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Development of a multipathogen oligonucleotide microarray for detection of *Bacillus anthracis*

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Abstract

An oligonucleotide microarray system has been specifically designed to detect and differentiate *Bacillus anthracis* from other bacterial species present in clinical samples. The pilot-scale microarray initially incorporated probes to detect six common species of bacteria, which were fully evaluated. The microarray comprised long oligonucleotides (50–70-mer) designed to hybridise with the variable regions of the 16S rRNA genes. Probes which hybridised to virulence genes were also incorporated; for *B. anthracis*, these initially included the *pag*, *lef*, *cap* and *vrr*A (for partial genotyping) genes. Hybridisation conditions were initially optimised to be run using 5×SSC, 0.1% SDS, 50 °C for 16 h. The detection limits of the microarray were determined under these conditions by titration of chromosomal DNA and unlabelled amplicons followed by hybridisation to determine the levels of sensitivity that could be obtained with the microarray. Two different amplification methodologies were also compared—specific-primer based PCR and random PCR (with the labelling stage incorporated). Higher sensitivity was obtained using specific PCR primers, however, since one of the desired outcomes of a microarray-based detection system was the high discrimination that it offered, random amplification and labelling was used as the amplification method of choice. The length of hybridisation was investigated using a time-course, and 1–2 h was found to give optimal and higher signals than 16 h incubation. These results indicate that microarray technology can be employed in a diagnostic environment and moreover, results may be obtained in a similar time-scale to a standard PCR reaction, but with the advantage that no a priori knowledge of the infectious agent is required for detection.

Keywords: Microarray; Multipathogen; Detection; *Bacillus anthracis*; Anthrax; Bioterrorism

1. Introduction

The requirement for multiplex detection of a wide range of potential biowarfare pathogens has never been greater. Considerable investment has been made to develop multiplex assays that can fulfil this function. DNA microarrays have received considerable attention due to the ability to simultaneously analyse the expression of a very large number of genes [20,25]. Recently, this huge potential has generated considerable interest for its utility as a diagnostic tool. Several workers have recently reported developmental microarrays to differentiate bacterial species using small-subunit (16S) ribosomal RNA gene sequences [14,17,23]. These approaches rely on the well-documented regions of high and low homology between the 16S rRNA genes from different species, which have received attention for their utility as phylogenetic tools [10,16,19]. In each case, single oligonucleotide targets are derived from different hypervariable regions of this gene. The availability of databases having large numbers of 16S rRNA gene sequences, make this particular gene attractive for this purpose. Other ribosomal RNA gene species could also potentially be used, however, the datasets for 5S and 23S rRNA genes are very much smaller than for 16S rRNA genes [11,12].

Such diagnostic microarrays have application in two main areas. First, the requirement for the parallel, multiplexed detection of a wide range of microbial strains, coupled with identification to the species or genus level is an

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application most suited to environmental microbiology [2]. Such requirements may also extend to quantification of microorganisms within a mixed population of bacteria and in complex milieu. The other major use for such microarrays is in clinical diagnostics. Here the requirements are somewhat different from those of environmental studies because the need is to detect single pathogenic species, often present in low numbers from normally sterile backgrounds or possibly from a background of commensal organisms [2,14].

Low-density microarrays have also recently been employed in the construction of a viral detection platform [22]. The absence of ubiquitous genes makes the construction of such arrays more complex than for bacterial pathogens. However, Wang et al. [22] describe an approach based on the design of between 6 and 10 oligonucleotide capture probes for each particular virus. The targets employed were designed using an in *silico* approach to maximise the selectivity of the capture probes and hence endow the highest discriminatory power to the array. This particular microarray contained approximately 1600 capture probes and has been evaluated using both purified nucleic acid and material directly extracted from clinical specimens.

Crucial to the successful construction of such identification microarrays is the design of the oligonucleotide probes within the microarray. These probes must be 'tuned' such that all have similar optimal melting temperatures (T_m) and the absence of significant secondary structure, which will limit their effectiveness. Several different approaches have been taken to overcome these differences [13–15] all of which involve additions to the hybridisation buffer to equalise the melting points of different oligonucleotides by AT base pair stabilisation. In our study, we have optimised the T_m values of the oligonucleotide probes at the design stage such that the hybridisation conditions should be compatible. We report here a pilot study in which probes for six pathogenic bacteria have been fully evaluated, with particular reference to the detection of *Bacillus anthracis*.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

All bacterial strains used were obtained from the culture collection of the Special Pathogens Reference Unit (HPA Porton Down). Bacterial strains (except *Mycobacterium tuberculosis* and *Neisseria lactamica*) were grown on Columbia blood agar plates at 37 °C. Unpurified DNA for hybridisation was extracted by emulsifying a fresh colony into 50 μl Brain Heart Infusion broth, heating at 37 °C for 1 min and then at 95 °C for 10 min. Cell debris was removed by centrifugation (13,000×*g*; 5 min; 4 °C). The supernatant containing bacterial DNA was used immediately. DNA from *M. tuberculosis* and *N. lactamica* were kindly provided by Drs Richard Vipond and Andrew Gorringe, respectively. Purified DNA from *B. anthracis* strains was prepared as described by Green et al. [7].

2.2. Design of oligonucleotide probes

Oligonucleotide probes were designed which would hybridise with 16S ribosomal RNA (16S rRNA) genes to provide preliminary detection of different bacterial pathogens. 16S rRNA gene sequence data was obtained from the EMBL/Genbank database and also from the comparative RNA web site (http://www.rna.icmb.utexas.edu) [4]. Probes that were capable of detecting DNA from *B. anthracis* were designed by aligning the 16S rRNA genes from a variety of *Bacillus* spp. (including *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus mycoides*) using the 'Pretty' program from the GCG package (www.accelrys.com) to derive a consensus sequence for each strain. Consensus sequences were similarly derived for other species of bacteria, (e.g. *Enterobacteriaceae*, *Pseudomonas*, *Mycobacterium*, *Streptococcus*, *Neisseria*), aligned using 'Pretty' and regions of high and low conservation determined. In the regions of low conservation (variable regions), probes were designed which would discriminate between bacterial species. A minimum of two probes were designed for each species based on the variable regions in the 5′ half of the 16S rRNA gene. Putative 50-mer oligonucleotide targets were evaluated in silico using the BLAST (Basic Local Alignment Search Tool) program of both the GCG package and NCBI databases. 50-mer sequences were accepted, rejected or modified on the basis of the BLAST search results, to ensure that targets with the highest possible selectivity were obtained. Virulence gene probes were designed using the ROSO online oligonucleotide design programme (http://pbil.univ-lyon1.fr/roso) [18] and were extensively evaluated in silico as above prior to synthesis. Finalised sequences were synthesised commercially, spotted and evaluated by hybridisation as described below. Probe sequences that were used in this study are shown in Table 1.

2.3. Microarray printing

Microarrays were printed using a BioRobotics Microgrid II spotter onto aminosilane-coated glass slides (UltraGAPS; Corning). Oligonucleotides were dissolved to a final concentration of 40 μM in sterile, nuclease-free water. Immediately prior to printing, oligonucleotides were mixed with an equal volume of DMSO. After printing, arrays were air-dried, baked at 80 °C for 3 h and stored dry at ambient temperature in the dark until required.

2.4. Nucleic acid amplification and labelling

Nucleic acid was amplified either by PCR using consensus 16S rRNA or other gene-specific primers, in a reaction which contained: 10×buffer (Promega Corp.); $MgCl_2$, 1 mM; primers, 400 nM; dNTP's, 100 μM each;