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ORIGINAL PAPER

Establishment and preliminary application of oligonucleotide microarray assay for detection of food-borne toxigenic microorganisms

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Abstract Rapid, high-throughput and accurate detection and identification of food-borne toxigenic microorganisms is crucial for food safety nowadays. An oligonucleotide microarray was designed and established and was applied to detect common food-borne toxigenic microorganisms in this study. PCR amplification of marker genes and 16S rRNA gene of 14 toxigenic bacteria and fungi using specific primers and oligo probes residing in these genes were employed and designed to fabricate the microarray. Optimization of hybridization conditions was implemented. The optimal conditions for hybridization were 51 °C for 30 min. Furthermore, the ratio of biotin labeled to unlabeled primer for PCR amplification was also optimized to enhance specific hybridization of the microarray. Specificity, sensitivity (710 CFU/mL), and reproductivity assessment confirmed the practicability of the microarray. Finally, this microarray was successfully applied to detect 6 common toxigenic microorganisms from 328 food samples. The established microarray may provide potential for rapid detection and identification of toxigenic microorganisms from foods.

Keywords Toxigenic microorganism · Oligonucleotide microarray · Food-borne · Detection · Optimization

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Introduction

Food-borne diseases are major public health problem, which caused more than 200 different diseases known to be transmitted from the animal reservoir via food or which contaminates the food on the processing line [1]. It was reported that food-borne microorganisms have caused 76 million cases and 5,000 deaths each year in the United States [2]. In China, hundreds of thousands of cases were documented every year, around 38–56 % of which are resulted from pathogenic bacteria [3]. Most food-borne toxigenic microorganisms, including *Staphylococcus aureus*, *Salmonella* spp., *Campylobacter jejuni*, *Yersinia enterocolitica*, *Vibrio parahaemolyticus*, *V. cholerae*, *Escherichia coli* O157:H7, *Bacillus cereus*, *Clostridium perfringens*, *Shigella* spp., *Listeria monocytogenes*, *C. botulinum*, *Aspergillus flavus*, *A. parasiticus*, could secrete toxin during growth process and then released by certain substances of the lytic body after cell death [4]. The pathways of toxigenic microorganism infection have been increasing to some extent due to the globalization and application of new natural food production processes, changes in people's eating habits and other causal factors, which has been causing mass poisoning events. Recently, most poisoning accidents are caused by toxigenic microorganisms, the toxicity of which even exceeds synthesized chemical poisons [5–7]. Therefore, development of rapid detection and characterization methods for toxigenic microorganisms has attracted increasing and intensive attentions throughout the world.

Detection and isolation of toxigenic microorganisms from food are often difficult due to the high number of contaminating and indigenous bacteria and a low number of the pathogenic bacteria of concern. Furthermore, due to the differences of life cycle and genetic backgrounds

between bacteria and fungi, it is difficult to simultaneously detect and characterize toxigenic bacteria and toxigenic fungi effectively. Most toxigenic microorganism detection approaches at present still rely on traditional culture methods that inevitably present the following drawbacks [8]: (1) less sensitive to mutant strains; (2) serious serotype cross-reactivity; (3) requirement for subjective experience of judgment; (4) low detection limit; (5) complicated operation and time-consuming test cycle adverse to fast port detection requirements. Moreover, conventional PCR method is prone to false positives, and thus, this method cannot achieve the requirements of quantitative analysis and massive detection [8]. Though multiplex PCR can detect a variety of toxigenic microorganisms simultaneously, it cannot achieve parallel detection of all toxigenic microorganisms due to the constraints of primer design, which hinder the widespread application of this technique [9]. Fluorescent PCR is characterized by high detection sensitivity, but it is difficult to establish high-throughput, rapid and specific detection methods for toxigenic microorganisms based on this technique [10]. Therefore, only a fraction of all food-borne infections are ever diagnosed and officially reported using traditional methodologies.

Microarray is a cutting-edge biotechnology developed in the 1990s for specific detection of nucleic acid on the basis of molecular hybridization techniques and microcrystalline science [11]. It possesses incomparable advantages against traditional detection method: miniaturization, high throughput, parallel processing, rapid analysis, high accuracy, high sensitivity, and quantitative analysis [12]. It is capable of rapid online detection of contaminated toxigenic bacterial and fungal virulence genes, and therefore, it reflects the potential food toxin pollution problems in time. Since a large number of microbial toxins are many structurally similar compounds derived, the existing methods have inevitable shortcomings regarding specificity, sensitivity, scope of application, and testing cost [12]. Microarray has the advantage over conventional techniques of high-throughput analysis; by reverse hybridization, it can simultaneously fulfill parallel analysis of multiple genes and sequence, synchronization detection and identification of a variety of toxigenic microorganisms. Consequently, it has been applied in many fields, including detection of food-borne bacterial pathogens [3], gene expression profiling of skin cancer [13], and environmental microorganisms [14]. Detailed review on the DNA microarray was given by Bryant et al. [15].

In this study, we used primers designed to amplify the marker genes and 16S rRNA gene of 14 toxigenic bacteria and fungi. Oligonucleotide probes were designed and an oligonucleotide microarray was designed and fabricated. Hybridization conditions were optimized and quality assessment was completed for the optimized microarray. The optimized microarray was applied to detect 6 common toxigenic microorganisms from 328 food samples.

Materials and methods

Bacterial and fungal strains and genomic DNA isolation

Bacterial and fungal strains listed in Table 1 for developing microarray method in this study are standard strains. Microorganism cultures were tenfold serially diluted and viable plate counts were determined by plating on standard plate count agar in triplicate. Bacterial strains were cultivated according to Wang et al. [3]; fungal cultures were grown, according to Leinberger et al. [16], in Sabouraud agar for 72 h at 30 °C. Fungal cultures were washed with 10 mL of sterile saline (0.9 %) to obtain conidia. For the detection of food-borne microorganisms from raw foods, cultures were pre-enriched by homogenizing 25 g of solid samples in 225 mL of nutrient broth (Becton–Dickinson, Kansas, USA) or 25 mL of liquid samples in 225 mL of universal pre-enrichment broth (Becton–Dickinson, Difco) and then incubating the preparations at 37 °C overnight, with the exception of fungal strains which were cultured as discussed above. Genomic DNA from all samples was extracted using a soil DNA extraction kit (Mobio). The concentration of the DNA extracted was determined using a UV spectrophotometer (Ultrospec 3100 pro, Amersham Bioscience), and its quality was checked using the 260 nm/280 nm ratio and by gel electrophoresis. The DNA was stored at −20 °C until being used.

Table 1 Strains used in this study

Strain	Collection No.	Source
Staphylococcus aureus	ATCC33591	D
Salmonella spp.	CMCC(B)50017	B
Campylobacter jejuni	ATCC33291	D
Yersinia enterocolitica	CMCC(B)52204	B
Vibrio parahaemolyticus	VPL4-90	B
Vibrio cholera	Vb0	E
Escherichia coli O157:H7	NCTC12900	B
Bacillus cereus	CMCC(B)63303	B
Clostridium perfringens	ATCC13124	D
Shigella spp.	51334-20	A
Listeria monocytogenes	54004-2	A
Clostridium botulinum	/	/
Aspergillus flavus	CGMCC 3.0117	C
A. parasiticus	CGMCC 3.0124	C

A, CMCC (B); B, NCTC; C, CGMCC; D, ATCC; E, Guangdong Huankai Microbial Sci & Tech. Co., Ltd.

Design of PCR primer and gene specific oligonucleotide probes

Primers were designed based on the specific genes for toxin products or characterized genes of toxigenic microorganisms retrieved from NCBI (Table 2). Multiple sequence alignment was conducted using Clustal X 1.83 and conserved regions were selected for design of primer and oligonucleotide probes complementary to the non-biotin-labeled strand. A homology search was done using Blast GenBank to confirm the uniqueness of the sequence. Each oligonucleotide sequence was attached to an amino group at the 5′ end to allow covalent bonding with an aldehyde slide (the slides were chemically treated by uniform surface of reactive aldehyde groups that react to primary amines that are attached to the single-strand nucleic acid). All DNA oligos were synthesized by Shanghai Invitrogen Biotech Company Ltd (Life Technologies Corporation, Shanghai).

To select adaptive probes to construct the chip matrix, all candidate probes (3 probes for each strain) were spotted and target gene PCR products of all strains used in this study were hybridized. According to the detection results, 1–2 probes of each target were used for further manipulation. The PCR mixture contained 25 μL Premix Ex Taq (TaKaRa, China), 20 pmol each specific primer, 50 ng of genomic DNA template, eventually distilled water was added to adjust a final 50 μL reaction volume. Following an initial incubation at 95 °C for 5 min, target amplification was achieved by 35 cycles at 94 °C for 30 s, Tm (Table 2) for 30 s, 72 °C for 30 s, and terminated with a cycle of 72 °C for 10 min incubation. The PCR product was verified by agarose electrophoresis and visualization with ethidium bromide.

Microarray chip preparation and hybridization

A tail composed of 19 T bases was added on each 5′ end of oligonucleotide probe, including the positive control probe.

Table 2 Target genes and PCR primers used in this study

Strain	Sequence	Tm (°C)	Fragment (bp)	Accession no.
Staphylococcus aureus	femA-F: aaa aaa gca cat aac aag cg	57	132	DQ352458
	femA-R: gat aaa gaa gaa acc agc ag			
Salmonella spp.	invA-F:gtg aaa tta tcg cca cgt tcg ggc aa	64	284	EU348367
	invA-R: tca tcg cac cgt caa agg aac c			
Campylobacter jejuni	VS1-F: gat atg tat gat ttt atc ttg c	56	358	CP000814
	VS1-R: gaa tga aat ttt aga atg ggg			
Yersinia enterocolitica	ail-F: tta atg tgtacg ctg gga gtg	62	425	AY004311
	ail-R:gga gta ttc ata tga agc gtc			
Vibrio parahaemolyticus	tlh-F: aaa gcg gat tat gca gaa gca ctg	60	450	AY578148
	tlh-R: gct act ttc tag cat ttt ctc tgc			
Vibrio cholerae	ompW-F:cac caa gaa ggt gac ttt att gtg	64	588	X51948
	ompW-R: gaa ctt ata acc acc cgc g			
Escherichia coli O157:H7	rfbE-F: att gcg ctg aag cct ttg	55	499	CP001368
	rfbE-R: cga gta cat tgg cat cgt g			
Bacillus cereus	16s-F: cgc tgg cgg cag gcc taa cac atgc	62	500	L37587.1
	16s-R: cgc ggc tgc tgg cac gga gtt agc c			
Clostridium perfringens	cpα-F: aga tat gaa tgg caa aga gga aac	55	125	AB477855
	cpα-R: gct atc aac ggc agt aac att ag			
Shigella spp.	ipaH-F: gtt cct tga ccg cct ttc cga tac cgt c	65	629	EU743832
	ipaH-R:gcc ggt cag cca ccc tct gag agt ac			
Listeria monocytogenes	prfA-F: gat aca gaa aca tcg gtt ggc	55	274	EU294567
	prfA-R: gtg taa tct tga tgc cat cag			
Clostridium botulinum	16s-F: cgc tgg cgg cag gcc taa cac atg c	55	500	L37587
	16s-R: cgc ggc tgc tgg cac gga gtt agc c			
Aspergillus flavus/ A. parasiticus	nor-F: acc gct acg ccg gca ctc tcg gca c	55	400	AY371490.1
	nor-R: gtt ggc cgc cag ctt cga cac tcc g			
	omt-F: ggc ccg gtt cct tgg ctc cta agc	65	1,024	L22091.2
	omt-R: cgc ccc agt gag acc ctt cct cg			

Each probe was diluted to a concentration of 30 pmol/μL and added into two volumes of spotting Buffer, respectively. 15 μL of each probe solution was then spotted to each specific position on the microarray polymer substrate using an automatic spotting machine DR. Fast spot system (DR. Chip Biotech, Taiwan) and immobilized by a UV Cross-linker SCIENTZ 03-II (Ningbo Scientz Biotechnology Co., Ltd., Taiwan).

The hybridization reaction between each DNA template and probe was carried out with DR. Chip DIY^{TM} Kit (DR. Chip Biotech, Taiwan). The procedures followed the manual and are briefly described below. The PCR product was denatured at 100 °C for 5 min and cooled in an ice bath for 5 min. To the microarray chamber was added 200 μL of DR. Hyb^{TM} Buffer (containing the 5′ end-biotinylated oligonucleotide complementary to the sequence of positive control probe) and 15 μL of denatured PCR product, incubated at 45 °C with vibration for 45 min using DR. Mini Oven (DR. Chip Biotech, Taiwan), and washed twice with 250 μL Wash Buffer. The blocking reaction was then performed by mixing 0.2 μL of Strep-AP (Streptavidin conjugate alkaline phosphates) and 200 μL of Blocking Reagent at room temperature for 30 min, and washing twice with 250 μL Wash Buffer. The colorimetric reaction was then implemented by adding 4 μL of NBT/BCIP and 196 μL of Detection Buffer in the chamber, developing in the dark at room temperature for 5 min, and washing twice with distilled water. Fluorescent images of the microarray were generated by scanning the slides by using a DR. AIM READER (DR. Chip Biotech, Taiwan). Moreover, the hybridization result was also indicated as the developed pattern on the microarray, which was read directly with the naked eyes.

Optimization of hybridization conditions

Different hybridization temperatures ranging from 45 to 54 °C, to be specific, four gradients: 45, 48, 51, and 54 °C, were tested to determine the optimal hybridization temperature with target gene PCR products of *C. botulinum*, *Salmonella* spp., *Shigella* spp., and *L. monocytogenes*. Then, hybridization time of 30, 45, and 60 min was optimized under the resultant optimal temperature, respectively. Furthermore, four concentration ratios of labeled to unlabeled primer, 1:1 (5 μM:5 μM), 2:1 (10 μM:5 μM), 3:1 (15 μM:5 μM), and 4:1 (20 μM:5 μM), were also tested to maximize the biotin labeling efficient of PCR products. During this test, the same PCR condition as that of amplification of target genes above was utilized.

Quality control of chip hybridization

Specificity, sensitivity, reproductivity tests, in combination of conventional counting method, were conducted to evaluate the microarray method established here. Fourteen target genes (*S. aureus femA*, *C. jejuni* 16s, *Shigella* spp. *ipaH*, *E. coli* O157:H7 *rfbE*, *V. parahaemolyticus tlh*, *V. cholerae ompW*, *Salmonella* spp. *invA*, *C. jejuni vs1*, *C. perfringens cp* α, *A. flavus nor/omt*, *A. flavus nor/omt*, *L. monocytogenes prfA*, *Y. enterocolitica ail*, *B. cereus* 16s RNA gene) were used for specificity evaluation; and *Shigella* spp. was selected for sensitivity test. *Salmonella* spp. was used for reproductivity experiment which was repeated four times.

Detection of food-borne toxigenic microorganism

The established DNA microarray method was applied to food-borne toxigenic microorganism detection in Zhuhai, China. Five categories of 328 food samples were tested and 6 common toxigenic microorganisms including *S. aureus*, *L. monocytogenes*, *Salmonella* spp., *Shigella* spp., *V. cholerae*, and *V. parahaemolyticus* were monitored (Table 4). Food samples that were used in the experiments were purchased from a supermarket. For the detection of bacteria from raw foods, cultures were pre-enriched by homogenizing 25 g of sample 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) and then incubating the preparations at 37 °C overnight, and then DNA were extracted. In the mock-contamination experiments, only those food samples that were confirmed to be pathogens negative by both culture and PCR methods were used. Food samples were mock contaminated in the following way: Food samples (25 g) were inoculated with 10^2–10^6 CFU of a strain before homogenization and were enriched directly without the need for pre-enrichment or selective enrichment steps. The isolates were confirmed using traditional methods. DNA extraction, PCR amplification, and hybridization were carried out as described above.

Results

Primer validation and oligonucleotide probe selection

The efficiency of the primer sets used was first assessed by homology search through Blast GenBank to confirm the uniqueness of the sequences (data not shown); then, PCR was employed to amplify the corresponding target genes. As is shown in Fig. 1, all PCR products are in accordance with the expected fragments, respectively. Sequencing results ultimately validate the obtained PCR sequences (data not shown).

After the primers were validated, three candidate oligonucleotide probes for each species were designed

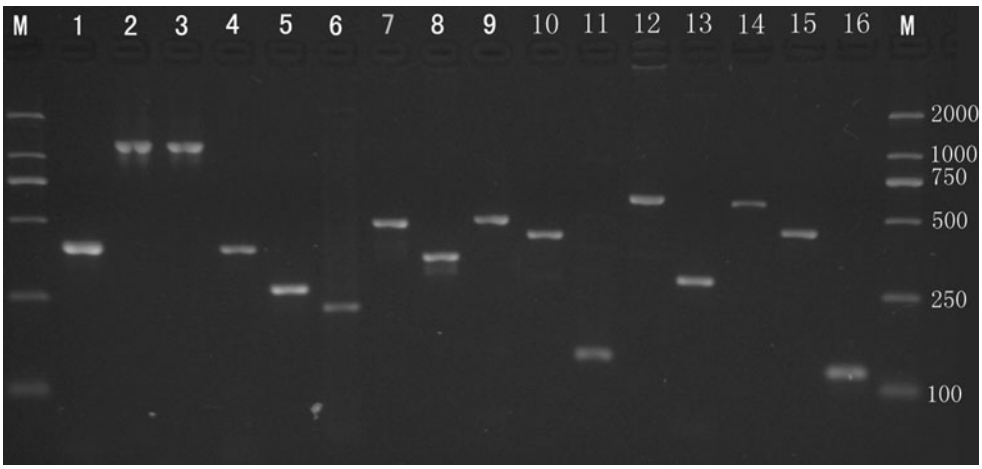

Fig. 1 Target genes amplified by PCR. M, DL2000 Ladder Marker; 1, *Aspergillus flavus nor*; 2, *A. flavus omt*; 3, *A. parasiticus omt*; 4, *A. parasiticus nor*; 5, *prfA*; 6, *bot*; 7, *rfbe*; 8, *vsl*; 9, 16s; 10, *ail*; 11, *fema*; 12, *ipah*; 13, *inva*; 14, *ompW*; 15, *tlh*; 16, *cpα*

according to the target fragments amplified by their corresponding primers. All candidate probes were spotted and the microarray was then established as Fig. 2l shown. One to two probes with specific and favorite hybridized signal were chosen for further investigation (Table 3). Probe 5′-AGCGATTCCTTGCTCCTGAGCAAC-3′ specifically hybridized with *E. coli* O55:H7 str. 3256-97 strain *dnak* gene (GenBank accession no. NZ_AEUA01000126.1) was used as negative control, no probe was used in blank control, and specific probe against *Legionella mip* gene was used as positive control (Table 3).

Optimization of hybridization conditions

To optimize the temperature for microarray hybridization, four target genes of *Salmonella* spp., *L. monocytogenes*, *C. botulinum,* and *Shigella* spp. were hybridized and characterized, respectively. As Fig. 2a–d shown, under low hybridization temperature (45 and 48 °C), though the hybridization signal was detected to be stronger than others, nonspecific hybridization was also detected. When the temperature was elevated to 54 °C, lowest signal was observed. Thereby, 51 °C was the best option due to the relative high specificity and strong detection signal.

With respect to the optimized hybridization time, prolonging hybridization time (45 min and 60 min) could enhance the detectable signal, but also generated nonspecific hybridization (Fig. 2f, g). The principle for hybridization time selection is compromised between signal strength and specificity. So we selected 30 min for hybridization, as Fig. 2e shown.

The ratio of biotin labeled to unlabeled primers could significantly affect the PCR and eventually impacted the efficiency of hybridization. As discussed above, we mainly considered signal strength and hybridization specificity to optimize the ratio. We found that the ratio of biotin labeled to unlabeled primers used to amplify *rfbE* of *E. coli* O157:H7 and *prfA* of *L. monocytogenes* should be 2:1, while others should be 1:1 (Fig. 2h–k). Consequently, further hybridization tests were conducted under the optimized conditions determined here.

Quality evaluation of the optimized microarray

After the hybridization conditions have been optimized, subsequent assessments were carried out in three aspects, including specificity, sensitivity, and reproductivity. We tested all stains to monitor the specificity of this optimized microarray, and *Salmonella* spp. was used for reproductivity test, while *Shigella* spp. was used for sensitivity detection in combination with culture test.

As Fig. 3 shown, the optimized microarray exhibited high specificity with the targets, each target hybridized with their corresponding probes specifically. No signal of off-target was detected which revealed low cross-linked hybridization was occurred.

Shigella spp. was used for sensitivity detection. Freshly cultured bacteria were serially diluted and then plate counting was performed (Fig. 4f–g). In addition, hybridization of different dilution gradients was shown in Fig. 4a–e. DNA was extracted and amplified by PCR. The PCR products were hybridized with the oligonucleotide probes on the oligonucleotide microarray. The results show that the sensitivity of the oligonucleotide microarray is 710 CFU/mL (Fig. 4c, f).

To evaluate the reproductivity of the assay, *Salmonella* spp. was detected using the described oligonucleotide microarray. Different batches of *Salmonella* spp. targets were compared under the same conditions as above (Fig. 5). No difference was observed between different batches (Fig. 5a–c) or among batch (Fig. 5d–h), which revealed good reproductivity and stability of this microarray. The experiment was repeated four times, applying the same conditions optimized. The coefficient of variation

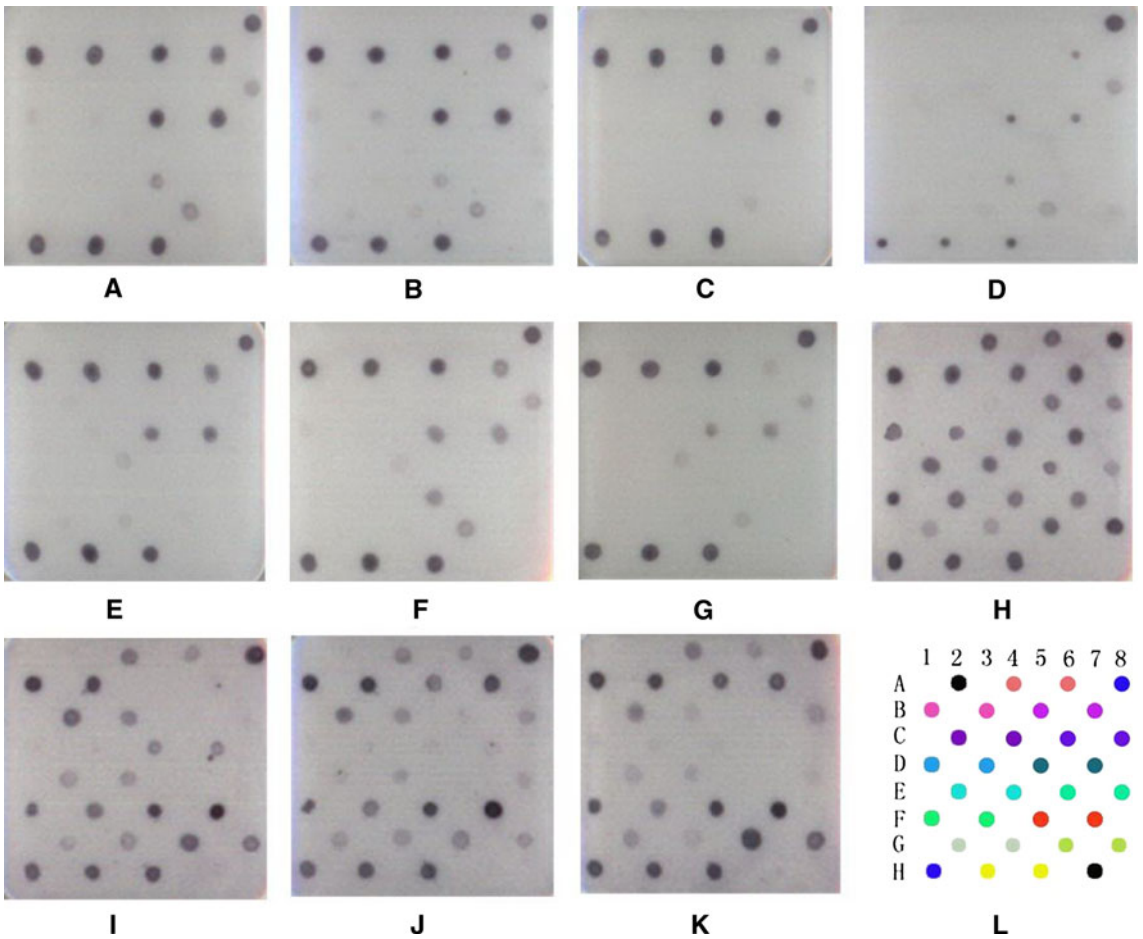

Fig. 2 Construction and optimization of gene chip hybridization. **a–d** optimization of hybridization temperature of 45, 48, 51, and 54 °C, respectively; **e–g** Optimization of hybridization time of 30, 45, and 60 min, respectively; **h–k** Optimization of biotin labeled/unlabeled primer ratio of 1:1, 2:1, 3:1, and 4:1, respectively; **l** construction of gene chip, *panel A2*: negative control; *panel H7*: blank control; *panel A8* and *H1*: positive control; *panel A4* and *A6*: *S. aureus*; *panel B1* and *B3*: *C. botulinum*; *panel B5* and *B7*: *Shigella* spp.; *panel C2* and *C4*: *E. coli* O157:H7; *panel C6* and *C8*: *V. parahaemolyticus*; *panel D1* and *D3*: *V. cholerae*; *panel D5* and *D7*: *Salmonella* spp.; *panel E2* and *E4*: *C. jejuni*; *panel E6* and *E8*: *C. perfringens*; *panel F1* and *F3*: *A. flavus/A. parasiticus omt*; *panel F5* and *F7*: *L. monocytogenes*; *panel G2* and *G4*: *Y. enterocolitica*; *panel G6* and *G8*: *A. lavus/A. parasiticus nor*; *panel H3* and *H5*: *B. cereus*

(CV %) of the signal-to-noise ratio was less than 10 % in all cases, and the average CV % was 9.3 %.

These observations demonstrated that the methodology possessed high specificity under optimized PCR amplification and hybridization conditions.

Application to food-borne toxigenic microorganism detection

Probes of *L. monocytogenes*, *Salmonella* spp., *Shigella* spp., and *V. cholerae* were used to detect samples of chicken, pork rear foot, pork neck bone; probes of *L. monocytogenes*, *Salmonella* spp., *Shigella* spp., *V. cholerae,* and *V. parahaemolyticus* were used for *Tilapia mossambica*; probes of *Shigella* spp., *V. cholerae,* and *V. parahaemolyticus* were used for clam, razor fish, buns shellfish samples; probes of *S. aureus*, *L. monocytogenes*, *Salmonella* spp., and *Shigella* spp. were used for biscuit, chocolate, and raw milk samples.

As Table 4 shown, 2 of the 23 chicken samples were found to be positive for *L. monocytogenes* with a positive rate of 8.60 %; 1 was positive for *Salmonella* spp. with a positive rate of 4.30 %; no signal for *Shigella* spp. and *V. cholerae* was detected. Remarkably, relatively high positive rates of shellfish samples ranging from 9.20 to 17.70 % were detected for *V. parahaemolyticus* in this study, among which 11 of 62 razor fish samples with the highest positive rate of 17.70 % were observed, while no signal for *Shigella* spp. and *V. cholerae* was detected. Other sample situations can be retrieved from Table 4. The PCR products obtained by amplification of the DNA extracted directly from food samples were hybridized to the microarray. Further confirmation was obtained by conventional microbiological means. All of the strains

Table 3 Oligonucleotide probes used in this study

Probe	Sequence (5′ → 3′)	Target	Length (bp)
SA2	GCTCATTTGCATCAAGTTGTTG	*femA*	22
SA3	TTTGCTCATTTGCATCAAGTTG	*femA*	22
CB1	TATAAGAGAATCGCATGATT	16s	20
SH2	CATTGCCCGGGATAAAGTCA	*ipaH*	20
SH3	CACATGGAACAATCTCCGGA	*ipaH*	20
EC2	GTGACAACCATTCCACCTTC	*rfbE*	20
VP1	ATCTCAAGCACTTTCGCACG	*tlh*	20
VP3	GATGCGTGACATTCCAGAAC	*tlh*	20
VC2	GTGTAATTCAAACCCGCACC	*ompW*	20
VC3	TACCACACAGAAGCGTTGAG	*ompW*	20
SA2	AATACCGGCCTTCAAATCGG	*invA*	20
CJ2	TGAAGAAAGCGCAAGAAGAGTA	*vsl*	22
CJ3	AACTTGGCTAAAGGCTAAGGCT	*vsl*	22
CP2	ACGGCAGTAACATTAGCAGG	*cpα*	20
ASo2	GAGAATCCAACCAAGGCATG	*omt*	20
ASo3	CCTTTCGTCTGCTGCAAGAA	*omt*	20
LM1	GATTAACGGGAAGCTTGGCT	*prfA*	20
YE2	AGCAGCACCCAGTAATCCAT	*ail*	20
YE3	TGGAAGCGGGTTGAATTGCA	*ail*	20
ASn1	ATCATGTGTGCCTGGAGATG	*nor*	20
ASn2	GGTTGCCTGAAACAGTAGGA	*nor*	20
BC1	TGCTAGTTGAATAAGCTGGCACCT	16s	24
LE	ATAGCATTGGTGCCGATTTGGGAAG	*mip*	25
NC	AGCGATTCCTTGCTCCTGAGCAAC	–	24

Species of probes: SA2-3, *S. aureus*; CB1, *C. botulinum*; SH2-3, *Shigella* spp.; EC2, *E. coli* O157:H7; VP1, 3: *V. parahaemolyticus*; VC2-3, *V. cholerae*; SA2, *Salmonella* spp.; CJ2-3, *C. jejuni*; CP2, *C. perfringens*; ASo2-3, *A. flavus/A. parasiticus*; LM1, *L. monocytogenes*; YE2-3, *Y. enterocolitica*; ASn1-2, *A. flavus/A. parasiticus*; BC1, *B. cereus*; LE (positive control), *Legionella*; NC (negative control), *E. coli* O55:H7 str. 3256-97 strain

used for microarray assay were in agreement with the result of the traditional methods.

Discussion

Changes in consumer preferences toward less obviously processed 'natural' foods, but with a reluctance to give up convenience, have encouraged food manufacturers to adjust their food product preparation using less salt and low temperature storage condition under which many toxigenic microorganisms grow better and lead to many so-called food-borne diseases [5]. These food-borne-disease-caused toxigenic microorganisms have led to great health problem and caused a loss up to $8–10 billion in US each year in the 1990s [6] including bacteria [7] such as *S. aureus*, *Salmonella* spp., *C. jejuni*, *Y. enterocolitica*, *V. parahaemolyticus*, *V. cholerae*, *E. coli* O157:H7, *B. cereus*, *C. perfringens*, *Shigella* spp., *L. monocytogenes*, *C. botulinum*, and fungi [5] like *Aspergillus*, *Fusartum,* and *Pentcillium*. Methods for rapid detection and identification of food-borne pathogens are vital to food processing, food safety, and public health.

Due to the intrinsic properties of food production, rapid, accurate, timely and efficient detection methods for toxigenic microorganism are paramount for the prevention of food-borne epidemics and thus have been intensively developed for food quality control. Oligonucleotide microarrays are widely used techniques thanks to its sensitivity and specificity, as well as rapid property, in gene expression profiling, infectious and genetic disease diagnoses, genotyping, etc. [17, 18]. Recent investigations on food-borne pathogens or toxigenic microorganisms have attracted increasing concentration [3, 19].

The efficacy of microarray technique depends significantly on several factors including PCR primers for target genes amplification and probes for chip hybridization [3]. It is of great importance to select proper target genes and design specific primers and probes. The ultimate resolution of oligonucleotide microarray depends on the level of conservation of the marker gene applied [4]. Though the 16S rRNA gene is the most widely used marker, it does not allow for resolution even at the species level in many cases [4]. Alternative universal marker genes with higher resolution include *femA*, *ipaH*, *rfbE*, *nor*, and so on (Table 2) [20–22]. Specificity is thus the vital issue for oligonucleotide microarray with respect to the above factors. Although complex multiple PCR satisfies the fast detection demand, it often cannot overcome nonspecific detection trouble [3]. Specificity of primers used in PCR amplification may be easily obtained when using a simple PCR, though it hinders the rapidity requirement for high-throughput assay. Here, we designed specific primers based on the conserved domain of their corresponding target genes, and uniqueness of the primer sequences was confirmed by Blast and PCR amplification (Fig. 1). No nonspecific band was observed, and sequencing result finally validated the primers used. Furthermore, we also optimized the ratio of biotin labeled to unlabeled primer because PCR amplification of the target genes is required to focus the labeling to the target genes, an optimized labeling efficiency can decrease the accumulation of nonspecific hybridization signal, which is largely affected by such ratio [4]. Certainly, the final performance of the above means of optimization must be confirmed by the hybridization reaction in which optimized probes should be used. After designing three candidate probes for their corresponding species, we tested and selected the best probe(s) of the matching species to fabricate the chip for further food-borne toxigenic microorganism detection. Oligonucleotide

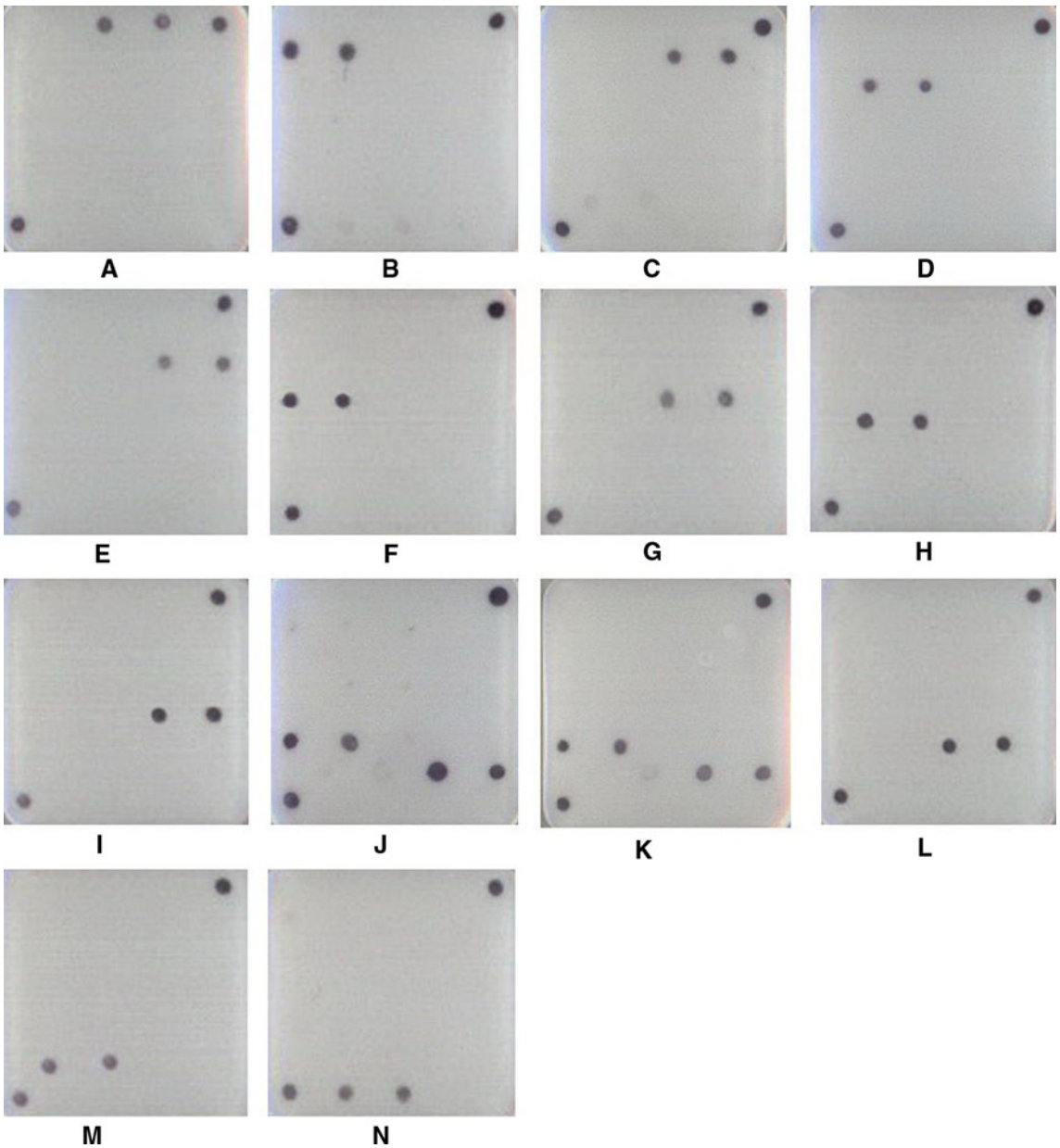

Fig. 3 Specificity of chip hybridization. **a–n** are *S. aureus femA*, *C. jejuni* 16s RNA gene, *Shigella* spp. *ipaH*, *E. coli* O157:H7 *rfbE*, *V. parahaemolyticus tlh*, *V. cholerae ompW*, *Salmonella* spp. *invA*, *C. jejuni vs1*, *C. perfringens cp* α, *Aspergillus flavus nor/omt*, *A. flavus nor/omt*, *L. monocytogenes prfA*, *Y. enterocolitica ail*, *B. cereus* 16s RNA gene, respectively

probes should be fine-tuned for nearly identical melting temperatures, which can be partly achieved by designing probes of the similar length or by modifying their melting temperature by changing their lengths [23]. Based on that, all probes used were designed to reside in the amplified target with typical length of 20–25 nt (Table 3). They all generated good hybridization signal with their target genes; each species was clearly distinguished through hybridization between DNA templates and probes on oligonucleotide microarrays (Fig. 3). Other factors affecting the practicality of oligonucleotide microarray may contain hybridization temperature and time used. It is reported that hybridization kinetics were significantly different for specific and nonspecific binding of labeled target to surface-bound oligonucleotides on microarray [24]. Proper time of hybridization (30 min) can increase the ratio of the perfect match to the cross-hybridization with a well detected hybridization signal under the optimized hybridization temperature (Fig. 2c), as the optimal specificity-to-signal intensity ratio was observed in Fig. 2e.

The quality assessment of a practical oligonucleotide microarray relied on specificity, sensitivity, and

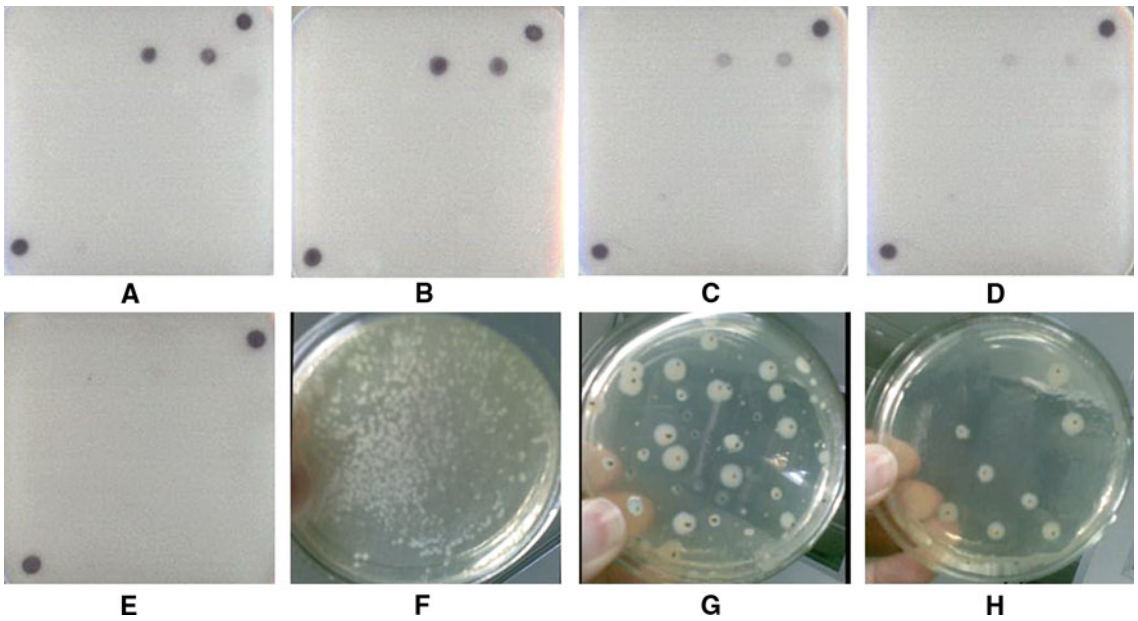

Fig. 4 Sensitivity of the optimized microarray. **a–e** hybridization of 10^{-5}–10^{-10} diluted samples, respectively; **f–g**, CFU count of 10^{-7}–10^{-9} diluted samples

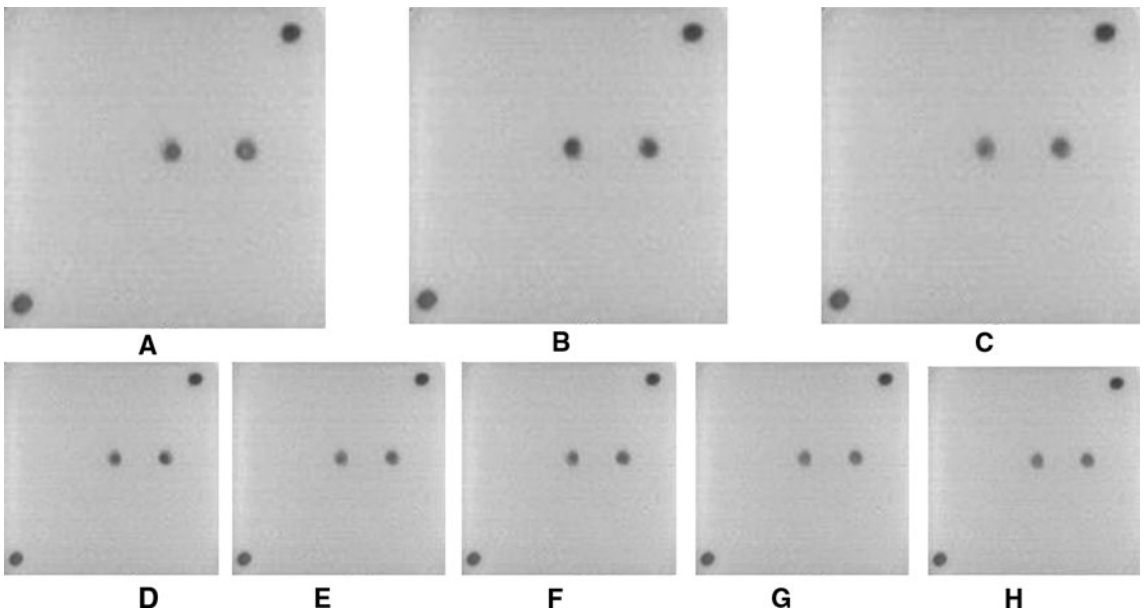

Fig. 5 Reproductivity test of the optimized microarray. **a–c** are three different batches of *Salmonella* spp. targets; **d–h** are *Salmonella* spp. targets from the fourth batch

reproductivity. The ultimate specificity is discrimination between a perfect match and a single mismatch target [4]. Hybridization using highly specific probe could yield elegant reproductivity and sensitivity [25]. As discussed above, primer and probe design, hybridization time and temperature used, and ratio of biotin labeled to unlabeled primer could affect the specificity of a microarray. Besides, enzyme-mediated detections are very sensitive to end-position mismatches [4]. The sensitivity is the lowest concentration giving a positive signal and is reported to be 10–100 times higher than the agarose gel [26]. Wang et al. [3] developed an oligonucleotide microarray for the detection of food-borne bacterial pathogens with a detection limit of 10^2 CFU/mL bacteria from which most PCR products amplified could not be visualized on the ethidium bromide-stained agarose gel. The detection limit of our microarray (710 CFU/mL) here is in agreement with Wang et al. [3], as shown by Fig. 4. To validate the reproductivity

Table 4 Optimized microarray applied to detect 6 common toxigenic microorganisms from five categories of 328 food samples

Category	Sample	Sample copy	*Staphylococcus aureus*		*Listeria monocytogenes*		*Salmonella* spp.		*Shigella* spp.		*Vibrio cholerae*		*Vibrio parahaemolyticus*	
			Positive no.	Positive rate (%)	Positive no.	Positive rate (%)	Positive no.	Positive rate (%)	Positive no.	Positive rate (%)	Positive no.	Positive rate (%)	Positive no.	Positive rate (%)
Frozen product	Chicken	23	–	–	2	8.60	1	4.30	0	0.00	0	0.00	–	–
	Pork rear foot	71	–	–	5	7.00	3	4.20	0	0.00	0	0.00	–	–
	Pork neck bone	19	–	–	1	5.00	0	0.00	0	0.00	0	0.00	–	–
	Tilapia mossambica	24	–	–	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
	Clam	76	–	–	–	–	–	–	0	0.00	0	0.00	7	9.20
Shellfish	Razor fish	62	–	–	–	–	–	–	0	0.00	0	0.00	11	17.70
	Buns shellfish	75	–	–	–	–	–	–	0	0.00	0	0.00	8	10.70
Biscuit	/	62	0	0.00	–	–	0	0.00	0	0.00	–	–	–	–
Chocolate	/	92	1	1.10	–	–	0	0.00	0	0.00	–	–	–	–
Raw milk	/	37	0	0.00	–	–	0	0.00	0	0.00	–	–	–	–

"–" not used for detection; "/" non-specified sample

of this microarray, batch-to-batch (Fig. 5a–c) and repeated experiments (Fig. 5d–h) were compared. An ideal reference should be available in large quantities, sufficient to satisfy long-term requirements, and reproducible such that different batches are indistinguishable from one another [27]. Consistent observations could be found among batches and repeated hybridizations (Fig. 5), demonstrating high reproductivity of this microarray.

Application of the oligonucleotide microarray to food-borne toxigenic microorganisms with genetic markers in bacterial genomes is characterized by its higher sensitivity over conventional methods which have a detection limit of one cell per 25 g sample [28]. By oligonucleotide microarray, *E. coli* O157:H7 was detected from less than one cell equivalent of genomic DNA [29]. Fifteen strains of *Salmonella*, *Shigella*, and *E. coli* were also identified using six marker genes (*slt-I*, *slt-II*, *eaeA*, *rfbE*, *fliC*, and *ipaH*) through DNA microarray [30]. Here we utilized an oligonucleotide microarray, which could supply similar sensitivity of 10^2 CFU/mL as pervious study under optimized conditions [3], to detect several common toxigenic microorganisms. With this technique, *S. aureus*, *L. monocytogenes*, *Salmonella* spp., *Shigella* spp., *V. cholerae*, and *V. parahaemolyticus* could be well detected and clearly discriminated in most of the 328 food samples (Table 4). Interestingly, relatively higher positive rates of shellfish for *V. parahaemolyticus* were observed (Table 4). *V. cholerae*, *V. parahaemolyticus*, and related vibrios, which can cause cholera-like diarrhea, were reported to show a spatial and temporal distribution characteristic of *Vibrio* species in an estuary [31]. Therefore, it is not surprising to find this species in the three kinds of shellfish. Kim et al. [32] found that *V. cholerae* ATCC 14547 and *V. vulnificus* ATCC 33815 did not react with any of the 10 probes of *V. parahaemolyticus*, which were designed to be specific for *V. parahaemolyticus*; only *V. parahaemolyticus* showed positive results and was thus discriminated from the other *Vibrio* species. Similar observation might be also obtained in our study since no positive signal of *V. cholerae* was detected (Table 4).

In summary, the oligonucleotide microarray hybridization protocol described herein provides a rapid, sensitive, specific, and high-throughput means for the detection and identification of food-borne toxigenic microorganisms. The specific target spectra produced by this gene chip may be gradually expanded through addition of newly designed oligonucleotide probes into the microarray. The accuracy may also be improved by increasing and readjusting the oligonucleotide probes in the array.

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Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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