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Development of DNA Microarray for Pathogen Detection

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Abstract Pathogens pose a significant threat to humans, animals, and plants. Consequently, a considerable effort has been devoted to developing rapid, convenient, and accurate assays for the detection of these unfavorable organisms. Recently, DNA-microarray based technology is receiving much attention as a powerful tool for pathogen detection. After the target gene is first selected for the unique identification of microorganisms, species-specific probes are designed through bioinformatic analysis of the sequences, which uses the information present in the databases. DNA samples, which were obtained from reference and/or clinical isolates, are properly processed and hybridized with species-specific probes that are immobilized on the surface of the microarray for fluorescent detection. In this study, we review the methods and strategies for the development of DNA microarray for pathogen detection, with the focus on probe design.

Keywords: microarray, pathogen, target gene, probe

INTRODUCTION

Pathogens have been and are becoming an even more significant threat to humans, animals, and plants. Therefore, a considerable effort has been devoted to developing rapid, sensitive, and specific assay systems to detect these organisms. The most common method of detection is the culture based test, which utilizes proper growth media for the identification of microbial species. However, because the culture-based method, or the current 'golden standard' test, typically takes between 24~48 hrs to obtain the results, this method is not appropriate in situations where rapid diagnostic decisions are required. Also, due to the necessity of the special culture media, it is difficult to identify some bacteria, such as *Legionella* spp. and *Mycobacteria* spp. Furthermore, the culture-based assay has a relatively low sensitivity (30~50%); for example, the probability of identifying a particular bacterium from the sepsis patient is less than 50% [1]. Additionally, the use of antibiotics remarkably reduces the success rate of cultivating bacteria. Immune-serological tests are also commonly used in the diagnosis of infectious diseases. Such tests include the bacterial aggregation test, the complement fixation test, the enzyme linked immunosorbent assay (ELISA), the radioimmunoassay, the immunofluorescent test, and the immunoblot. Other diagnostic methods are based on molecular biological techniques such as the Southern blot and polymerase chain reaction (PCR). Because these methods identify the pathogens by extracting only a small amount of DNA and amplify the target sequences without cultivation, the diagnostic procedure is rapid and convenient.

DNA microarray has become an indispensable tool for sensitive and high-throughput analysis of transcriptome (cDNA or oligonucleotide microarray) and DNA sequence variation (oligonucleotide microarray) [2]. Oli-

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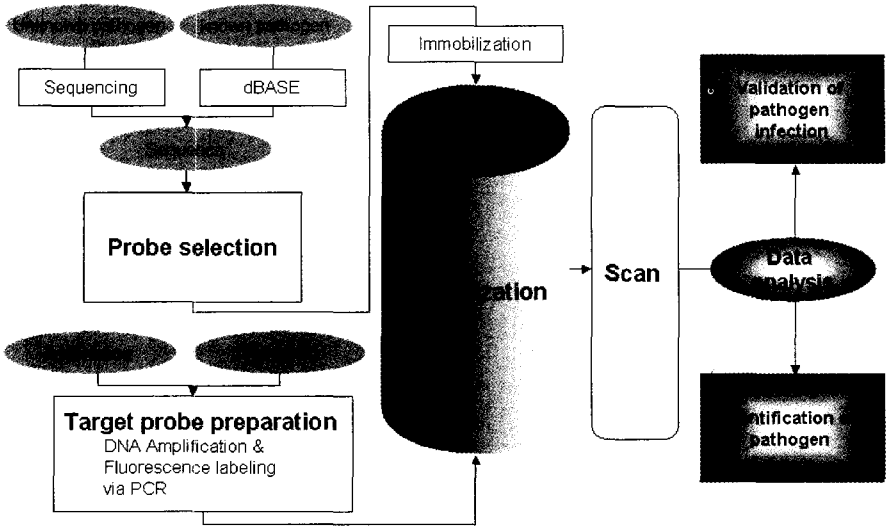

Fig. 1. Overall outline and strategy of the DNA chip for pathogen detection. Species-specific probes are designed by various selection tools from the genome sequences of the pathogen. The sample is hybridized with the probes that were immobilized on the solid substrate, which is followed by fluorescent detection of other means.

gonucleotide microarrays are particularly useful in the detection of pathogens. In an oligonucleotide microarray, probes for different genes can be deposited or directly synthesized on the solid substrate in a patterned manner [3]. Oligonucleotides offer greater specificity than cDNAs, and this is mainly because of the uniformity in probe length, which significantly reduces the chances of cross-hybridization and enables the ability to distinguish single base mismatches. Wilson *et al.* [4] developed the Multi-Pathogen Identification (MPID) microarray for high confidence identification of eighteen pathogens. Using this detection system, these eighteen pathogens were screened for their presence by the examination of specific diagnostic regions and by creating a fingerprint that is specific to each microbe. Wang and his colleagues [5] developed an oligonucleotide microarray that contains oligonucleotide probes designed from the 16S rDNA sequences for the detection of 20 predominant human intestinal bacterial species in fecal samples. Rudi *et al.* [6] developed several new methods in the field of nucleic acid-based microbial community analyses. All of these methods cover both the sample preparation and detection approaches. The assay combines the specificity obtained by enzymatic labeling of DNA probes with the possibility of detecting several targets simultaneously by DNA array hybridization. Also, Rudi *et al.* [6] has developed a novel approach for multiplex quantitative PCR. The multiplex quantitative PCR was combined with the DNA array-based detection method.

TARGET GENE SELECTION

Selecting proper target genes is very important in nucleic acid based diagnosis. Various targets have been used for identifying pathogens, and they include: (i) parts of DNA encoding a particular toxin or virulence factor, (ii) special sequences that are revealed by subtraction hybridization among closely related species, (iii) small, mostly species-specific, non-coding DNA regions such as insertion elements, and (iv) relatively well conserved nucleic acid sequences that also serve as phylogenetic markers. For the fourth target, there are 16S rDNA, 23S rDNA, 16S-23S rDNA internal transcribed spacer region (ITS), and other genes that code for β-galactosidase, RNA polymerase, elongation factors Tu, F1F0 ATPase, RecA protein, and Hsp60 heat shock protein, *rpoB* gene.

Since George Fox *et al.* [7] recognized, as early as 1980, the importance of rDNA in bacterial taxonomy for identifying evolutionary relationships among species, rDNA has been widely used as a microorganism specific genetic marker [8]. There are many advantages for targeting probes to the 16S/18S rRNA of the small subunit of the ribosome (SSU rRNA) or to the 23S/28S rRNA of the large subunit of the ribosome (LSU rRNA). These advantages include the large amount of rRNA in most cells, the apparent lack of lateral gene transfer, and a good length of about 1500 and 3000 nucleotides for 16S (or 18S) and 23S (or 28S), respectively, with a range of very conserved and quite variable sites [9]. Another important advantage is the availability of huge rRNA databases. However, 16S rDNA may be difficult to discriminate a large number of microbial strains because its sequence is conserved [10], which offers very little sequence diversity. Nonetheless, progress along this route with systems of limited diversity has been recently reported [11]. Alternatively, 23S rRNA genes and the ITS region have attracted attention as target sequences. Because 23S rDNA and ITS have regions with highly specific sequences, unlike 16S rDNA, pathogens can be easily discriminated from one another [3,12]. Park *et al.* [13] illustrated the usefulness of the ITS region as a use-

ful genetic marker for identifying *mycobacteria* at the species level. Anthony *et al.* [14] introduced a rapid detection and identification system that uses universal PCR primers to amplify a variable region of bacterial 23S rRNA, which is followed by reverse hybridization of the products to a panel of oligonucleotides. Other target genes have also been used as genetic markers for pathogen detection. Volokhov *et al.* [15] analyzed four *Campylobacter* species, *Campylobacter jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*, by using the microarray-based assay after PCR amplification of the specific regions in five target genes (*fur*, *glyA*, *cdtABC*, *ceuB-C*, and *fliY*). In order to quantify the target genes in biological samples using DNA microarrays, Cho *et al.* [16] employed reference DNA to normalize the variations in spot size and hybridization. This method was tested using nitrate reductase (*nirS*), naphthalene dioxygenase (*nahA*), and *Escherichia coli* O157 O-antigen biosynthesis genes as model genes and lambda DNA as the reference DNA. Until recently, pathogen detection that utilizes a specific target gene sequence has been limited due to sequence availability, but this limitation has been overcome because more complete sequences of the many microbial genomes are becoming available.

PROBE SELECTION AND DESIGN

Currently, nucleic acid probes allow the most taxonomically precise and quantitative description of microbial community structures. It has been suggested that assays based on nucleic acid probes have the potential to be fully quantitative and also can be useful even for unculturable microorganisms. Quantitative blot- or dot-blot hybridization with nucleic acid probes and PCR-based sequencing techniques were suggested for community composition analysis [17,18,19].

The key step in the microarray-based technology of pathogen detection is to determine suitable sets of oligonucleotide probes. Oligo design is a computationally intensive process as one must consider various factors, such as guanine/cytosine (G/C) content, melting point, secondary structure, and sequence specificity, in addition to potential splicing variants for each gene because these factors affect the sensitivity and specificity of hybridization. Maximizing the specificity and the sensitivity are often conflicting goals in terms of achieving probe design. Nonetheless, the following general strategies can be employed.

1. Positive probes: Given a selected subset S_1 in a database S_0 of sequences, find for each sequence in S_1 at least one positive probe p which hybridizes only with specific sequence S within S_1; it may however cross-hybridize with some sequences B, where $B \subseteq S_1$ if this cannot be avoided. High specificity means that the number of non-target matches is minimized, while high sensitivity indicates that a maximum number of selected target sequences is covered.

2. Negative probes: Given the positive probes identified above, determine the fewest possible negative probes that, together, hybridize with all sequences in B but with none in S_1. In this case, high specificity means that no sequences in S_1 may cross-hybridize with any negative probe, while high sensitivity means that a maximum number of sequences in B must be covered. The following constraints are further imposed onto the probe selection process:

1) Minimum and maximum length of the probes can be defined.

2) The melting points of the probe-target hybrids must be similar. This can be accomplished by specifying a range of percentage of G/C content.

3) Probes should not contain self-complementary regions that are longer than four sequential nucleotides.

A series of microarrays were developed for the detection of human pathogens by designing species-specific probes based on the rDNA sequences considering the procedures and constraints mentioned above [20]. In more detail, our strategy for the generation of oligonucleotide probe sets is as follows: First, each rDNA sequence of a pathogen was collected from GenBank (http://ncbi.nlm.nih.gov/Genbank), TIGR (http://www.tigr.org), and other databases [10,12,21] or through our own direct DNA sequencing. Second, suitable probe candidates for each species were designed from various regions of the DNA. Multiple alignments were performed with rDNA sequences of all pathogens of interest in order to select species-specific variable regions. Third, each species-specific probe was designed by using the oligo design programs that were available (see below). Fourth, each designed probe was checked for cross-reactivity with the probes designed for other species by the BLAST search tool. Finally, optimal probes were confirmed by real hybridization experiments on microarrays with all the rDNA amplified from reference pathogens to increase their sensitivity and to reduce cross-reactivity. This procedure for the probe design is outlined in Fig. 2.

There are several primer/oligo design programs [22], which may be helpful in probe analysis and design.

- Primer3 [23] (http://www.broad.mit.edu/genome_software/other/primer3.html) is a commonly used software for designing primers in the development of microarrays.
- OligoArray [24] (http://berry.engin.umich.edu/oligoarray/) is a program that helps design gene specific probes that are free of the secondary structure for the development of a genome-scale oligonucleotide microarray. In this program, probe selection is based on three criteria: oligonucleotide melting point, specificity to a single target, or at least to the shortest list of possible targets, and the inability to fold into a stable secondary structure at the hybridization temperature.
- OligoChecker (http://brainarray.mhri.med.umich.edu/OligoChecker/index.htm) is a fully automatic solution for verifying the specificity of batch oligo and designs against the whole transcriptome.
- GST-PRIME [25] is a useful program for retrieving and assembling gene sequences even from complex genomes by using the NCBI public database. Then,

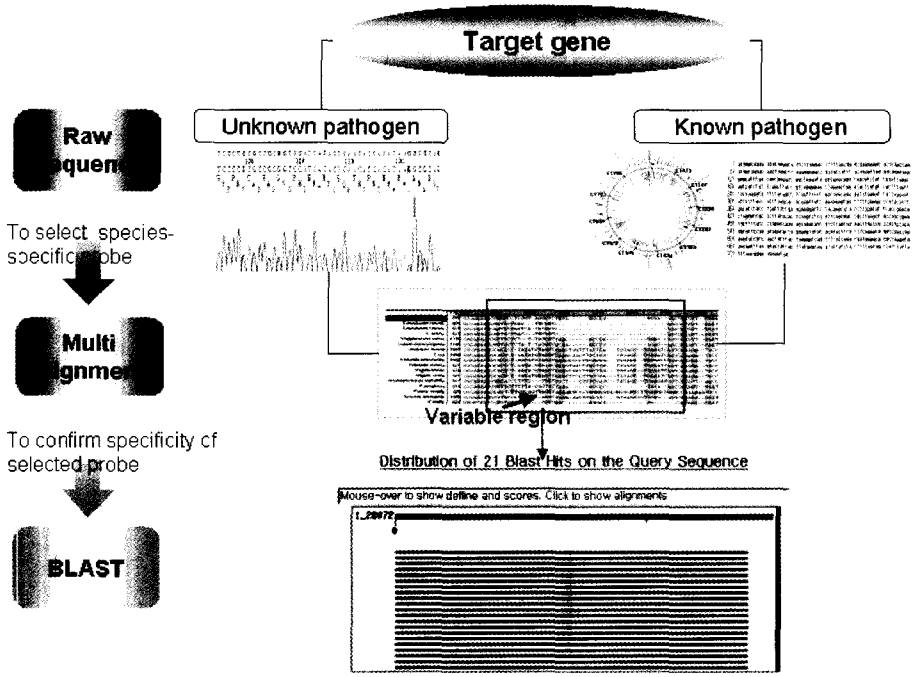

Fig. 2. Schematic representation of the probe design. After selecting the target genes as appropriate genetic markers for pathogen detection, species-specific probes usually based on the sequences of the variable regions are designed by bioinformatic analysis.

the designing sets of primer pairs can be used in gene amplification.

- Array Designer (http://www.premierbiosoft.com/dnamicroarray/dnamicroarray.html) is a software that can be used to design hundreds of primers for the development of oligonucleotide microarrays.
- ProMide (http://oligos.molgen.mpg.de/) is a collection of command-line tools that can be used for probe selection and microarray design.
- Visual OMP (http://www.dnasoftware.com/vo-microcase.html) is a software for probe design, which was optimized to maximize the specificity of the probes. This program has been integrated in an engine for visualizing target and oligo structures, thermodynamics modeling, and built-in BLAST and ClustalW.
- Sarani Gold (Genome Oligo Designer, http://www.strandgenomics.com/SOLUTIONggS/PRODUCTS/SARANI/sar_over.htm) is a software for automatic large-scale design of optimal oligonucleotide probes for microarray development. This program can analyze thousands of gene sequences simultaneously and can select the best available probes with uniform thermodynamic properties and minimal similarity to non-specific genes.

An interesting sequencing-independent method that generates oligonucleotide probes was recently developed by Bertilsson *et al.* [26]. This method allows the synthesis of oligonucleotide probes that target a variable region in the 16S rDNA without any information about the target sequence. The method relies on two consecutive PCR amplifications of bacteria-specific primers that include a highly variable region in the 16S rDNA. Double stranded PCR is carried out with a one-side primer, which consists of 5'-biotin and 3'-ribonucleotide. The biotinylated PCR product is bound to streptoavidin-coated magnetic beads and is washed at 85°C to remove the complementary strand. The biotinylated primer is detached by alkaline hydrolysis of the 3'-ribonucleotide, and it is used to amplify the single stranded product as shown in Fig. 3.

SAMPLE PREPARATION AND HYBRIDIZATION

In order to prepare a sample that can be hybridized with target probes, which were immobilized on the solid substrate, the DNA is boiled or asymmetric PCR is carried out. In general, DNA is chemically modified by incorporating fluorescent nucleotides or end-labeled by using a one-side primer with 5'-fluorescein. The amplified products are hybridized on a slide in hybridization solution. After washing, the slides are air-dried, and by using the confocal laser scanner, they are scanned under a wavelength that is compatible in fluorescent excitation.

rRNA DATABASES

Sequencing rRNA genes generally provides the most accurate information when identifying microorganisms. The quality of data present in the reference database is important to the success of the probe design. Apart from well-established databases such as GenBank, researchers can also consult several other databases that specialize in ribosomal genes, which include the Ribosomal Database

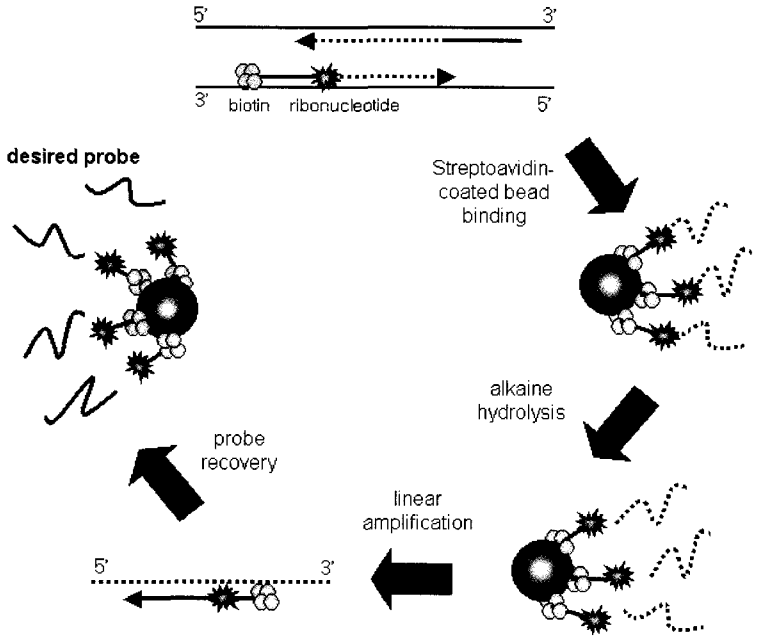

Fig. 3. Outline of the PCR-based method of generating population-specific oligonucleotide probes without sequence information. Target samples are amplified with the primer having 3'-biotin and 5'-ribonucleotide bound on the bead coated streptoavidin, and they are hydrolyzed by alkaline solution. Single stranded DNA is re-amplified, followed by binding to the bead and hydrolysis, where the desired probe can be obtained.

Project (RDP) and the European ribosomal subunit RNA sequence database. The Ribosomal Database Project (RDP, http://rdp.cme.msu.edu/html/), which was previously described by Maidak *et al.* [12], has currently made tens of thousands of rRNA sequences available. European Ribosomal RNA Database (http://rrna.uia.ac.be/ssu/) contains the complete or nearly complete set of SSU and LSU rRNA sequences in an aligned format [21]. The alignment also takes into account the secondary structure information. The Small RNA Database (http://mbcr.bcm.tmc.edu/smallRNA/smallrna.html) contains sequences that are based on actual sequence determination and not on the basis of oligonucleotide catalogs. Other RNA databases that can be consulted include the Comparative RNA web site (http://www.rna.icmb.utexas.edu/RNA/GRPI/introns.html), the Ribosomal DNA Primer Database (http://rrna.uia.ac.be/primers/database.html), and the RNase P Database (http://jwbrown.mbio.ncsu.edu/RNaseP/home.html). The RNase P Database is a compilation of RNase P sequences, sequence alignments, secondary structures, and three-dimensional models.

PROBLEMS IN PROBE DESIGN

The current barrier to the commercialization of DNA microarrays for pathogen detection is again the limited ability in designing specific probes. Each of the databases mentioned above contains errors, which questions their value and ability in diagnosing pathogens. Much effort is being devoted to correcting these errors, and attempts are being made to generate high quality databases. The fancy databases include MicroSeq, which is managed by Applied Biosystems (Foster City, CA, USA) and RIDOM (http://www.ridom-rdna.de/).

Another possible limitation may be the diversity of target genes in different microorganisms. Even if species-specific probes were designed based on current data sets and were found to be specific to all of the test organisms that were examined, there is still a chance that cross-hybridization with yet unknown microorganisms can occur.

PROSPECTS

The DNA microarray or DNA chip generally comprised a glass surface on which multiple DNA probes with known identities are fixed for molecular hybridization with DNA samples, which allows the simultaneous analysis of thousands of gene in a short assay time. The assay provides high accuracy by using the species-specific probes. Probes made of peptide nucleic acids (PNA), which have very strong affinity for complementary DNA sequence, can further improve the specificity. Therefore, the PNA probes can more effectively discriminate the pathogens at the level of single-base mismatches.

To detect the specific microbes in the patients and animals with infectious disease or to detect in various environmental habitats such as rivers, lakes and soil, the development of an enhanced method that requires only a small amount of the specific sample is necessary. The

scale-down of a DNA chip from the micro-scale to the nano-scale can be one of the scenarios. Additionally, in the use of the microarray-based diagnostic techniques, the simplification of the steps during sample preparation and hybridization with probes will be continuously pursued for the sake of saving time and handling effort. Ultimately, these diagnostic systems will be fabricated as a lab-on-a-chip. Other potential application is a system using nanocrystal tagged oligonucleotide probes, which are attached to a fixed substrate in such a way that the nanocrystals can only fluoresce when the DNA probe couples with the corresponding target genetic sequence. This system provides several advantages. Probes can be labeled in advance, and an excellent quantum dot signal can yield increased sensitivity. This assay compatile with any DNA extraction technique and enables robust multiplexed analysis that is unchanged by exreme conditions or roughhandling.

In conclusion, in the near future, DNA microarrays will become a major tool for pathogen detection. When considering the commercialization of DNA microarrays or chips for diagnostics, there are still additional issues to be addressed. Sample handling cannot be overlooked as the "garbage in garbage out" principle also holds here. The key factor in the successful introduction of the new technologies in clinical laboratories is the automation of procedures. Hence, it is important to automate the handling of samples, integrate the system further, significantly reduce the analyzing time, and the overall costs to a level that most clinical laboratories can afford. With various useful techniques, the improved microarray-based system will allow the clinician and patients to experience a more rapid and accurate diagnosis which in turn will allow for immediate clinical treatment in the near future.

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