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A low density oligonucleotide microarray for the detection of viral and atypical bacterial respiratory pathogens

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A B S T R A C T

Acute respiratory tract infections are a major cause of morbidity and mortality worldwide and exert a considerable economic burden on healthcare systems. Acute respiratory tract infections of the upper and lower respiratory tract are caused by a wide variety of viral and bacterial pathogens, which require comprehensive laboratory investigations. Conventional serological and immunofluorescence-based diagnostic methods for acute respiratory tract infections lack sensitivity when compared to polymerase chain reaction (PCR)-based approaches and the development of new diagnostic methodologies is required, to provide accurate, sensitive and rapid diagnoses.

In the present study, a PCR-based low density oligonucleotide microarray was developed for the detection of 16 viral and two atypical bacterial pathogens. The performance of this DNA microarray-based analysis exhibited comparable sensitivities and specificities to multiplex real-time reverse transcription polymerase chain reactions (rtPCRs) confirming the potential diagnostic utility of the method. In contrast to routine multiplex PCR, the microarray incorporates an intrinsic redundancy as multiple and non-identical probes per target on the array allow direct intra-assay confirmation of positives. This study demonstrates that microarray technology provides a viable alternative to conventional serological-based approaches and multiplex PCR for pathogen identification in acute respiratory tract infections.

1. Introduction

Acute respiratory tract infections are the most common reason for general practitioner visits and acute admission to hospital during the winter season irrespective of age or gender (Elliot et al., 2008). In 2002, lower respiratory tract infections accounted for 3.9 million deaths worldwide; 6.9% of all deaths that year (WHO, 2004). Viral and bacterial co-infections are frequently observed using PCR-based investigation, which in many cases are not detected by current serological-based and direct antigen detection methods. Therefore, the development of new diagnostic methods is required to enable the detection of a broad range of pathogens. Indeed an accurate, sensitive and rapid differential diagnosis can influence patient management, reduce potentially inappropriate antibiotic use and aid infection control measures in institutional settings.

The current serological-based methods for the diagnosis of acute respiratory tract infections are limited in assay specificity and sensitivity, often resulting in underdiagnosis, e.g. antigen detection of the *Adenoviridae* (Arnold et al., 2008). However, viral antigen detection tests are used increasingly because of low demand on equipment and cost effectiveness (Grandien, 1996). In addition, virus isolation is slow, expensive and labour intensive; often requiring 1–5 days incubation to detect or confirm the absence of cytopathic effects. In addition, with decreasing resources fewer clinical laboratories have the appropriate facilities and expertise to undertake culture-based investigations (Koenig et al., 2001). Culture-based methods are also used for bacterial detection and are also hampered by slow turnaround times for reporting of results and insufficient sensitivity (Peters et al., 2004). Serological profiling is also used often, however, this can require more than two weeks for antibodies to develop and sequential samples, which are often not collected and are usually

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required to confirm the diagnosis (Nilsson et al., 2008; Wong et al., 2008).

Advances in molecular diagnostic methods have enabled rapid diagnosis of infectious disease (Murata, 2008). Molecular techniques offer several advantages over conventional serology-based methods, including speed, ease of standardisation, automation and high assay sensitivities and specificities (Dong et al., 2008). To date, DNA microarray technology has been used predominantly for gene expression profiling studies in basic research applications (Hegde et al., 2000). Clinical presentations of acute respiratory tract infections are diverse and non-specific; therefore an efficient laboratory assay that can detect a panel of common respiratory pathogens would be advantageous. In addition, large-scale screening may identify co-infections, which would not have been detected previously. Microarray-based analysis is a cost-effective approach that yields reproducible results and can allow replicate analyses in a single assay run. This is achieved by spotting of solid phase arrays with multiple copies of the same probe, alternative probes within the same target amplicon and/or mismatch probes allowing intra-assay confirmation of results that is not readily possible with multiplex rtPCR-based approaches.

A novel, non-fluorescent, low-cost, low density oligonucleotide microarray format has been developed which facilitates the use of DNA microarrays for clinical diagnostic purposes. The ArrayTube™ (AT) platform offers a fully automated system for clinical diagnosis. Several studies have used this platform for a variety of applications (Borel et al., 2008; Sachse et al., 2006, 2005). The AT system represents a cost-effective platform involving 2.4 mm × 2.4 mm glass biochips, integrated into the bottom of standard 1.5 ml plastic microtubes. The chip may contain a number of different probe types depending on the specific application and allows all steps of the hybridisation reaction to be conducted within the AT vessel, obviating the requirement for a separate hybridisation chamber or other additional laboratory equipment. The hybridised target is visualised by enzyme-catalysed precipitation and the microtubes are read using a simple low cost, transmission scanner. A previous study revealed that specific hybridisation to virus-specific oligonucleotide probes can be obtained from a single PCR amplifiable target copy (Ehricht et al., 2006).

The development of an oligonucleotide microarray-based system for the detection, differentiation and subtyping of 18 viral and bacterial respiratory pathogens simultaneously, comprising 16 viruses and two atypical bacteria is described. PCR-derived amplicons from external quality assessment panels and known positive samples were used as targets to establish probe sensitivity. A validation panel was assembled, comprised of throat swab specimens, collected from adults who presented with symptoms of respiratory disease to a tertiary care hospital during the 2007–2008 winter season, to evaluate the potential role of the microarray assay compared to multiplex rtPCRs.

2. Materials and methods

2.1. Oligonucleotide probe selection

Oligonucleotide probe genomic target regions are listed in Table 1. Oligonucleotide probes were evaluated using Primer3 (http://frodo.wi.mit.edu/). Probes on the array were between 18 and 35 nucleotide bases in length and were modifications of existing TaqMan assays. The most important parameter for modification of existing published TaqMan probes was that they would have similar T_m values so that hybridisation would be uniform for all denatured amplicons on the array. All probes have a T_m value close to the average of all probes and T_m values were calculated using Primer3 which uses the SantaLucia method for T_m calculation (SantaLucia, 1998). The 18 pathogens included Adenoviruses, Bocavirus, *Chlamydia* (*Chlamydophila*) *pneumoniae*, Coronaviruses types 229E, OC43, NL63, HKU1, Human metapneumovirus (hMPV) types A and B, Influenza A, Influenza B, Influenza C, *Mycoplasma pneumoniae*, Parainfluenza viruses 1–4, respiratory syncytial virus (RSV) types A and B and Rhinoviruses.

2.2. DNA microarray production

Oligonucleotide probes (Metabion, Martinsried, Germany), ~18–35 bp in length, were spotted in fourfold redundancy on glass arrays using a proprietary technology (ArrayTube, Clondiag Technologies, Jena, Germany).

2.3. Pathogen target labelling by end-point PCR

Biotinylation end-point PCR primer sequences and multiplex set-up are shown in Table 2. The OneStep RT-PCR kit (Qiagen, Crawley, UK) was used according to the manufacturing instructions with the modified addition of 0.15 mM dTTP and 0.2 mM dATP, dGTP, dCTP and 0.05 mM biotin-21-dUTP per reaction (Annecis Ltd., Lancaster, UK) for target labelling. PCR conditions were 1 cycle of 50 °C for 30 min, 1 cycle of 95 °C for 15 min, 50 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min and final extension for 1 cycle of 72 °C for 10 min on a PTC-200 thermocycler (MJ Research, Waltham, MA, USA).

2.4. DNA microarray hybridisation

The microarrays were washed in nuclease-free water (Promega, Madison, WI, USA) and hybridisation buffer [250 mM NaPOi, pH 7.2 (Camida Ltd., Tipperary, Ireland); 4.5% SDS; 1 mM EDTA, pH 8.0; 1× SSC] (Sigma, Dublin, Ireland) for 5 min each at 55 °C at 550 rpm in the Thermomixer comfort (Eppendorf AG, Hamburg, Germany). A 0.5 μl volume of each target solution in a final volume of 100 μl of hybridisation buffer was heated at 95 °C for 5 min and then chilled on ice. The biotinylated target reactions were hybridised to the microarrays at 55 °C for 1 h at 550 rpm. DNA arrays were washed in solutions of decreasing stringency [2× SSC, 0.01% Triton X; 2× SSC; 0.2× SSC] (Sigma, Dublin, Ireland) for 5 min each at 30 °C at 550 rpm. Blocking was performed using 2% BSA in 6XSSPE, 0.005% Triton X (Sigma, Dublin, Ireland) for 15 min at 30 °C at 550 rpm. A 1 in 5000 dilution of streptavidin-horseradish peroxidise (SA-HRP) conjugate (Pierce, Dublin, Ireland) was added for 15 min at 30 °C at 550 rpm. Washing was repeated in solutions of decreasing stringency [2× SSC, 0.01% Triton X; 2× SSC; 0.2× SSC] (Sigma, Dublin, Ireland) for 5 min each at 30 °C at 550 rpm to remove unbound SA-HRP conjugate. Visualisation of the hybridised targets was achieved by incubation with 100 μl tetramethyl bendzidine (TMB) (KPL Ltd., MD, USA) at 20 °C for 10 min.

2.5. DNA microarray data analysis

Hybridisation signals were measured at 20 °C after 10 min using the ATR03 AT DNA microarray transmission scanner (Clondiag Technologies, Jena, Germany). Quantitative staining values (QSVs) with local background correction were obtained for each probe spot via the Iconoclust software, version 2.3 (Clondiag Technologies, Jena, Germany). The criteria for assignment of hybridisation patterns were as follows:

Background-corrected signal intensities were given as NI = 1–*M*/BG, with NI being Normalised Intensity, *M* is Mean spot intensity, and BG local background intensity. Spot intensities were measured as light transmission, with *M* values ranging from 1 for complete transmission (background, weak spots) to 0 for complete absorption (dark spots). Normalised intensities ranged