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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

Table 1
Oligonucleotide probe sequence and genomic target region information.

Oligonucleotide probe name	Genomic region	Probe sequence	Oligonucleotide probe name	Genomic region80	Probe sequence
Adenovirus-01[a]	Hexon gene	AGACCCGGGCTCAGGTACTCC	Influenza B-01	NS gene	ATGGCCATCGGATCCTCAACTCACTCT
Adenovirus-05[a]	Hexon gene	GGCTGAAGTACGTCTCGGTGGC	Influenza B-02	NS gene	ATGGCCATCGGATCCTCAATTCACTCT
Adenovirus-06[a]	Hexon gene	CCCGGGCTCAGGTACTCCGAG	Influenza C	Matrix	CTCTTCCTTCTGATTTTTTCAAA
					GCAACTTCTAGTTTGAAAAAATCAGAAGGAAGAGA
Adenovirus-07[a]	Hexon gene	CAGGCTGAAGTACGTATCGGTGGC	Parainfluenzavirus1-01	HN	CCTATGACATCAACGACAACAGGAAATCATG
Adenovirus-09[a]	Hexon gene	TGAAGTAGGTGTCTGTGGCGCG	Parainfluenzavirus-2	HN	ACCTAAGTGATGGAATCAATCGCAAAAGC
Adenovirus-10[a]	Hexon gene	TGAAGTAGGTGTCTGTTGCACGGG	Parainfluenzavirus-3-01	HN	TGGATGTTCAAGACCTCCATACCCGA
Bocavirus[b]	Non-capsid protein 1	AGCTCAGGGAATATGAAAGACAAGCATCG	Parainfluenzavirus-3-02	HN	TGGATGTTCAAGACCTCCATATCCGAG
Enterovirus-01[c]	5′ NCR	CCCAAAGCCACGGGACGCTAG	Parainfluenzavirus-3-03	HN	TGGGTGTTCAAGACCTCCATATCCGA
Enterovirus-2[c]	5′ NCR	GCCCAAAGCCACAGGACGCTAG	Parainfluenza-4	Fusion	CCMATCACAAGCTCAGAAATYCAAAGTCGT
hMPV-A[d]	Fusion	CAACATTTAGAAACCTTCTGTTGAATTGACTGAAG	Respiratory syncytial virus A[f]	NP	CACCATCCAACGGAGCACAGGAGAT
hMPV-B[d]	Fusion	CTGCCGCACAACATTTAGGAATCTTCTG	Respiratory syncytial virus B[f]	NP	TGCTATGTCCAGGTTAGGAAGGGAAGAC
Human coronavirus 229E[e]	NP	CCCTGACGACCACGTTGTGGTTC	Rhinovirus-01[g]	5′	TCCTCCGGCCCCTGAATGCG
			Rhinovirus-02[g]	NCR	TCCTCCGGCCCCTGAATGTG
Human coronavirus NL63[e]	NP	ATTGCCAAGGCTCCTAAACGTACAGGT	Rhinovirus-03[g]	5′	GGACAGGGTGTGAAGAGCCGC
			Rhinovirus-04[g]	NCR	GGACAGGGTGCGAAGAGCCG
Human coronavirus OC43[e]	NP	TTCCGCCTGGCACGGTACTCC	*Chlamydophila pneumoniae*-0[h]	omp A	CTACTGGAACAAAGTCTGCGACCAT
Human coronavirus HKU1-01[e]	Replicase 1b	TGTGTGGCGGTTGCTATTATGTTAAGCCT	*Chlamydophila pneumoniae*-02[h]	omp A	AGCTACTGGAACAAAGTCTGCGACCA
Human coronavirus HKU1-02[e]	Replicase 1b	TGAAATAGTTATGTGTGGCGGTTGCTATTATGT	*Mycoplasma pneumoniae*-01[h]	p1 adhesion	TCGGCCCCGATCGCCCTC
Influenza A	Matrix	ACGCTCACCGTGCCCAGTG	*Mycoplasma pneumoniae*-02[h]	p1 adhesion	CCAAGCAGGGCTTTCAAAAGGAAGCT
			K-*ras*-01	Exon 1	TGCCTACGCCACAAGCTCCAACTAC

Probe sequences used for the amplification and labelling of the clinical samples are listed above. All probe sequences were designed in this study or modified from published sequences. NP, nucleoprotein; HN, haemagglutinin-neuraminidase; NCR, non-coding region; NS, non-structural.

[a] Heim et al. (2003).
[b] Neske et al. (2007).
[c] Corless et al. (2002).
[d] Kuypers et al. (2005).
[e] Dare et al. (2007).
[f] Gunson et al. (2005).
[g] Lu et al. (2008).
[h] Gullsby et al. (2008).

Table 2
Multiplex biotinylation end-point PCR and rtPCR set-up.

Multiplex PCR	Virus	Forward primer (5′–3′)	Reverser primer (5′–3′)
Tube 1	Influenza A	AAGACAAGACCAATYCTGTCACCTCT	TCTACGYTGCAGTCCYCGCT
	Influenza B	ATGATCTTACAGTGGAGGATGAAGAA	CGAATTGGCTTTGRATGTCCTT
	Influenza C	GGCAAGCGACATGCTGAAYA	TCCAGCTGCYTTCATTTGCTTT
Tube 2	Parainfluenza-2	ATGAAAACCATTTACCTAAGTGATGGA	CCTCCYGGTATRGCAGTGACTGAAC
	Parainfluenza-3	CCAGGGATATAYTAYAAAGGCAAAA	CCGGGRCACCCAGTTGTG
	Parainfluenza-4	CAGAYAACATCAATCGCCTTACAAA	AGCAAYTGTACCTATGACTGCC
Tube 3	hMPV-A	GCYGTYAGCTTCAGTCAATTCAA	TCCAGCATTGTCTGAAAATTGC
	hMPV-B	GCYGTYAGCTTCAGTCAATTCAA	GTTATCCCTGCATTGTCTGAAAACT
	Parainfluenza-1	GTGATTTAAACCCGGTAATTTCTCA	CCTTGTTCCTGCAGCTATTACAGA
	Mycoplasma pneumoniae	CAGACGGTCGCGGATAACG	AACCAGGTGAGGTTGCCAATG
Tube 4	Coronavirus 229E	CAGTCAAATGGGCTGATGCA	AAAGGGCTATAAAGAGAATAAGGTATTCT
	Coronavirus OC43	CGATGAGGCTATTCCGACTAGGT	CCTTCCTGAGCCTTCAATATAGTAACC
	Coronavirus NL63	ACGTACTTCTATTATGAAGCATGATATTAA	AGCAGATCTAATGTTATACTTAAAACTACG
	Coronavirus HKU1	TCGCCTTGCGAATGAATGTGC	TTGCATCACCACTGCTAGTACCAC
Tube 5	RSV-A	AGATCAACTTCTGTCATCCAGCAA	TTCTGCACATCATAATTAGGAG
	RSV-B	AAGATGCAAATCATAAATTCACAGGA	TGATATCCAGCATCTTTAAGTA
	Rhinovirus	CPXGCCZGCGTGGC	GAAACACGGACACCCAAAGTA
Tube 6	Adenovirus	GCCACGGTGGGGTTTCTAAACTT	GCCCCAGTGGTCTTACATGCACATC
	Bocavirus	GCACAGCCACGTGACGAA	TGGACTCCCTTTTCTTTTGTAGGA
	K-ras	GCCTGCTGAAAATGACTGAATATAAAC	TGATTCTGAATTAGCTGTATCGTCAAG
	Chlamydophila pneumoniae	AGGCGTTGCTTTCCCCTTGCC	GATAGAGAGGCTCCTACTTGCCAT

Primer sequences and the multiplex set-up for the biotinylation end-point PCR used for labelling of the targets is indicated above. HMPV, human metapneumovirus; RSV, respiratory syncytial virus.

between 0 and 1. QSVs were reported as the median of all four replicate signals for each probe.

2.6. Multiplex rtPCR analysis

Real-time PCR primer sequences and multiplex set-up are shown in Table 2 and carried out using the Invitrogen Superscript III one-step qPCR system according to the manufacturer's instructions (Invitrogen, Paisley, UK). For an internal control for the extraction efficiency, sample addition and the absence of PCR inhibitors in each specimen we amplified a human gene (k-ras) from all throat swabs.

2.7. PCR product preparation

Biotinylated PCR products were purified using the High Pure PCR product Purification Kit according to manufacturers instructions

Table 3
Oligonucleotide microarray probe specificity.

Oligonucleotide probe	Probe	BG (QSV)	Signal-to-noise ratio (*x*factor)	Negative control BG (QSV)	Staining control (QSV)
Influenza A	0.72		*x*198		
Influenza B[a]	0.88, 0.92	0.00363	*x*242, 253	0.0020	0.84
Influenza C[a]	nt		nt		
Parainfluenza-2	0.86		*x*500		
Parainfluenza-3[b]	0.85, 0.81, 0.73	0.00172	*x*494, *x*471, *x*424	0.0007	0.87
Parainfluenza-4	nt		–		
hMPV-A	0.69		*x*191		
hMPV-B	0.62		*x*171		
Parainfluenza-1	0.90		*x*249	0.0001	0.85
Mycoplasma pneumoniae[a]	0.79, 0.68	0.00361	*x*219, *x*188		
Coronavirus 229E	0.95		*x*137		
Coronavirus OC43	0.55	0.00692	*x*79.8	0.0070	0.92
Coronavirus NL63	nt		nt		
Coronavirus HKU1[a]	nt		nt		
RSV-A	0.88		*x*518		
RSV-B	0.90	0.0017	*x*529	0.0004	0.90
Rhinovirus[c]	0.76, 0.75, 0.76, 0.73		*x*447, *x*441, *x*447, *x*429		
Adenovirus-1,5,6,7,9,10[d]	0.87, 0.83		*x*116, *x*111		
Bocavirus	0.89		*x*119		
K-ras	0.83	0.0075	*x*111	0.0016	0.90
Chlamydophila pneumoniae[a]	0.73, 0.78		*x*107		
			*x*97, *x*104		

Multiplex end-point PCR reactions were set up as above, using known positive samples for the viral and bacterial pathogens. Purified biotinylated PCR products were hybridised to the diagnostic DNA microarray. Signal intensities for all probes along with signal intensities of the internal staining controls are listed above. nt, not tested; QSV, quantitative staining control.

[a] 2 probes per virus.
[b] 3 probes per virus.
[c] 4 probes per virus.
[d] 5 probes per virus.

and eluted in 50 μl (Roche Applied Science, Mannheim, Germany). DNA concentration was determined using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, DE, USA) and all DNA target solutions normalised to 50 ng/μl. A series of 10-fold dilutions (5000–5 pg/μl) were prepared and subjected to DNA microarray analysis.

2.8. Specimen collection and nucleic acid extraction

Throat swabs were collected from adult patients with respiratory symptoms presenting at an emergency department in a tertiary care hospital in Dublin during the 2007–2008 winter season. Swabs were stored in viral transport medium at −80 °C before processing. DNA and RNA were extracted from respiratory specimens using the QIAamp Virus BioRobot MDx kit on the BioRobot MDx workstation, according to the manufacturer's instructions (Qiagen, Crawley, UK).

3. Results

We have developed and validated a diagnostic PCR-based low density oligonucleotide microarray for the detection of 16 viral and 2 bacterial respiratory pathogens. In initial studies the oligonucleotide microarray probe specificity was assessed using known positive specimens (i.e. either external quality assessment samples or clinical material confirmed by rtPCR and/or sequencing) and then analytical sensitivity was evaluated by serial dilution of biotinylated target amplicons and hybridisation to the array. Table 3 lists the QSVs for each of the individual pathogen probes, the mean internal staining controls, the background signal for the assay, the background signal for an equivalent 'negative' assay as well as the factor by which the signal is greater than the background level. Excluding Coronavirus OC43 which had a QSV of 0.55, the remaining probes exhibited QSVs between 0.62 and 0.95, with the median QSV being 0.77. All internal staining controls had a QSV of ≥0.84. The minimum factor by which the probe signal was greater than the background was a factor of 80 (coronavirus OC43) and the maximum was 529 (RSV subtype B). Certain pathogens that had multiple probes of differing sequences showed a greater specificity for a single probe sequence, e.g. Parainfluenza-3; 0.85, 0.81 and 0.73 for all 3 probes respectively. The analytical sensitivity of the microarray was evaluated using a serial dilution of individual targets of known concentration. The majority of the probes exhibited a reduced sensitivity below 50 pg of target DNA/cDNA and a complete loss of signal below 5 pg of target DNA for all probes (data not shown). Some signal was observed at 5 pg of target DNA for the probes RSV subtype A, Rhinovirus-03 and -04, with Rhinovirus-03 still yielding a QSV of approximately 0.63.

A cohort of clinical specimens were tested by both multiplex rtPCR and DNA microarray analysis ($n = 50$) to assess the performance of the DNA microarray for clinical investigation. The results are summarised in Table 4 and representative images of five typical microarray experiments alongside barplots of hybridisation signals and rtPCR amplification plots are represented in Fig. 1. Experimental images shown are positive for Parainfluenza-3, *M. pneumoniae*, Influenza A, Influenza B as well as an RSV-A/Rhinovirus co-infection. A total of 20/50 and 25/50 clinical specimens tested positive for a respiratory pathogen(s) using DNA microarray analy-

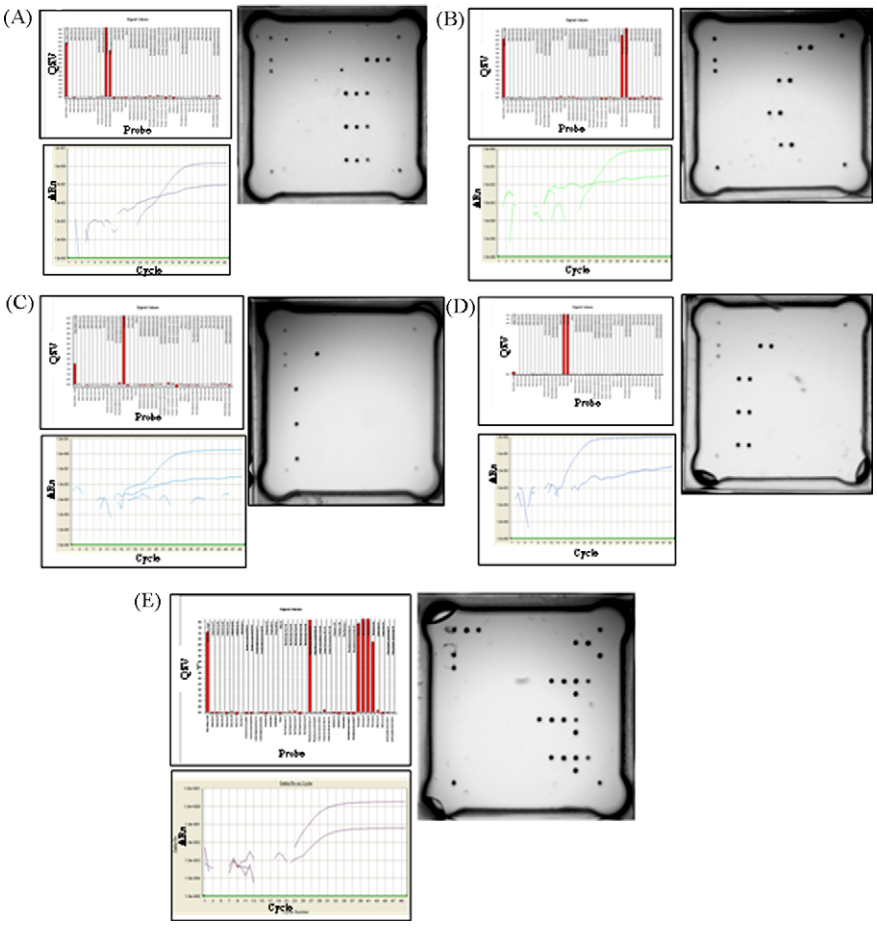

Fig. 1. Examination of clinical samples using spotted DNA microarray analysis. Microarray images, barplots of hybridisation signals and rtPCR amplification curves are shown for five typical samples. Positive hybridisation signals include (A) Parainfluenza-3, (B) *M. pneumoniae*, (C) Influenza A, (D) Influenza B and (E) an RSV-A/Rhinovirus co-infection.

Table 4
DNA microarray analysis of clinical specimens.

Virus	AT test	rtPCR	
	Positive	Positive	C_t values
Influenza A	4	4	26.69, 29.41, 34.63, 35.95
Influenza B	3	3	22.19, 26.76, 36.91
Influenza C	0	3	45.57[a], 45.74[a], 45.94[a]
Parainfluenza-3	0	1	47.16[a]
Parainfluenza-4	0	1	40.70[a]
hMPV	4	4	32.61, 32.65, 33.91, 35.07
M. pneumoniae	2	2	36.81, 38.42
Coronavirus OC43	0	1	48.15[a]
Coronavirus NL63	0	1	40.02[a]
Coronavirus HKU1	0	1	48.79[a]
RSV A	5	5	19.76, 24.93, 27.41, 27.88, 30.36
Rhinovirus	2	4	29.80, 32.03, 46.65[a], 47.82[a]
No. of co-infections	5		
Total positive	20/25	25/25	20/50

Results for the DNA microarray-based analyses of clinical specimens are listed above, along with rtPCR results.
[a] C_t values >40 are equivocal results and are unlikely to be clinically relevant.

sis and rtPCR respectively. Positive results of the 50 clinical samples were: four Influenza A (5.6%), three Influenza B (4.2%), three Influenza C (4.2%), one parainfluenza-3 (1.4%), one parainfluenza-4 (1.4%), four human metapneumovirus (5.6%), two *M. pneumoniae* (2.8%), one Coronavirus OC43 (1.4%), one Coronavirus NL63 (1.4%), Coronavirus HKU1 (1.4%), five RSV A (7%) and four Rhinovirus (5.6%). Table 4 illustrates the clinical sensitivity of the DNA microarray-based assay. Real-time PCR C_t values are listed for all positive specimens alongside DNA microarray results. All six targets not detected by DNA microarray analysis were weakly positive via rtPCR analysis, and had C_t values >40 with 4/6 having C_t values of $\geq$45. For all of the clinical samples tested, the QSVs were $\geq$0.60 and had a background of $\leq$0.10.

We next attempted to determine the clinical relevance of the findings from the rtPCR and microarray analysis. Only the specimens positive by DNA microarray analysis and with C_t values <40 in rtPCR were included in this analysis (n = 17) (Table 5). Among these 17 positive test results two patients had a concomitant bacterial respiratory tract infections (*M. pneumoniae*). Antibiotic treatment had been implemented in 13 of the remaining 15 patients and 8 patients had been admitted to hospital. Two patients with an admitting diagnosis of pneumonia and an infiltrate on chest X-ray were positive for *M. pneumoniae* and their antibiotic treatment was therefore considered appropriate. As the majority of other patients neither had an elevated peripheral white cell count nor significant chest X-ray infiltrates, it seems plausible that if a positive viral test result would have been available for clinicians less antibiotics would have been prescribed.

4. Discussion

Current diagnostic methodologies for the detection of respiratory pathogens are cell culture isolation, antigen/antibody detection and immunofluorescence and conventional/rtPCR. However, not all of these methodologies have the sensitivity, specificity and turnaround time required to have an impact on clinical management. Variations in sample quality may impact on the viability of virus for culture and also the number of available cells for immunofluorescence investigation, whereas PCR is less impacted by poor sample quality. Therefore, molecular techniques are required for an accurate and rapid diagnosis. PCR tests are considered very sensitive, and our data has shown that the combined PCR and DNA microarray-based assay was capable of detecting and identifying 18 pathogens from throat swab specimens in a single run. Our study also shows that the DNA microarray assay for respiratory pathogens has: (i) the sensitivity required for the testing of clinical samples and (ii) the high specificity and capacity to achieve identification and differentiation of a pathogen(s) in a single test in a single working day.

Importantly, the redundant spotting of probes and multiple non-identical probes per target on the array allows a direct intra-assay confirmation of positives. This ability to perform replicate analyses in a single assay run provides a major advantage over real-time technologies that are limited by the number of available dyes and the current detection technology platforms. In addition, precipitation staining utilised in the microarray approach described herein does not have problems with respect to signal stability, quenching effects and cross-talk between detectors which can be encountered with rtPCR. Most importantly, microarrays have a significantly greater multiplexing capability than rtPCR as presently a maximum of five targets can be analysed per well which in best practice would employ one filter for the internal control leaving only four filters to detect pathogens per reaction. In addition, there is increasing concern regarding the accuracy of the multiple formats compared to the amplification in a simplex reaction.

The PCR-based DNA microarray assay failed to detect a number of samples that were reported as weak positives by rtPCR;

Table 5
Clinical details of patients with positive throat swab results.

	Age	Sex	Viral isolate	WCC	Temperature	CXR infiltrate	Diagnosis	Admitted	Antibiotic treatment
1.	61	M	RSV/Rhino	10.7	36.4	No	Inf. Exac.	Yes	Yes
2.	97	F	hMPV	9.9	37	No	LRTI	Yes	Yes
3.	76	F	RSV	13.3	38.7	No	LRTI	Yes	Yes
4.	85	M	Flu B	2.9	36.3	No	LRTI	Yes	Yes
5.	88	F	hMPV	17.3	38.1	Yes	LRTI	Yes	Yes
6.	46	F	hMPV	–	37.4	No	RTI	No	Yes
7.	84	M	hMPV	6.5	36.5	Yes	RTI	Yes	Yes
8.	65	F	Flu A	2.8	38.1	No	RTI	No	Yes
9.	75	F	*M. pneum*	8.8	–	Yes	LRTI	Yes	Yes
10.	67	F	RSV/Rhino	6.9	36	No	–	–	–
11.	16	F	Flu A	6.5	40	No	Flu/tonsillitis	No	Yes
12.	18	M	*M. pneum*	5.9	36.3	Yes	LRTI	No	Yes
13.	23	M	Flu B	2.4	38.5	No	Viral illness	No	No
14.	77	M	RSV	17.2	38.5	Yes	LRTI	Yes	Yes
15.	36	F	Flu A	5	38.2	No	Inf. Exac.	Yes	Yes
16.	25	M	Flu A	6.4	38.6	No	Viral RTI	No	No
17.	21	F	Flu B	13.2	37.7	Yes	Viral RTI	No	Yes

Clinical details of the 17/50 patients that had a positive test result are shown above. Two viral co-infections (RSV/Rhino) and two bacterial infections (*M. pneum*) were identified. *Abbreviations*: RSV (respiratory syncytial virus), Rhino (Rhinovirus), hMPV (human Metapneumovirus), Flu B (Influenza B), Flu A (Influenza A), *M. pneum* (*Mycoplasma pneumoniae*), Inf. Exac. (infective exacerbation of chronic obstructive pulmonary), LRTI (lower respiratory tract infection), and RTI (respiratory tract infection).

however these positives with C_t values >40 are of dubious clinical significance. This technology allows for differential diagnoses and an advantage of the microarray is that it provides more clinically relevant information. This technology will inevitably impact on the management strategy of respiratory infections and potentially could be employed to limit outbreaks in institutions as well as to decrease empiric antibiotic usage where the pathogen responsible can be shown to be viral. The ability to attach large numbers of probes on each solid phase and the comparatively low cost per pathogen tested of such an oligonucleotide microarray-based assay compared to multiplex rtPCR suggests it may be a potential alternative for clinical testing. Furthermore, the increased capacity of such testing will broaden the approach to diagnosis and patient management. Timely molecular test results can contribute to good antimicrobial stewardship and help prevent secondary complications associated with antibiotic treatment, such as intravenous line infections or potential toxic/allergic side effects. Of the 7 patients with an Influenza A or B positive test result, two had been admitted to general medical wards. The admission of influenza patients into open wards poses a significant infection control hazard as this could potentially result in influenza outbreaks. Molecular testing of respiratory specimens could help initiate appropriate isolation and infection control measures to protect other patients.

To date, high-density spotted DNA microarrays have almost exclusively been used for research purposes for a broad spectrum of applications (Dankbar et al., 2007; Gruden et al., 2008; Scaria et al., 2008; Sidders et al., 2007). However, this technology is also being employed in the clinical laboratory for diagnosis of viral pathogens in clinical specimens and can potentially become a frontline screening assay supplanting quantitative PCR in the management of herpesvirus and adenoviral infections in the immunocompromised patient population (Muller et al., 2009). While high-density *in situ* synthesised DNA microarrays can offer a greater analytical sensitivity and can accommodate a greater number of probes for a more advanced approach to analysis, e.g. resequencing microarrays (Lin et al., 2007) and genotyping (Lodes et al., 2007), low density DNA microarray-based analysis is a cost-effective assay concept. In the future, combining microarray hybridisation with high-throughput, automated DNA extraction and amplification will have a significant impact on high-throughout analysis. Regarding the role of spotted DNA microarrays in diagnostics, we have shown that a suitable low density platform is available for the development of diagnostic assays. Though less sensitive, this may paradoxically be of benefit in the identification of clinically significant levels of pathogen. The cost and ease of automation incorporating liquid handling and multiplexing capability makes this platform an attractive alternative technique for the standardisation purposes required in clinical diagnostics. This is specifically relevant when current commercial assays do not meet the test algorithms for a particular clinical laboratory and this may have an impact on the approach to specimen testing in the clinical laboratory and hospital setting. This will inevitably aid the detection, monitoring and therapeutic treatment of disease. DNA microarray-based analysis may thus be a potential alternative for clinical testing, providing more information on pathogens allowing better assessment of the viral aetiopathogenic agent(s) responsible for respiratory illness in the case viral and bacterial of co-infections.

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