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Using PCR amplification to increase the confidence level of *Salmonella typhimurium* DNA microarray chip hybridization

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Abstract

In order to design and validate a method to identify virulence genes of *Salmonella typhimurium* using DNA microarray, a protocol was developed to label the isolated bacterial DNA directly and to use PCR amplification of limited numbers of genes to validate the hybridization signals. Therefore, a DNA microarray chip of 71 virulence genes of *S. typhimurium* was developed and evaluated using 10 isolates. Each gene was represented by 65 bp oligonucleotide probes (oligoprobes) and immobilized on the surface of chemically modified slides. Whole DNA genomes were digested with *Hin*f1 and *Sau*3AI, labeled with a fluorescent tag of Cy3 and then hybridized. The presence of virulence genes in 10 strains of *S. typhimurium* was established by measuring a fluorescent signal above the background noise of the chip. PCR amplification of 10 genes (*orgA*, *ORF319, ttrB, rmbA, misL, spi4F, spi4H, spi4N, rRNA,* and *purR*) of *S. typhimurium* was used as a standard to verify the confidence level of the DNA microarray chip. In conclusion, using PCR amplification to increase the confidence level of the microarray hybridization data was successful.

Keywords: DNA microarray; *Salmonella typhimurium*; PCR

1. Introduction

Salmonella spp. are normally found in the intestinal tracts of humans and other animals. They are Gram-negative facultative anaerobic non-endospore-forming rods that are usually motile and which ferment glucose to produce acid and gas. *Salmonella* spp. are pathogenic and are the cause of human and animal diseases [14]. There are two species within the genus *Salmonella*: *S. enterica* and *S. bongori*. Within *S. enterica* there are six subspecies and each of these subspecies are further differentiated, serologically, into individual serovars. There are more than 2400 serovars within *S. enterica*. In the United States, *Salmonella typhimurium* was the most commonly isolated serovar (29.4%) from 1968 to 1998 [1]. It was isolated more than twice as much as the next most commonly occurring serovar, *S. enteritidis* (14.2%), during this same period.

The high throughput of gene sequencing in *Salmonella* has revealed the presence of large blocks of virulence genes called pathogenicity islands. The concentration of virulence genes in certain blocks of the genome probably indicates that the acquisition of the genes occurred by horizontal transfer from inter and intra-species. This transfer of the newly acquired genes was preserved later by vertical transfer from mother to daughter cells in a single pathogenic clone [20]. *Salmonella enterica* serovar *typhimurium* was shown to have five pathogenicity islands distributed throughout the *t*-RNA regions [9].

Salmonella pathogenicity island 1 (SPI-1) encodes for several proteins that are involved in invading epithelial cells and inducing apoptosis in macrophages. Most of these genes are involved in regulatory function, adhesion, and type III or a contact-dependent secretion system [20]. SPI-2 genes are required for replication within the macrophages and for survival in mice when the bacterial cells are taken up by a macrophage. SPI-3 is much smaller that SPI-1 and SPI-2

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and is believed to augment the function of the two pathogenicity islands. The island encodes for an *mgtBC* operon that is involved in magnesium transport, which allows *S. typhimurium* to survive in a low Mg^{2+} environment [9]. SPI-4 encodes for *SigDE*, which was found to play a role in the invasion process into epithelial cells and can also be secreted by SPI-1 [18,19]. Finally, SPI-5 encodes *sopE* and it is flanked by the genes for tail-fiber proteins from a P1-like phage. The *sopE* gene produces a 25 kDa protein molecule that is secreted by SPI-1 and is required for maximal invasion activity [20].

Salmonella genotyping using molecular and non-molecular methods has been well documented in the literature [17]. Some of these methods, like pulsed-field gel electrophoresis (PFGE), are considered reliable strategies only when combined with plasmid profiling and ribotyping to differentiate between strains [17]. Fluorescent amplified fragment length polymorphism (FAFLP) [16] and multi-locus enzyme electrophoresis (MLEE) [10], are alternative methods to study the genetic diversity between the *Salmonella* strains but these methods lack the ability to serotype strains.

Recently, molecular identification of *Salmonella* by DNA microarray chip technology has demonstrated some advances in high throughput screening to rapidly determine a complete genetic profile of pathogenic microorganisms [3–5,11]. In general, microarray chips, which can hold thousands of oligonucleotide probes per chip, show potential for simple and efficient genotyping of many organisms in a single hybridization experiment [3–5,11,22]. In the last 10 years, DNA microarray technology has contributed a wealth of information on gene expression as well as gene identification.

Our ability to study a few genes has been increased to investigate thousands of genes in a single set of experiments. But with the number of genes to be identified or studied increasing significantly, irreproducibility among researchers and platforms in analyzing DNA microarray data detracts from the potential usefulness of this technology. In three recent studies of gene expression microarray reproducibility [8,13,15], it was found that there are large differences in data analysis between 10 laboratories even when they used the same chip platform [13]. In addition, Larkin et al. [15] used 11 quantitative RT-PCR analyses for randomly selected genes to understand the discrepancies between different hybridization data of two tested chips containing the same genes but produced by different labs. Only one gene, based on the RT-PCR data, gave compatible confirmation of the data obtained from the two chips. The other 10 genes appeared to have different expression profiles on the two chips. Therefore, it is quite obvious that microarray technology needs to be validated and 'debugged' for robust reproducible results across different lab settings.

In this work, our goals were to develop a protocol to bypass multiplex PCR amplification, to increase the number of genes fabricated on the chip, and to have a reproducible method to test the confidence level in microarray hybridization data. The 71-associated virulence genes of *S. typhimurium* located throughout the pathogenicity islands were selected to build a DNA microarray chip. Ten of these virulence genes were amplified by PCR and used to examine the confidence level of the DNA microarray data in 10 *S. typhimurium* strains.

2. Material and methods

2.1. Bacterial strains and DNA preparation

Biochemical tests were conducted to confirm all the strains (see Section 3). Brain Heart Infusion (BHI) medium was used to grow the 10 *S. typhimurium* strains. Genomic bacterial DNA was isolated using Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN) and it was used in PCR amplification and chip hybridization.

2.2. PFGE

Agarose plugs of bacterial strains were prepared and digested as described previously [2], and PFGE was performed with the CHEF MAPPER system (BioRad Laboratories, Hercules, CA) in 1% agarose gel and 0.5× TBE buffer at 14 °C. The running time was 19 h at 6 V/cm, with initial and final switch times of 2.16 and 63.8 s, respectively. Genomic DNA of CDC strain H9812, digested with *Xba*I, was used as reference ladder CHEF DNA Size Standard. The gel was stained with ethidium bromide (1 μg/ml), and the gel image captured with the Gel Doc 2000 system (BioRad) was analyzed with the BioNumerics fingerprinting software (Applied Maths, Sint-Martens-Latem, Belgium).

2.3. Design of PCR primers and gene-specific oligonucleotide probes

Sequences of PCR primers used in the current study were newly designed for *orgA*, *ORF319*, *ttrB*, *rmbA*, *misL*, *spi4F*, *spi4H*, *spi4N*, *16s rRNA*, and *purR* (Table 1). Individual gene-specific oligoprobes were designed on the basis of unique sequences found in the target genes using ArrayDesigner software (Premier Biosoft International, Palo Alto, CA) (Table 2). The specificity of designed oligoprobes was assessed by using a BLAST search of homologous sequences available in GenBank. The goal of PCR amplification was to test the specificity and sensitivity of the chip hybridization. When the positive or negative signals in the chip were found not to match the PCR amplification, some of the PCR products were sequenced (see Section 3). All oligonucleotides were synthesized by Operon Technologies, Inc., Alameda, CA.

2.4. Microchip fabrication

Microchips were prepared using a contact microspotting robotic system, PixSys 5000 (Cartesian Technologies, Inc., Irvine, CA). The average size of the spots was 50–100 μm. The concentration of each oligoprobe before printing was adjusted