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zjs / DOCHEAD=ARTICLE zjss / DOCTOPIC=BACTERIOLOGY
FROM COVER SHEET: Editor: Cockerill Article No.: T Section: Bacteriology

JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2009, p. 000 Vol. 47, No. 10
0095-1137/09/$08.00+0 doi:10.1128/JCM.00366-09

Detection of *Enterobacter sakazakii* and Other Pathogens Associated with Infant Formula Powder by Use of a DNA Microarray▽†

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Received 18 February 2009/Returned for modification 4 May 2009/Accepted 23 July 2009

Pathogen detection is critical to the process of generating and testing powdered infant formula (PIF). An obstacle associated with PIF microbial surveillance is that most current procedures are time-consuming and labor-intensive. We have developed a rapid, DNA microarray-based detection technique to identify 10 different pathogenic bacteria associated with PIF contamination based on the 16S–23S rRNA gene internal transcribed spacer (ITS) sequences and *wzy* (O antigen polymerase) gene. Using this procedure, *Enterobacter sakazakii*, *Salmonella* species, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Serratia marcescens*, *Acinetobacter baumannii*, *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Escherichia coli* O157 were identified. One hundred eighty-five strains were used to validate the microarray assay (including 134 target pathogen strains and 51 closely related bacteria). Twenty-seven probes reproducibly detected multiple pathogens with high specificity and sensitivity (0.100 ng genomic DNA or 10^4 CFU/ml). Twenty-one real PIF samples were tested by the microarray with 100% accuracy. The data presented reveal that the designed oligonucleotide microarray is a promising method for basic microbiology, clinical diagnosis, food safety, and epidemiological surveillance.

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Pathogen detection is a critical parameter linked to the safety of powdered infant formula (PIF). Because PIF effectively supports the growth of numerous pathogens, it can become easily contaminated (9, 15). Various studies examining PIF contamination have identified various pathogenic bacteria (9, 16, 22); e.g., powdered milk produced by Wyeth (in 2002) was contaminated with *Enterobacter sakazakii*, which led to fatality rates of 33 to 80% in infected children (18). In 2005, an outbreak associated with *Salmonella*-contaminated PIF in France affected more than 141 children (4). These and other similar episodes have prompted research aimed at improving pathogen detection to guarantee PIF safety.

FAO/WHO Expert Consultations (held in 2004 and 2006) concluded that the primary microorganisms associated with PIF contamination were *E. sakazakii*, *Salmonella enteritidis*, *Enterobacter agglomerans*, *Hafnia alvei*, *Klebsiella pneumoniae*, *Citrobacter koseri*, *Citrobacter freundii*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Escherichia coli*, *Serratia* spp., *Acinetobacter* spp., *Bacillus cereus*, *Clostridium difficile*, *Clostridium perfringens*, *Clostridium botulinum*, *Listeria monocytogenes*, *Staphylococcus aureus*, and coagulase-negative staphylococci (http://www.who.int/foodsafety/publications/micro/mra10/en/index.html). *Enterobacter agglomerans*, *Enterobacter cloacae*, *Hafnia alvei*, and coagulase-negative staphylococci (normally referring to *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*), whose pathogenicity is weak, were rarely isolated by the Entry-Exit Inspection and Quarantine Bureau. *Clostridium difficile*, *Clostridium perfringens*, and *Clostridium botulinum* are anaerobes, and both their cultivation and strain collection are difficult. The internal transcribed spacers (ITS) of *Citrobacter koseri* and *Citrobacter freundii* are not available. Therefore, in this report, 10 important and often-isolated pathogens, *E. sakazakii*, *Salmonella* species, *K. pneumoniae*, *K. oxytoca*, *S. marcescens*, *A. baumannii*, *B. cereus*, *L. monocytogenes*, *S. aureus*, and *E. coli* O157, were employed to design a microarray-based approach for the detection of PIF-associated pathogens.

Conventional methods for the detection of these pathogens involve their isolation in pure culture combined with biochemical tests that often are laborious, time-consuming, and difficult to quantify (6); however, the use of molecular methods, such as PCR, real-time PCR, and immunoassays, has facilitated pathogen detection (2). Despite these improvements, only the detection of *E. sakazakii* and *Salmonella* species in PIF have been reported (17). Recently, DNA microarray-based assays have been introduced and developed as potential strategies for facilitating the high-throughput and specific screening of pathogen-associated DNA sequences (3).

In this study, we targeted the detection of the 16S–23S rRNA gene ITS regions for nine pathogen targets and the O

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† Supplemental material for this article may be found at http://jcm.asm.org/.

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▽ Published ahead of print on ●●●●●●●●.

AUTHOR: Publication of this article cannot proceed without the signature of the person who read and corrected the proof on behalf of all the authors: signature date

TABLE 1. Target pathogen strains used for microarray analysis

Bacterium	No. of strains and source	Total no.
E. sakazakii	3,[a] 3,[b] 15[c]	21
Salmonella species	11,[d] 2,[e] 6[c]	19
K. pneumoniae	4,[e] 1,[a] 1,[f] 2,[g] 8[c]	16
K. oxytoca	3,[a] 1,[b] 6[c]	10
S. marcescens	3,[h] 4,[e] 1,[a] 4[c]	12
A. baumannii	1,[a] 1,[e] 11[i]	13
B. cereus	2,[h] 2,[e] 2[c]	6
L. monocytogenes	2,[e] 1,[j] 2,[f] 2[c]	7
S. aureus	1,[h] 4,[e] 3,[c] 14[i]	22
E. coli O157	1,[d] 1,[k] 3,[l] 3[m]	8

[a] ATCC.
[b] Czech Collection of Microorganisms (CCM), Czech Republic.
[c] Environmental isolates from Tianjin Entry-Exit Inspection and Quarantine Bureau, China.
[d] Institute of Medical and Veterinary Science (IMVS), Adelaide, Australia.
[e] National Center for Medical Culture Collection (CMCC), China.
[f] Center for Veterinary Culture Collection (CVCC), China.
[g] National Collection of Type Cultures (NCTC), United Kingdom.
[h] Institute of Microbiology, Chinese Academy of Sciences (IMCAS).
[i] Clinical isolates from General Hospital of Tianjin Medical University, China.
[j] Agricultural Culture Collection of China (ACCC).
[k] Chinese Center for Disease Control and Prevention (CDC).
[l] National Reference Laboratory for *E. coli*, Berlin, Germany.
[m] Robert Koch-Institut (RKI), Germany.

unit-processing gene *wzy* (O antigen polymerase) for *E. coli* O157. A total of 185 bacterial strains were used to validate the microarray assay. Twenty-seven specific probes and two primer pairs reproducibly and specifically were used to identify bacterial genomic DNA in samples with as little as 0.1 ng DNA or 10^4 CFU/ml for pure culture.

MATERIALS AND METHODS

Bacterial strains. The target bacterial strains tested in this study are described in Table 1. *Listeria* species strains were cultured in trypticase soy-yeast extract broth (TSB-YE) medium, and the other were inoculated into 2YT medium. All strains were grown overnight at 37°C with shaking.

Preparation of PIF samples. For mock samples, respective bacterial cultures were serially diluted, and 10^0 CFU was mixed with 100 g of PIF and 900 ml of 2YT medium (*L. monocytogenes* was mixed with 900 ml of TSB-YE medium); for test samples, only 100 g of PIF and 900 ml medium were mixed. The mixture was incubated at 37°C for 5 h, and then 10 ml was used to inoculate 100 ml of enterobacteria enrichment broth and meat infusion broth (*L. monocytogenes* was inoculated in Fraser medium). These three selective media were incubated at 37°C overnight with shaking, and genomic DNA then was extracted from 1.5 ml of the overnight cultures.

DNA isolation. Genomic DNA was extracted using the Bacteria Genomic DNA Purification kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol.

Primer and probe design. The primer pair wl-5793 (5′-TGT ACA CAC CGC CCG TC-3′) and wl-5794 (5′-GGT ACT TAG ATG TTT CAG TTC-3′), which is specific to the ITS region, was designed as previously described (29). The *wzy* primer pair wl-5593 (5′-TCA GCG GCT AAG TTG ATT-3′) and wl-5594 (5′-ATT TGC TCC CAT GTC TCC-3′) was designed using Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA). The ITS probe specificity was examined by comparing the sequences in GenBank to our sequencing database using BLAST. The *wzy* probes were designed by OligoArray 2.0 software and were based on sequences available in GenBank. For each bacterial target, one to four specific capture probes were designed. In addition, one probe, based on the conserved region of 16S rRNA gene, was used as the positive control, one probe containing a 40-poly(T) oligonucleotide tail was used as the negative control, and one 3′-Cy3-labeled probe was used as the positional reference and printing control. Each probe was chemically synthesized and 5′-amino modified with a space linker of 15 poly(T) oligonucleotides. All of the probes are listed in Table 2.

***Serratia* sp. ITS sequences.** PCR amplicons were cloned into the pGEM-T Easy vector (Promega, MA) and transformed into *E. coli* DH5α. Transformants (white colonies grown on an ampicillin plates containing isopropyl-beta-D-thio-galactopyranoside and 5-bromo-4-chloro-3-indolyl-â-D-galactopyranoside) were selected randomly. Plasmid DNA was isolated using the conventional alkaline lysis method, digested with EcoRI, and visualized on agarose gels to confirm the presence of the corresponding inserts. Sequences were verified using an ABI 3730 automated DNA sequencer. Seven to 16 transformants per strain were examined.

Target DNA amplification and labeling. The primer concentrations were optimized based on the final intensity of the microarray hybridization signals. Duplex PCR mixtures contained 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 2.5 mM $MgCl_2$, 400 μM deoxynucleoside triphosphates, 0.15 μM ITS, 0.2 μM primer for each *wzy* gene, 2.5 U *Taq* DNA polymerase, and 50 to 100 ng of DNA template in a final volume of 50 μl. PCR conditions used consisted of an initial denaturation 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 1 min, with a final extension at 72°C for 5 min. PCR products were purified with the Microcon Centrifugal Filter Devices kit (Millipore Corporation, MA). To label the PCR products, the two reverse primers were used during the PCR, and 0.3 μl of 25 nM Cy3-dUTP was added. Ten microliters of the purified products generated from the duplex PCR described above was added as the template, and PCR was carried out as described above.

Microarray preparation. Probes were dissolved in 50% dimethylsulfoxide (DMSO) to a final concentration of 1 μg/μl and coated onto aldehyde group-modified glass slides (CapitalBio Corporation, Beijing, China) using a SpotArray72 (Perkin-Elmer Corporation, CA). Each probe was spotted in triplicate, and coated slides were dried and stored at room temperature in the dark. Each glass slide contained eight individual arrays framed with an 8-sample cover slip that constituted individual reaction chambers (see Fig. S1 in the supplemental material).

Microarray hybridization and data analysis. All labeled PCR products were precipitated using 100% cold ethanol, centrifuged at 13,000 × *g* for 10 min, washed with 75% ethanol, and dried at room temperature. The dried, labeled DNA was diluted in 16 μl hybridization buffer (25% formamide, 0.1% sodium dodecyl sulfate [SDS], 6×SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA {pH 7.7}]), and then hybridized with the prepared microarray at 40°C for 12 h. After hybridization, the slide was washed with solution A (1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% SDS) for 3 min, solution B (0.05× SSC) for 3 min, and solution C (95% ethanol) for 1.5 min. The microarray then was dried under a gentle air stream and scanned with a laser beam of 532 nm using a GenePix biochip scanner 4100A (Axon Instruments, CA) set to the following parameters: photomultiplier tube gain, 600; pixel size, 5 μm. The signal-to-noise ratio (SNR) was calculated for each spot using the Bactarray Analyzer software developed in house with the threshold set at 3.0. A signal was considered positive when all probes to a respective target gene generated hybridization signals above the SNR threshold.

Nucleotide sequence accession numbers. The ITS sequences of *Serratia* spp. were deposited in GenBank under accession numbers GQ332578 to GQ332604.

RESULTS

***Serratia* sp. ITS regions.** A total of 27 ITS sequences from *Serratia* spp. were obtained, including 11 from five *S. marcescens* strains, 4 from one *S. odorifera* strain, 8 from one *S. rubidaea* strain, and 4 from one *S. fonticola* strain. ITS regions were analyzed using tRNA-ScanE software (http://lowelab.ucsc.edu/tRNAscan-SE/). Two distinct ITS types were identified (see Table S1 in the supplemental material): ITS^{glu} (with the $tRNA^{Glu}$ gene) and $ITS^{ile+ala}$ (with $tRNA^{Ile}$ and $tRNA^{Ala}$ genes). For ITS^{glu}, a 403-bp product was detected in all five *S. marcescens* strains, 358- and 380-bp products from *S. rubidaea*, a 454-bp product from *S. odorifera*, and a 463-bp product from *S. fonticola*. For $ITS^{ile+ala}$, a 471-bp product was detected from *S. marcescens*, a 571-bp product from *S. odorifera*, 559- and 672-bp products from *S. rubidaea*, and 556- and 602-bp products from *S. fonticola*. Alignments of the ITS sequences described above revealed significant interspecies variations (0.608 to 0.794) but low

TABLE 2. Oligonucleotide probes used

Bacterium and probe no.	Target gene	T_m[a] (°C)	Sequence (5′–3′)	GenBank accession no.
E. sakazakii				
OA-1976	ITS	69.8	436-GTCAGAGTCTCTCAAACTCGCAGCACG-462	AY748357
OA-1978		68.9	465-ACAGACACGCTGCTGTATTTCTCCGTAAT-493	
Salmonella species				
OA-1979	ITS	67.3	239-GAGGTTCTGACTACACGATGGGGCTAT-265	AF046814
K. pneumoniae				
OA-2355	ITS	67.6	163-ATTTGAAGAGGTTGCAAACGATGGG-188	EU623376
OA-2356		67.4	140-GGCCTACCAAATTTGCGAAGCAA-163	
K. oxytoca				
OA-2351	ITS	64.5	40-CTGATAGATGTAAAGAAGCAAGACGGC-66	EU623150
OA-2352		73.8	62-ACGGCTGCGAAGTCGCGACACCT-84	
OA-2354		66.0	178-TGAAAGGCACAACCAACCGATATCT-202	
S. marcescens				
OA-2568	ITS	69.9	CAAGCTTCCGACCCACCCGGT	This study
OA-2569		69.7	GCGGCGGTCTCAAGTATCTGACGAT	
OA-2570		64.4	CTTAAAGATGACTTCCGAGTCATGTTTAAG	
OA-2571		64.2	CGAACGATGGAAACATCTTCGG	
B. cereus				
OA-2391	ITS	67.7	25-ATCAATATAAGTTTCCGTGTTTCGTTTTCG-55	AM062662
OA-2393		63.4	321-TTCTTTGAAAACTAGATAACAGTGTAGCTCAT-352	
A. baumannii				
OA-2530	ITS	67.3	342-TCATTATCACGGTAATTAGTGTGATCTGACG-372	AY601825
OA-2531		67.4	359-AGTGTGATCTGACGAAGACACATTAACTCATT-390	
OA-2532		67.4	524-GTACGGTGCTTAAGTGCACAGTGCTCTA-551	
L. monocytogenes				
OA-2094	ITS	72.4	140-AGGCACTATGCTTGAAGCATCGCGC-164	U57912
OA-2095		61.8	271-AAGAAATACAAATAATCATACCCTTTTACG-300	
OA-2096		62.8	257-TTTCTTTCTGACATAAGAAATACAAATAATCATA-290	
S. aureus				
OA-1973	ITS	70.4	219-GCTTATGCGAGCGCTTGACAATCTATTCT-247	U11774
OA-1974		69.0	335-TAAAGCAGTATGCGAGCGCTTGACTAAA-362	
OA-2006		66.5	546-ATGTTAACGTTTGACTTATAAAAATGGTGG-575	U11786
E. coli O157				
OA-1929	*wzy*	79.94	1632-AGGGAATAAAGCATCAAGACTTATTTTATGGAGAACGGTT-1671	AF061251
OA-1930		79.05	1580-TTTCCGACACCAGAGTTAGAAAAGGAATTAAAAGCAATAA-1619	
OA-1932		79.7	1310-GGTTATCGTTCTGAATTGGTGTTGCTCATTCTTCAATATA-1349	
OA-1994		79.07	1245-ATGATGAGCATAAATTCAAACAGAGGACCATCATATTTGT-1284	
Positive control				
OA-1993	16S rRNA	71.9	1380-TTGTACACACCGCCCGTCACACCAT-1404[b]	X80725
Negative control				
wl-4006			TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT[c]	
Positional and printing control				
Cy3			TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT_Cy3[d]	

[a] Melting temperature.
[b] The 16S rRNA gene-based probe. The 1380 to 1404 position in *E. coli* was used as the positive control.
[c] The probe containing the 40 poly(T) oligonucleotide tail was used as the negative control.
[d] The 3′-Cy3-labeled probe was used as the positional reference and printing control.

intraspecies polymorphisms (0.985 to 0.997), suggesting that the ITS can be used as a target for species-specific probe designs.

Optimization of duplicate PCR. Primer pair efficiency for 10 target pathogens was determined based on the amplicons of anticipated sizes by testing different primer concentrations (0.10 to 0.50 μM). Using the optimized ITS and *wzy* primer concentrations (0.15 μM ITS primers and 0.2 μM *wzy* primers), target sequences from 10 representative pathogenic strains were amplified (Fig. 1). F1

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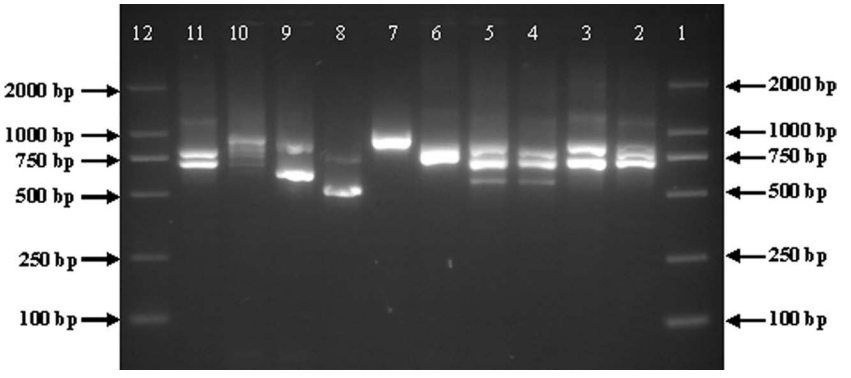

FIG. 1. PCR amplification of 10 pathogens. Genomic DNA from 10 representative strains was amplified with the two primer pairs wl-5793/wl-5794 and wl-5593/wl-5594, and amplified products were subjected to agarose gel electrophoresis. Lanes 1 and 12, DL2000 DNA marker; lane 2, *E. coli* O157 38/99; lane 3, *Salmonella* species strain M44; lane 4, *S. aureus* CMCC 26058; lane 5, *A. baumannii* ATCC 19606; lane 6, *K. pneumoniae* ATCC 10031; lane 7, *K. oxytoca* ATCC 49334; lane 8, *L. monocytogenes* CMCC 54001; lane 9, *B. cereus* AS1.196; lane 10, *E. sakazakii* ATCC 29544; and lane 11, *S. marcescens* ATCC 13880.

Probe specificity. A total of 185 strains (134 target pathogen strains and 51 closely related bacteria) (see Table S2 in the supplemental material) belonging to 15 different genera and 47 species were used to test the specificity of the designed probes. Probes that cross-hybridized or did not produce signals were removed from the test panel. From 269 hybridization reactions, 30 probes (including 27 species-specific probes, 1 positive control probe, 1 negative control probe, and 1 positional and printing control probe) were selected from the 79 probes initially screened (Table 2). The microarray procedure designed identified all 134 target strains specifically; none of the 51 closely related bacteria tested yielded positive signals, suggesting that the designed probes were species specific (Fig. 2A to K).

Microarray sensitivity. The sensitivity was further tested by hybridization with serially diluted genomic template DNA (0.001 to 10 ng). Representative hybridized results using *E. sakazakii* genomic template DNA are shown in Fig. S2A to D in the supplemental material. Based on the positive signals generated, the sensitivity of the assay using genomic DNA was 0.001 ng DNA for *A. baumannii*; 0.010 ng DNA for *Salmonella* species, *E. sakazakii*, and *E. coli* O157; and 0.100 ng DNA for *K. pneumoniae*, *K. oxytoca*, *S. marcescens*, *B. cereus*, *L. monocytogenes*, and *S. aureus*. The microarray also was tested against pure cultures of gram-negative and -positive organisms. Genomic DNA was isolated and used to test the sensitivity of the microarray. *E. sakazakii* ATCC 29544 was selected as a representative gram-negative bacterium and was serially diluted to 10^1 to 10^6 CFU/ml. The positive signals were obtained at 10^4 CFU/ml (see Fig. S2E to H in the supplemental material). *S. aureus* CMCC 46112 was selected as a representative gram-positive bacterium, and the probes were sensitive using 10^4 CFU/ml (see Fig. S2I to L in the supplemental material). Likewise, the remaining eight strains were identified using 10^4 CFU/ml pure culture (data not shown), suggesting that this was the minimal dose needed for detection.

Simultaneous detection of multiple pathogens. Since the detection of pathogens from a single sample would be carried out most efficiently if multiple pathogens could be detected simultaneously, genomic DNA from two pathogens (*A. baumannii* and *L. monocytogenes* or *E. coli* O157 and *B. cereus*) were mixed and used as templates to further test the specificity of the microarray assay. The data demonstrated that the probes were able to hybridize the target regions, further demonstrating the specificity of the designed probes to their respective species (data not shown). Mixing genomic DNA from three pathogens (*E. sakazakii*, *Salmonella* species, and *B. cereus* [Fig. 2L] or *S. marcescens*, *K. oxytoca*, and *S. aureus* [Fig. 2M]) also demonstrated that the array probes could detect multiple pathogens from a sample containing multiple genomic profiles.

Blinded testing. The specificity and sensitivity of the designed microarray detection system described was further tested using a double-blind approach. Coded DNA samples from 26 strains (see Table S3 in the supplemental material) were randomly selected and used to hybridize to the microarrays. These results revealed that five isolates were *E. sakazakii*, four isolates were *Salmonella* species, three isolates were *K. pneumoniae*, two isolates were *K. oxytoca*, two isolates were *A. baumannii*, two isolates were *S. marcescens*, two isolates were *B. cereus*, two isolates were *L. monocytogenes*, three isolates were *S. aureus*, and one isolate was *E. coli* O157. These results matched the identity according to the Vitek system and serotyping methods in the Tianjin Entry-Exit Inspection and Quarantine Bureau and General Hospital of Tianjin Medical University.

Detection of mock PIF samples. *E. sakazakii* ATCC 29544, *Salmonella* species strain M44 CMCC 50071, *K. pneumoniae* ATCC 10031, *S. aureus* CMCC 26058, and *E. coli* O157 IMVS 1332 (8) were used to inoculate PIF and cultured as mentioned in Materials and Methods. Pathogen-free PIF was used as a negative control. DNA was extracted from the respective samples and tested using the microarray probes described above. Hybridization results demonstrated that 25 g PIF contaminated by 3 CFU *E. sakazakii*, 3 CFU *Salmonella* sp. strain M44, 2 CFU *K. pneumoniae*, 6 CFU *K. oxytoca*, 9 CFU *S. marcescens*, 3 CFU *A. baumannii*, 5 CFU *B. cereus*, 3 CFU *L. monocytogenes*, 7 CFU *S. aureus*, and 4 CFU *E. coli* O157 DNA could be correctly detected (see Fig. S3A to J in the supplemental material).

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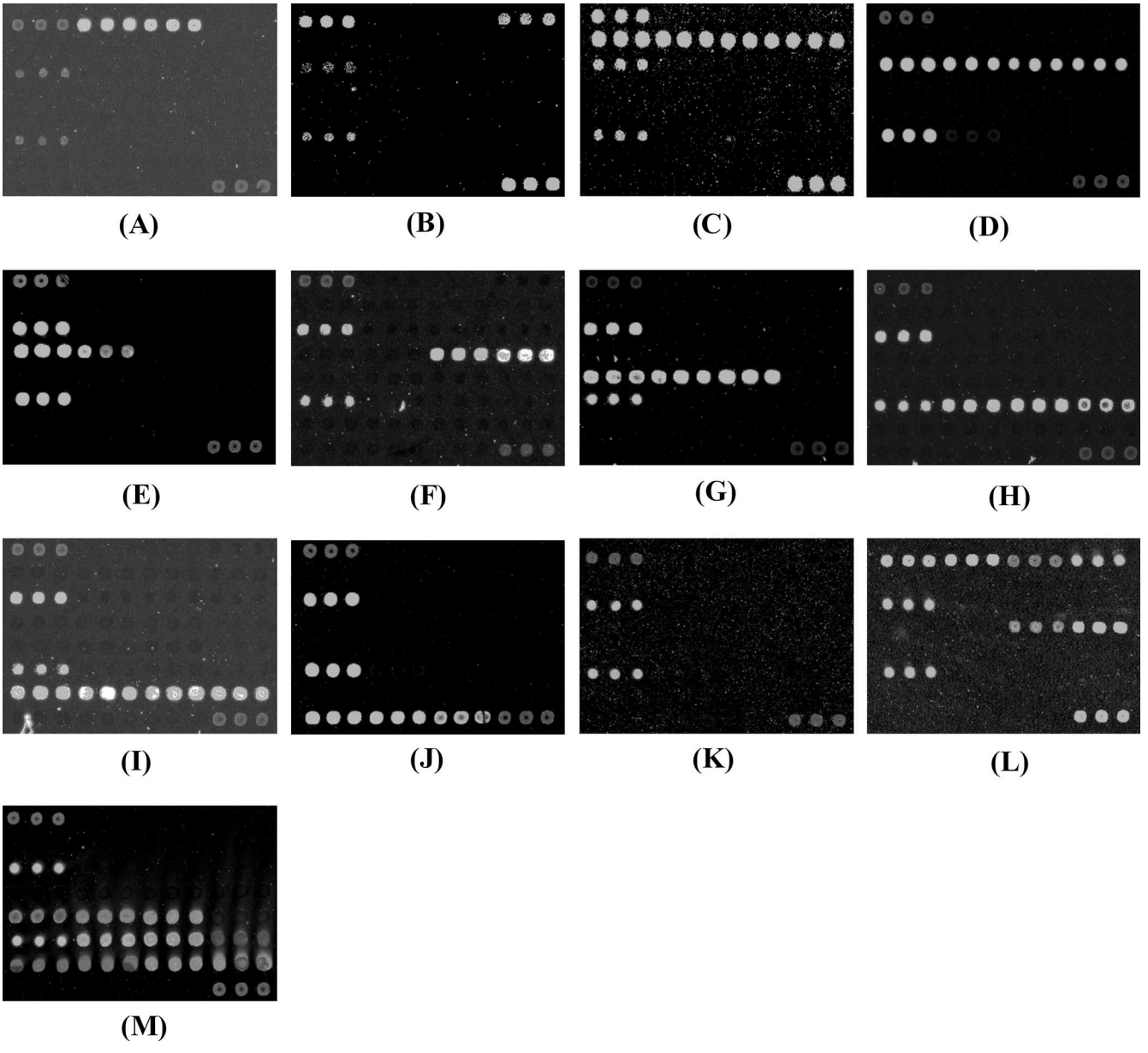

FIG. 2. Hybridization results. Cy3, which was used as the printing and positional control, is weak or invisible due to fluorescent attenuation. (A) *E. sakazakii* ATCC 29594; (B) *Salmonella* CMCC 50071; (C) *E. coli* O157 IMVS 1332; (D) *A. baumannii* ATCC 19606; (E) *K. pneumoniae* ATCC 10031; (F) *B. cereus* AS1.196; (G) *K. oxytoca* ATCC 49334; (H) *S. aureus* CMCC 26058; (I) *S. marcescens* ATCC 13880; (J) *L. monocytogenes* CMCC 54001; (K) *S. epidermidis* CMCC 26069; (L) *E. sakazakii* ATCC 29544, *Salmonella* sp. strain M44, and *B. cereus* AS1.196; (M) *S. marcescens* ATCC13880, *K. oxytoca* ATCC49334, and *S. aureus* CMCC26058.

Detection of PIF samples. A total of 21 batches of PIF from the United States ($n = 9$), Ireland ($n = 3$), France ($n = 2$), Argentina ($n = 2$), Holland ($n = 3$), and India ($n = 2$) were collected from the Tianjin Entry-Exit Inspection and Quarantine Bureau and detected according to inspection and quarantine trade standard methods. In the meantime, the PIF samples were detected by the designed microarray. The hybridization profiles showed that two samples were detected as *E. sakazakii* and *B. cereus*, respectively (see Fig. S3K to L in the supplemental material), which was affirmed by the Vitek system in the Tianjin Entry-Exit Inspection and Quarantine Bureau and 16S rRNA sequencing in our laboratory. The other 19 samples were signals of the positive control probes being detected, suggesting that there are other bacteria beyond the 10 pathogens studied. These results of 100% accuracy indicate that the microarray has the practical ability to detect and differentiate pathogens in PIF samples.

DISCUSSION

DNA microarrays currently are used for the detection of food-borne pathogens, since they are rapid, sensitive, specific, and allow for high-throughput analysis (25). Recently, various reports have demonstrated the efficacy of this approach in the

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detection of waterborne pathogens (21), marine fish pathogens (11), and other food-borne pathogens (7, 10, 19, 28, 30). To our knowledge, this study describes for the first time the application of DNA microarray technology for the rapid and reliable detection of pathogens associated with PIF contamination. The rRNA genes (i.e., 16S, 23S, and 5S) are ideal genetic targets that can be used for bacterial identification, since these sequences are highly conserved between species (14). The primary caveat associated with ribosomal sequences as targets is that their variable regions make the identification of closely related organisms imprecise (23). However, the ITS sequence is not subject to the same selective pressures as the rRNA genes, therefore targeting these sequences overcomes the specificity issues associated with rRNA sequences (1, 13). Sequence and length polymorphisms associated with ITS increasingly are being used as targets for bacterial species and subspecies identification (5, 32) and typing (20, 31), as well as being used in evolutionary studies (12, 24). Another advantage of targeting ITS sequences is that they are relatively short. Specific capture probes can be designed for the variable regions, and universal primers can be designed for the conserved 16S and 23S rRNA gene sequences, respectively.

Lin et al. reported the detection of food-borne pathogens using microarrays designed to target ITS sequences (19). *B. cereus*, *E. coli*, *L. monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella* species, *S. aureus*, and *Vibrio parahaemolyticus* were detected. In the present study, five additional pathogens, *E. sakazakii*, *K. pneumoniae*, *K. oxytoca*, *S. marcescens*, and *A. baumannii* were added to the panel of pathogens associated with PIF contamination.

Five *Bacillus* species, i.e., *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. mycoides*, and *B. weihenstephanensis*, are closely related organisms that also posses a high degree of homology at the DNA level, making them difficult to differentiate (26). In this report, the *B. cereus*-specific probes described correspond to the conserved ITS regions of all five of these species, therefore all five species could be identified by the probes designed in this study.

Another obstacle associated with microarray-related approaches is that the detection of different DNA sequences is subject to different levels of sensitivity (0.100 to 0.001 ng). One possible reason is that each species possesses different operon copy numbers (*rrn*) and ITS types. For example, *A. baumannii* has six RNA *rrn* operons and one ITS type ($ITS^{ile+ala}$, with $tRNA^{ile}$ and $tRNA^{ala}$ genes); *E. sakazakii* has seven *rrn* operons, and four of them are ITS^{glu}, the rest are $ITS^{ile+ala}$, and the specific probes were designed based on the ITS^{glu} sequences; *K. pneumoniae* has eight *rrn* operons, including three $ITS^{ile+ala}$, four ITS^{glu}, and one ITS^{none} (with no tRNA genes), and the specific probes were designed based on the $ITS^{ile+ala}$ regions. Therefore, their target sequences possess different copy numbers (six for *A. baumannii*, four of seven for *E. sakazakii*, and three of eight for *K. pneumoniae*), and the final amplicons among them are 56:32:21. Another possible reason for different levels of sensitivity is different amplification efficiencies. Although they employed the same primers, different templates hold different levels of DNA quality, DNA structure, and GC content, which have profound effects on the final amplicon amount. Our results also showed that the closely related bacteria *Salmonella* and *E. sakazakii* share the same DNA sensitivity, which is also the same as that for *K. pneumoniae*, *K. oxytoca*, and *S. marcescens*. For the sensitivity of the microarray as a whole, the lowest one, 0.100 ng bacterial genomic DNA, should be reported.

AQ: B

Another challenge associated with food pathogen detection is that organisms are surrounded by the biological matrix that comprises food or food products, and the nature of the food product can critically affect sample preparation. In this study, we employed preenrichment along with selective culture for the preparation of specific pathogens. Although this sample preparation method is laborious, it has its advantages. First, the two-step culture method is prone to yield more bacteria, which will increase the amount of genomic DNA available for analysis and enhance hybridization sensitivity. Second, the two-step culture method also will reduce contaminants of food samples. If the DNA obtained for hybridization was contaminated with food product, it could interfere or inhibit the PCR by reducing hybridization efficiency or producing a false-positive signal (27). In the second step, only 10 ml of the preenrichment was inoculated into 100 ml of selective culture, reducing the amount of food sample.

At present, the detection of food-borne pathogens is crucial for the safety of PIF because the consumers are infants and children, who are highly susceptible to outbreaks of life-threatening neonatal meningitis, bacteremia, and diarrhea illness (4). Although our initial efforts focused on only 10 pathogens, efforts now are under way to include most pathogens commonly associated with PIF contamination. A diagnostic tool with such a high-throughput value undoubtedly will facilitate the identification of pathogenic bacteria, thereby making preventive measures timely and also expediting epidemiologic investigations.

ACKNOWLEDGMENTS

This study was supported by grants from the National High Technology Research and Development Program of China (863 Program) (2006BAK02A14, 2006AA06Z409, and 2006AA020703) and the Tianjin Municipal Science and Technology Committee, China (07JCYBJC08500).

REFERENCES

1. **Barry, T., G. Colleran, M. Glennon, L. K. Dunican, and F. Gannon.** 1991. The 16s/23s ribosomal spacer region as a target for DNA probes to identify eubacteria. PCR Methods Appl. **1:**51–56.
2. **Bej, A. K.** 2003. Molecular based methods for the detection of microbial pathogens in the environment. J. Microbiol. Methods **53:**139–140.
3. **Brown, P. O., and D. Botstein.** 1999. Exploring the new world of the genome with DNA microarrays. Nat. Genet. **21:**33–37.
4. **Cahill, S. M., I. K. Wachsmuth, L. Costarrica Mde, and P. K. Ben Embarek.** 2008. Powdered infant formula as a source of *Salmonella* infection in infants. Clin. Infect. Dis. **46:**268–273.
5. **Chun, J., I. N. Rivera, and R. R. Colwell.** 2002. Analysis of 16S–23S rRNA intergenic spacer of *Vibrio cholerae* and *Vibrio mimicus* for detection of these species. Methods Mol. Biol. **179:**171–178.
6. **de Boer, E., and R. R. Beumer.** 1999. Methodology for detection and typing of foodborne microorganisms. Int. J. Food Microbiol. **50:**119–130.
7. **Eom, H. S., B. H. Hwang, D. H. Kim, I. B. Lee, Y. H. Kim, and H. J. Cha.** 2007. Multiple detection of food-borne pathogenic bacteria using a novel 16S rDNA-based oligonucleotide signature chip. Biosens. Bioelectron. **22:**845–853.
8. **Feng, L., W. Wang, J. Tao, H. Guo, G. Krause, L. Beutin, and L. Wang.** 2004. Identification of *Escherichia coli* O114 O-antigen gene cluster and development of an O114 serogroup-specific PCR assay. J. Clin. Microbiol. **42:**3799–3804.
9. **Forsythe, S. J.** 2005. *Enterobacter sakazakii* and other bacteria in powdered infant milk formula. Matern. Child Nutr. **1:**44–50.
10. **Gehring, A. G., D. M. Albin, S. A. Reed, S. I. Tu, and J. D. Brewster.** 2008. An antibody microarray, in multiwell plate format, for multiplex screening of

foodborne pathogenic bacteria and biomolecules. Anal. Bioanal. Chem. **391:** 497–506.

11. **González, S. F., M. J. Krug, M. E. Nielsen, Y. Santos, and D. R. Call.** 2004. Simultaneous detection of marine fish pathogens by using multiplex PCR and a DNA microarray. J. Clin. Microbiol. **42:**1414–1419.
12. **Gürtler, V.** 1999. The role of recombination and mutation in 16S–23S rDNA spacer rearrangements. Gene **238:**241–252.
13. **Gürtler, V., and V. A. Stanisich.** 1996. New approaches to typing and identification of bacteria using the 16S–23S rDNA spacer region. Microbiology **142:**3–16.
14. **Gutell, R. R., N. Larsen, and C. R. Woese.** 1994. Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. Microbiol. Rev. **58:**10–26.
15. **Iversen, C. F. S.** 2004. Isolation of *Enterobacter sakazakii* and other *Enterobacteriaceae* from powdered infant formula milk and related products. Food Microbiol. **21:**771–777.
16. **Iversen, C., M. Lane, and S. J. Forsythe.** 2004. The growth profile, thermotolerance and biofilm formation of *Enterobacter sakazakii* grown in infant formula milk. Lett. Appl. Microbiol. **38:**378–382.
17. **Joosten, H., E. Bidlas, and N. Garofalo.** 2006. *Salmonella* detection in probiotic products. Int. J. Food Microbiol. **110:**104–107.
18. **Lai, K. K.** 2001. *Enterobacter sakazakii* infections among neonates, infants, children, and adults. Case reports and a review of the literature. Medicine (Baltimore) **80:**113–122.
19. **Lin, M. C., A. H. Huang, H. Y. Tsen, H. C. Wong, and T. C. Chang.** 2005. Use of oligonucleotide array for identification of six foodborne pathogens and *Pseudomonas aeruginosa* grown on selective media. J. Food Prot. **68:**2278–2286.
20. **Maggi, R. G., B. Chomel, B. C. Hegarty, J. Henn, and E. B. Breitschwerdt.** 2006. A *Bartonella vinsonii berkhoffii* typing scheme based upon 16S–23S ITS and Pap31 sequences from dog, coyote, gray fox, and human isolates. Mol. Cell Probes **20:**128–134.
21. **Maynard, C., F. Berthiaume, K. Lemarchand, J. Harel, P. Payment, P. Bayardelle, L. Masson, and R. Brousseau.** 2005. Waterborne pathogen detection by use of oligonucleotide-based microarrays. Appl. Environ. Microbiol. **71:**8548–8557.
22. **Muytjens, H. L., H. Roelofs-Willemse, and G. H. Jaspar.** 1988. Quality of powdered substitutes for breast milk with regard to members of the family *Enterobacteriaceae*. J. Clin. Microbiol. **26:**743–746.
23. **Palys, T., L. K. Nakamura, and F. M. Cohan.** 1997. Discovery and classification of ecological diversity in the bacterial world: the role of DNA sequence data. Int. J. Syst. Bacteriol. **47:**1145–1156.
24. **Pérez Luz, S., F. Rodriguez-Valera, R. Lan, and P. R. Reeves.** 1998. Variation of the ribosomal operon 16S–23S gene spacer region in representatives of *Salmonella enterica* subspecies. J. Bacteriol. **180:**2144–2151.
25. **Rasooly, A., and K. E. Herold.** 2008. Food microbial pathogen detection and analysis using DNA microarray technologies. Foodborne Pathog. Dis. **5:**531–550.
26. **Rhodehamel, E. J., and S. M. Harmon.** 1998. Bacillus cereus. *In* Food and Drug Administration bacteriological analytical manual, 8th ed. (revision A), chapter 14. AOAC International, Gaithersburg, MD.
27. **Rossen, L., P. Norskov, K. Holmstrom, and O. F. Rasmussen.** 1992. Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. Int. J. Food Microbiol. **17:**37–45.
28. **Sergeev, N., M. Distler, S. Courtney, S. F. Al-Khaldi, D. Volokhov, V. Chizhikov, and A. Rasooly.** 2004. Multipathogen oligonucleotide microarray for environmental and biodefense applications. Biosens Bioelectron. **20:**684–698.
29. **Wang, M., B. Cao, Q. Yu, L. Liu, Q. Gao, L. Wang, and L. Feng.** 2008. Analysis of the 16S–23S rRNA gene internal transcribed spacer region in *Klebsiella* species. J. Clin. Microbiol. **46:**3555–3563.
30. **Wang, X. W., L. Zhang, L. Q. Jin, M. Jin, Z. Q. Shen, S. An, F. H. Chao, and J. W. Li.** 2007. Development and application of an oligonucleotide microarray for the detection of food-borne bacterial pathogens. Appl. Microbiol. Biotechnol. **76:**225–233.
31. **Wojciech, L., Z. Staroniewicz, A. Jakubczak, and M. Ugorski.** 2004. Typing of *Yersinia Enterocolitica* Isolates by ITS profiling, REP- and ERIC-PCR. J. Vet. Med. B Infect. Dis. Vet. Public Health. **51:**238–244.
32. **Xiong, L., F. Kong, Y. Yang, J. Cheng, and G. L. Gilbert.** 2006. Use of PCR and reverse line blot hybridization macroarray based on 16S–23S rRNA gene internal transcribed spacer sequences for rapid identification of 34 *mycobacterium* species. J. Clin. Microbiol. **44:**3544–3550.

AQ: C

AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES 1

AQA—References to Salmonella in this paper included "*Salmonella enteritidis*," "*Salmonella* sp. strain M44," and the general citation "*Salmonella*." The term "species" has been added to "*Salmonella*" when it stands alone. Is this correct, or should it be changed to either "*enteritidis*" or "sp. strain M44"?

AQB—Is 56:32:21 a ratio? If so, what do the numbers correspond to?

AQC—I am unable to locate ref. 15 on PubMed. Please check the information.