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Full Length Research Paper

Rapid detection of *Salmonella* species using an improved gel-based DNA microarray method

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***Salmonella*, widely distributed in nature, is a great human and animal health hazard of a class of pathogens. Culture-based methods may require many days to detect *Salmonella*. Traditional microbiology could advantageously be replaced by DNA microarray technology. We described an improved 3-D polyacrylamide gel-based DNA microarray assay based on *gyrB* gene (DNA gyrase B subunit gene) sequences that can be used for the identification of *Salmonella* species. Primers specific for a *gyrB* gene region common to all 13 samples were synthesized and used for PCR amplification of purified DNA. An oligonucleotide probe for specific *gyrB* gene regions was developed for the identification of 7 *Salmonella* species. Acrylamide-modified oligonucleotides solutions containing acrylamide monomer, glycerol, APS and probe were prepared at the desired concentration. The solutions were spotted on the modified glass slide by ink jet using a microarrayer and then the slide was transferred to a vacuum chamber with TEMED, after that the slide was used for hybridization with fluorescently labeled ssDNA derived from amplified sample DNA to yield a pattern of positive spots. This microarray produced unique hybridization patterns for species of *Salmonella* and could differentiate closely related bacterial species. The sample preparation and microarray method used in this study increased sensitivity and reduces time-to-result for detection of *Salmonella*. The described method allowed microarray monitoring for *Salmonella* contamination of food and manure for aquaculture.**

Key words: *Salmonella*, *gyrB gene*, PCR, DNA microarray, TEMED.

INTRODUCTION

Salmonella is a facultative intracellular pathogen that causes a variety of infectious diseases (Pui et al., 2010). Annually pathogens account for more than 25 million infections worldwide, resulting in approximately 200,000 deaths (Crump et al., 2004). Effective treatment of the infections requires the rapid detection and identification of *Salmonella*. The old standard for the detection of *Salmonella* usually involves pre-enrichment, selective enrichment, isolation on selective agar media and confirmation of presumptive positive colonies using biochemical and serological tests. This conventional cultural method is very expensive and time consuming (Schonenbrucher et al., 2008).

The identification of bacteria by molecular genetics can be advanced further by DNA microarray technology (Batchelora et al., 2008; Sugihara et al., 2009). Discrete zones of DNA targets less than 1 mm in diameter (spots) are immobilized to a planar surface and exposed to a sample containing labeled DNA fragments. Complementary fragments in the fluorescent dye-labeled sample hybridize to the target spots, and unbound or weakly bound fragments are removed through washing. The amount of hybridized DNA on each spot is measured

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Table 1. Strains used in the study.

Name	Strain	Accession number for partial *gyrB* gene seqence
Salmonella sub-genus II	CMCC50215	AB598750
Salmonella sub-genus III b	CMCC50381	AB598754
Salmonella enteritidis	CMCC50335	AB598753
Salmonella enteritidis var. danysz	CMCC50100	AB598752
Salmonella gallinarum	CMCC50770	AB598751
Salmonella manchester	CMCC50380	AB598748
Salmonella senftenberg	CMCC 50210	AB598747
Escherichia coli	ATCC 25922	AB598755
Streptococcus faecalis	CMCC 32223	AB598756
Enterobacter cloacae	CMCC 45301	AB598757
Enterobacter aerogenes	CMCC (B) 45103	AB598749
Citro Bacter freumdii	CMCC48001	AB598758
Shigella flexneri	CMCC 51229	AB598759

using a fluorescence scanner (Liu and Fratamico, 2006). The flat surfaces of rigid supports have limitations in improving the immobilization capacity due to a spatial effect (Mir and Southern, 1997; Peterson et al., 2002), and 3-D polyacrylamide gel-based DNA microarray platforms provide a high capacity for nucleic acids immobilization and a solution-mimicking environment for hybridization, which is being widely used. However, several technological bottlenecks still remain in these platforms, such as difficult microarray preparation and high fluorescent background, high price which limit their application. Fortunately, Xiao et al. (2006) improved the technology by improving the convenience in microarray preparation and reduced the background after hybridization (Xiao et al., 2006).

In this study, we used the improved gel-based DNA microarray method to improve the specificity of the detection of *Salmonella*.

The use of 16S rRNA gene sequences for the development of primers/probes is currently well established in bacterial characterization (Moyaert et al., 2008; Widmer et al., 1998). Nevertheless, it is difficult to separate closely related bacteria in the superfamily γ-proteobacteria (Kenichi, 2003). The gene *gyrB*, encoding the B-subunit of DNA gyrase, is estimated to evolve faster than that of 16S rRNA (Yamamoto and Harayam, 1995) and thus provides an alternative effective target gene for the identification of bacteria at the species or subspecies level (Wang et al., 2007). The *gyrB* gene has been used as the target sequence for detection and identification of several species of bacteria (Sugihara et al., 2003; Kasai et al., 2000; Yamada et al., 1999) by PCR based assays. In recent years, a microbial diagnostic microarray approach, using single nucleotide extension labeling with *gyrB* as the marker gene, was developed (Kakinuma et al., 2003; Järvinen et al., 2008).

Overall, the aims of the study were to develop an improved 3-D polyacrylamide gel-based DNA microarray method using *gyrB* gene for a wider detection and differentiation of *Salmonella* from the other bacteria. Our approach enabled the sensitive and specific detection of a broad range of *Salmonella*.

MATERIALS AND METHODS

Bacterial strains

Strains used in this study were obtained from National Center for Medical Culture Collections. The partial *gyrB* gene was sequenced from the 13 strains representing 6 species (Table 1). Bacteria were cultured on Luria-Bertani (LB) agar plates at 37℃ for 24 to 48 h.

Sequencing of the *gyrB* gene

Genomic DNA strains from pure cultures were purified using the E.Z.N.A. TM Bacterial DNA Kit (Omega Bio-Tek, USA), following manufacturer's instructions. The *gyrB* gene was amplified from the extracted DNA by PCR with universal primers UP1 and UP2r (Table 2). PCR was performed for 60 cycles at 96℃ for 1 min, 60℃ for 1 min and 72℃ for 2 min, and a termination cycle at 72℃ for 7 min. All amplified fragments were purified with QIA quick gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The PCR fragments were directly sequenced using an ABI PRISM Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3730 automated sequencer (Applied Biosystems) according to the manufacturer's instructions. Accession numbers of the *gyrB* sequences of the strains used in this study are listed in Table 1.

Oligonucleotide synthesis

The detection oligonucleotides were prepared based on the *gyrB* sequence of the *Salmonella* species and other different species. Alignment and neighbor-joining phylogenetic tree of the *gyrB* sequences were constructed using the ARB software package (Ludwig et al., 2004), which was subsequently also used for the probe design. The designed probe was S-Acryl: 5'-

Table 2. Primers used for detection and sequencing of *gyrB* sequences.

Primer	Direction	Primer sequence (5'–3')
UP1	Forward	GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA
UP2r	Reverse	AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT
S-for	Forward	AACGCCGATCCACCCGAA
S-rev	Reverse	GAATCGCCTGGTTCTTGC(TAMRA fluorescent dyes at 5'-terminal)

TTTTTTTTCTGCTGGCAGAATAC-3', and was modified with acrylamide group at its 5'-terminal.

Fluorescence labeling of the samples

Because the labeled sequences for microarray is usually less than 800 bp, internal primers (S-for, S-rev), producing DNA fragments of about 580–650 bp, were designed based on these preliminary sequence data, by using a primer design utility (Primer Premier 5.0) (Table 2). The reverse primer was labeled with TAMRA fluorescent dyes at its 5'-terminal. The asymmetric broad-range PCR was done by the two primers used in 50:1 molar ratio limiting amounts for allowing the accumulation of single-strand amplification. The asymmetric broad-range PCR was performed with a DNA Thermal Cycler 480 using a PCR buffer containing dATP, dGTP, dCTP and dTTP at 2 mM each, 10 pM S-rev (TAMRA fluorescent labeled) primer, 0.2 pM S-for primer, 50 ng of DNA sample, and 5 U of Taq DNA polymerase. PCR was performed for 30 cycles at 94℃ for 4 min, 56℃ for 1 min and 72℃ for 1-2 min, and a termination cycle at 72℃ for 7 min.

Preparation of the acryl-modified slides

Acryl-modified slides were prepared as described by Rehman et al. (1999). Glass slides were cleaned by soaking in 10% aqueous nitric acid for 2 h. Slides were then rinsed with water and acetone and air dried. The cleaned slides were soaked in 10% 3-methacryloxypropyltrimethoxysilane (Sigma) in acetone for 1 h, and were washed in acetone and air dried.

Attachment of the modified oligonucleotides to the acryl-modified slide surface

Acrylamide-modified oligonucleotides solutions containing 2 to 15% acrylamide monomer (29:1 acrylamide:bisacrylamide), 20 to 50% glycerol, 1% APS, 5μM probe were prepared at the desired concentration. The solutions were spotted on the modified glass slide by ink jet using a microarrayer (Capital Biochip Corporation, China). After spotting, the slide was placed onto a humid sealed chamber in which a well containing TEMED was deposited in advance. The pressure in the sealed chamber was reduced to about 1000 Pascal (Pa), and this pressure was maintained for 0.5 h at room temperature. Under this pressure, TEMED was vaporized and diffused onto the slide surfaces to induce the copolymerization between acrylamide groups and acryl groups.

Microarray hybridization

All the samples were amplified with forward primer and acrylamide modified reverse primer. After PCR amplification and gel electrophoresis test, PCR products were processed by ethanol precipitation, evaporation or left untreated. To obtain ssDNA for hybridization analysis, dsDNA on the slides was denatured in 0.1 M NaOH for 8 min. Hybridization Solutions (Clontech) containing TAMRA fluorescent labeled PCR products were prepared, spotted, and polymerized onto the slide as described earlier. During hybridization, the labeled-targets solution was applied to the microarray and spread evenly by placing a coverslip on top, and the hybridization was performed in a humid glass chamber sealed with plastic film at 37℃ for 4 to 5 h. After hybridization, the slide was subjected to electrophoresis under 5 to 30 V/cm for 8 min in Tris-borate-EDTA (TBE) buffer at room temperature. The position of the slide in the electric field was fixed, and was about 5 cm from the cathode. The immersion depth of the slide was 0.2 cm. After electrophoresis was completed, the slide was rinsed in water and dried under a stream of nitrogen. Images of the slides were captured by a scanner (ScanArray Lite, Packard BioScience Company, USA).

RESULTS

Design of *Salmonella* species-specific oligonucleotides for microarray analysis

The sequence alignment in Table 3 was used to identify regions that were both unique to particular *Salmonella* species and sufficiently different from all other species to avoid cross-hybridization. In addition, we designed the probe in such a way that the *Salmonella* species-specific base sequence was located in the center of the probe.

Hybridization results from the samples

Asymmetric broad-range bacterial *gyrB* PCR was positive from all the samples (data not show). The reaction patterns of the detection oligonucleotides on the micro-arrays for *Salmonella* are shown in Figure 1, in which the colors of the spots according to the fluorescence intensity represent the intensities of the reactions with the different sample PCR products. Positive spots could be differentiated from negative spots, and a clear, specific pattern of reactivity was observed for each seven *Salmonella* species and the samples, while *E. coli*, *S. faecalis*, *E. cloacae*, *E. aerogenes*, *C. Bacter freumdii*, *and S. flexneri* assays are negative. Thus, through such the platform, *Salmonella* can be distinguished from other bacteria.

DISCUSSION

Salmonella species are usually identified by time-consuming culture methods. Recently, the developments

Table 3. Nucleotide sequence alignment of the *gyrB* genes from the 13 species strains. Nucleotides identical to those in *Salmonella gyrB* are indicated with dots.

Name	Nucleotide sequence
Salmonella sub-genus II	CTGCTGAGCGAATAC
Salmonella sub-genus III b	…….
Salmonella enteritidis	…….
Salmonella enteritidis var. danysz	…….
Salmonella gallinarum	…….
Salmonella manchester	…….
Salmonella senftenberg	…….
Escherichia coli	……GCA……...
Salmonella faecalis	……G.A…….
Enterobacter cloacae	……C…….
Enterobacter aerogenes	……C…….
Citrobacter freumdii	……...T……..
Shigella flexneri	……GCA……...

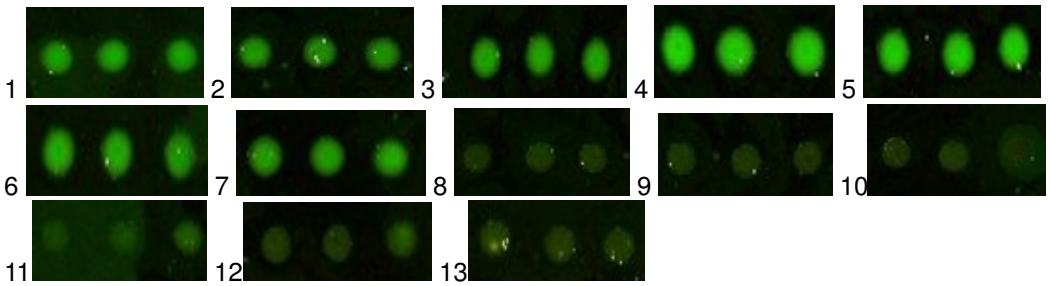

Figure 1. Hybridization results from the samples of acrylamide-modified oligonucleotides immobilized on acrylsilane-coated slides. (1) to (7): *S.* sub-genus II, *S.* sub-genus III b, *S. enteritidis*, *S. enteritidis* var. danysz, *S. gallinarum*, *S. manchester*, *S. senftenberg* hybridization. All seven *Salmonella* gave positive signals; (8) to (13): (8) to (13): *E. coli*, *S. faecalis*, *E. cloacae*, *E. aerogenes*, *C. Bacter freumdii*, *S. flexneri*. All six other samples gave negative signals. The slide was scanned at 70% laser power and 70% PMT gain.

of rapid diagnostic tests that use molecular genetic methods, such as PCR and microarray, have been reported. The microarray has proved to be a valuable tool for the specific detection of microorganisms directly from samples. Classical detection methods have some disadvantages: first, the number of organisms in a sample is not always large enough for the organisms to be detected by microscopic methods; second, the period required for culture of these organisms is long, and an identification test must be used (Fukushima et al., 2003). However, the microarray can solve these problems, since the oligonucleotide is designed based on the sequence unique to the species.

In this study we developed a microarray assay for the detection of *Salmonella* species that used the *gyrB* gene as the target. Rapid identification of *Salmonella* species is an important factor for the successful diagnosis of *Salmonella*. However, because of the variable nature of the sputum specimens there is a risk of false-negative results. The assay described here showed that no false-negative results were obtained by the microarray assay in this study. Meanwhile, the microarray assay targeting the *gyrB* gene could identify *Salmonella* at the species level and can even differentiate among closely related species.

Relevant reports have been made about detection of *Salmonella* species using the *gyrB* gene as the target (Kakinuma et al., 2003). However, in the assay we used the improved microarray method (Xiao et al., 2006) and the different oligonucleotide. To control the polymerization process, solutions containing acrylamide-modified oligonucleotide, acrylamide, glycerol and ammonium persulfate were spotted onto a functionalized glass slide, this sample handling was more convenient and much cheaper than for other nucleic acid immobilization methods, such as modification of the nucleic acids with amino groups in, which samples were purified using a commercial purification cartridge to remove the PCR medium and to condense DNA fragments. Then the slide

was transferred to a vacuum chamber with TEMED, so that TEMED was vaporized and diffused into the spots to induce polymeri-zation (Xiao et al., 2006). In this way, comparing with the previous identification, we used the microarray method of identification of *Salmonella*, saving not only time but also cost. In addition, the initial study suggested that our microarray method was at least as sensitive as and may be less subject to error than Kenichi' methods. And the microarray assay could also be used to analyze a sample for several kinds of bacteria at the same time.

In summary, the potential of the *gyrB* gene-based microarrays strategy for the parallel testing of different targets has been demonstrated. Future studies will focus on rapid detection of *Salmonella* species in different environmental samples, and defining the identification of bacterial species of this technology for application in diagnostics.

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