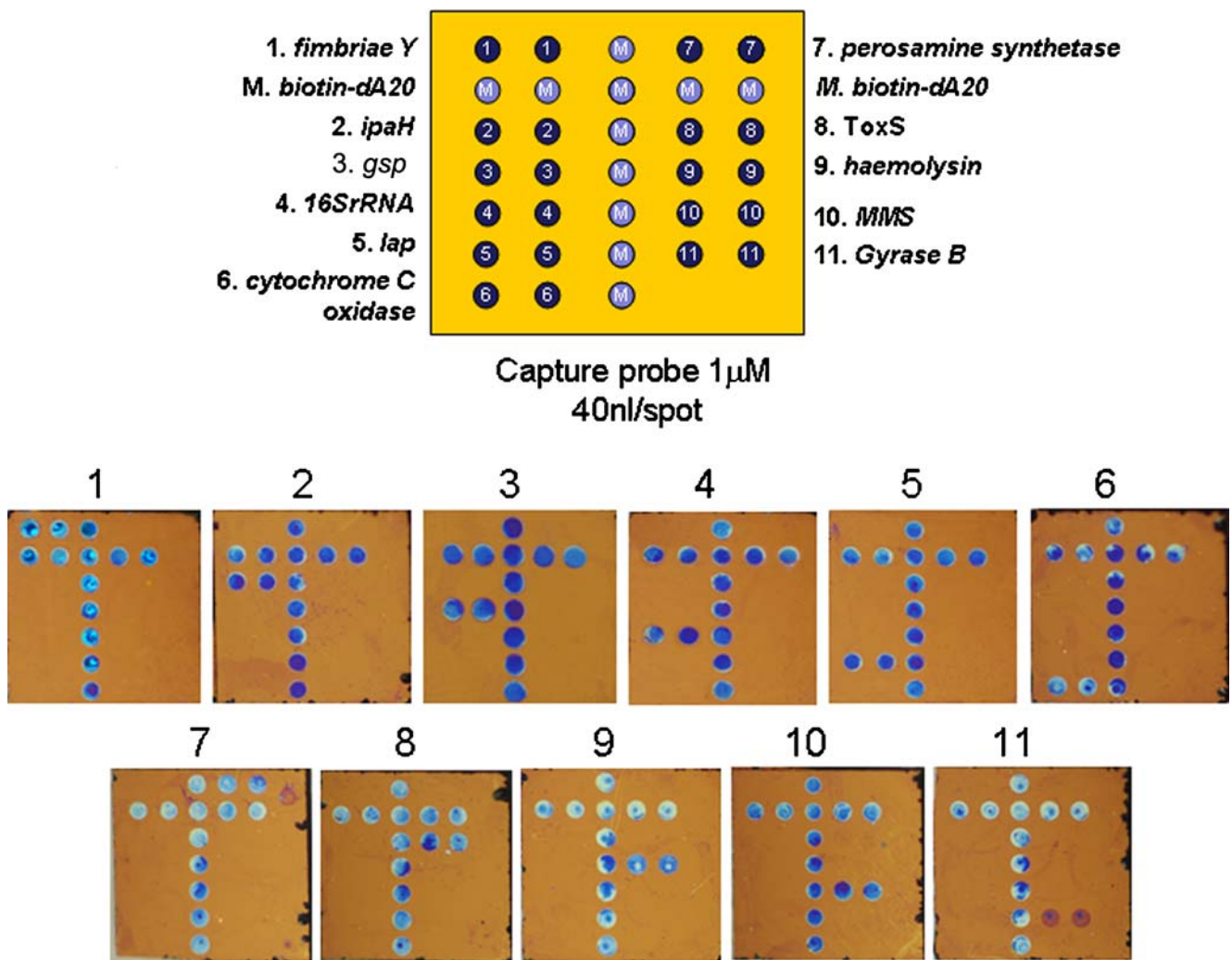


Fig. 3 Food-borne pathogen detection on a chip with capture probes spotted by a computer-controlled dispenser. Each spot comprised 40 nl of 1 μ M probe solution. Capture probes were printed in the order shown in the upper panel: M biotin-dA20 (positive control marker); spots 1 fimbriae Y (*Salmonella*); 2 ipaH (*Shigella*); 3 gsp (*Staphylococcus*); 4 16SrRNA (*Y. enterocolitica*); 5 lap (*L. monocytogenes*); 6 cytochrome C

oxidase (*Campylobacter jejuni*); 7 perosamine synthetase (*E. coli* O157: H7); 8 ToxS (*V. parahaemolyticus*); 9 hemolysin (*V. cholerae*); 10 MMS (*E. sakazakii*); 11 gyrase B (*P. aeruginosa*). Lower panel shows the detection of the food-borne pathogen-specific genes on thin-film biosensor chips. The PCR targets from genes 1 to 11, respectively, were applied on food-borne pathogen-detection chips

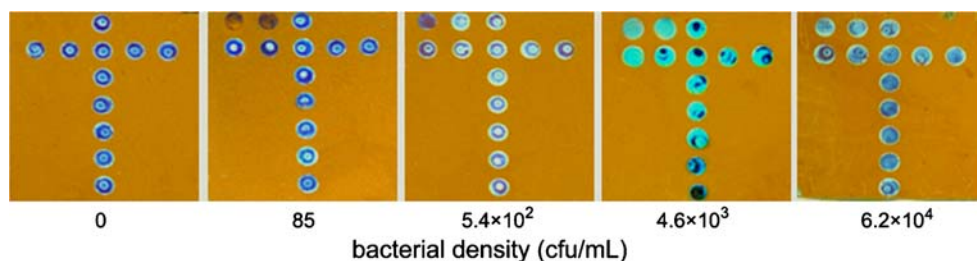


Fig. 4 Results of the sensitivity experiment. The results showed that 0 cfu per milliliter samples of *S. thompson* gave no visible signal, 8.5×10^1 cfu per milliliter samples of *S. thompson* gave weak signals, while samples of *S. thompson* with densities of 5.4×10^2 cfu per milliliter,

4.6×10^3 cfu per milliliter, and 6.2×10^4 cfu per milliliter gave strong signals. The bacterial densities are shown under the corresponding pictures

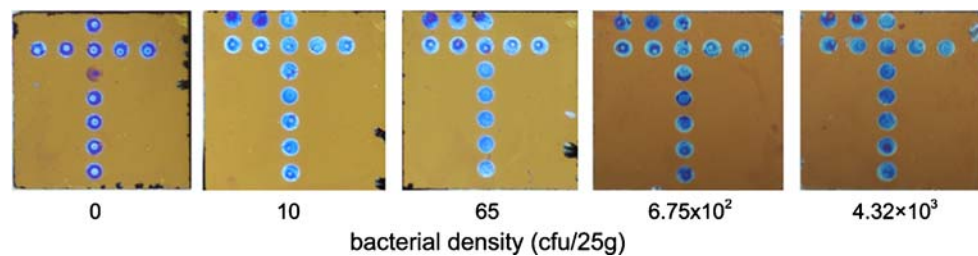


Fig. 5 Results of the model experiment. The results showed that 0 cfu per 25 g samples of *S. thompson* gave no signal, whereas the 10, 65, 6.75×10^2 , and 4.32×10^3 cfu per 25 g *S. thompson* samples gave visible signals. The bacterial densities are shown under the corresponding pictures

Amplification of 11 target gene fragments by PCR

The PCR products for the 11 species are shown in Fig. 2. The sizes of PCR products are the same as predicted (Table 1). Some extra bands above 103 bp may represent nonspecific amplification when using a high concentration of cells or DNA of *S. typhimurium* and *S. dublin* (lanes 1-1, 1-2, Fig. 2).

Detection of target genes from food-borne pathogens using optical thin-film biosensor chips

Chips spotted by robotic pipetting (40 nl per spot) as shown in Fig. 3 (upper) were used to detect food-borne pathogens. The DNA targets were successfully amplified by PCR (Fig. 2) and hybridized to the chips. For example, the *Fimbriae Y* gene targets were amplified from the *S. typhimurium* and *S. dublin* samples (Fig. 2). The DNA targets amplified from the *Fimbriae Y* gene of *S. typhimurium* were used for hybridization in a 100- μ l reaction, and the assay resulted in one set of colored dots (Fig. 3, lower panel, 1). The DNA fragments amplified from *S. dublin* showed the same pattern (data not shown). The other ten gene targets showed their own specific sets of colored dots (Fig. 3, 2–11). No false positives were observed among these tests. Our data indicates that one custom-designed chip can be readily used to detect the presence of these 11 food-borne pathogens. This chip may be further modified to accommodate all needs for the detection of commercialized food-borne pathogens.

Sensitivity and limitations of the assay

The results of the method sensitivity experiment are presented in Fig. 4, which shows that the change in hybridized signal is consistent with the increase in *S. thompson* density. There was no visible signal when the density of *S. thompson* was 0 cfu per milliliter, a weak signal when the density was 8.5×10^1 cfu per milliliter, and a strong signal equal to that of the positive control was detected when the density of *S. thompson* was 5.4×10^2 cfu per milliliter (Fig. 4).

Pork samples polluted with *S. thompson* were used to assess the practical detection sensitivity of the method. The results showed that as few as 10 cfu *S. thompson* were detected in a 25 g sample of pork (Fig. 5), which means that the practical detection limit of the method was 0.4 cfu per gram.

Discussion

Ribosomal DNA (rDNA) sequences have frequently been used for PCR amplification in biochip tests for food-borne pathogens (Abdullahi et al. 2005; Wang and Wang 1997). However, multiple sequence alignments of the published 16S and 23S rDNA nucleotide sequences showed that different pathogens have highly homologous sequences, and it was difficult to design probes for identifying food-borne pathogens, especially among the *Enterobacteriaceae* (data not shown). In this case, false-positive or false-negative results might be obtained. Therefore, in this study, all primers and probes for identifying 11 food-borne pathogens were designed against genes specifically found in the respective pathogens so as to prevent false-positive or false-negative results.

In order to simplify experiments, the annealing temperatures of all primers were set at 58°C. In this study, the lowest detection limit of the method was 8.5×10^1 cfu per milliliter, a much greater sensitivity than that of published methods (Gao et al. 2007; Wang et al. 2007). Moreover, the simple equipment requirements of this method make it possible to be widely used by diverse laboratories (Jenison et al. 2001). Taken together, the method is rapid, simple, specific, and sensitive and is suitable for the detection of food-borne pathogens.

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