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Development of a PCR probe test for identifying *Pseudomonas aeruginosa* and *Pseudomonas (Burkholderia) cepacia*

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Abstract

***Aims*—To develop a system of species specific polymerase chain reaction (PCR) and DNA hybridisation based on 16s ribosomal RNA sequences for the identification of *Pseudomonas aeruginosa* and *Pseudomonas (Burkholderia) cepacia* in sputum from children with cystic fibrosis.**

***Methods*—Most of the 16s rRNA sequences from strains of *Ps aeruginosa*, *Ps (Burkholderia) cepacia*, and *Ps putida* were determined. PCR primers and DNA probes were synthesised from suitable sequences and then evaluated on bacterial cultures and sputum samples.**

***Results*—About 1000 bases of sequence was obtained from strains of *Ps aeruginosa*, *Ps (Burkholderia) cepacia*, and *Ps putida*. PCR of bacterial cultures was species specific, but PCR on sputum resulted in some non-specific amplification products. The subsequent hybridisation reaction was species specifc.**

***Conclusion*—A species specific system of PCR and DNA hybridisation based on 16s rRNA sequences is applicable in clinical practice, and may aid the early diagnosis of respiratory tract infection with small numbers of *Ps aeruginosa* and *Ps (Burkholderia) cepacia* in patients with cystic fibrosis.**

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In patients with cystic fibrosis chronic colonisation of the respiratory tract by *Pseudomonas aeruginosa* is followed by a slow decline in their respiratory function.[1] Antibiotic treatment is associated with a temporary clinical improvement but does not eradicate the organism. It is reasonable to believe that very early detection of *Ps aeruginosa* infection would increase the chances of successful eradication. The presence of increased titres of serum IgA antibodies specific to *Ps aeruginosa* before the organism can be isolated from the sputum using conventional microbiological techniques[2] suggests that initial infection may be with small numbers of organisms. Chronic infection with *Ps aeruginosa*, however, can occur without an antibody response, and, conversely, the presence of serum antibodies does not necessarily implicate the lower respiratory tract as the site of infection. To produce a sensitive means of identifying infection with small numbers of *Ps aeruginosa* in the respiratory tract we have developed a system of species specific polymerase chain reaction (PCR) and DNA hybridisation which can be applied directly to sputum samples.

The incidence of infection with *Pseudomonas (Burkholderia) cepacia* is increasing in patients with cystic fibrosis. The prevalence in the United Kingdom is about 7%[3,4] but in some North American centres it has been reported to be as high as 20%.[5] *Ps (Burkholderia) cepacia* infection presents a major clinical problem both because its acquisition may be associated with a rapid deterioration in the patient's condition and because it is multiply drug resistant.[5–7] *Ps (Burkholderia) cepacia* tends to grow slowly and with a variable colony morphology on media commonly used for routine sputum culture, and it can therefore be obscured by overgrowth of *Ps aeruginosa* or confused with other respiratory pathogens, such as *Staphylococcus aureus*, which have a similar morphological appearance.[8,9] Species specific PCR and DNA hybridisation would make it possible to detect and distinguish between infection with either *Ps aeruginosa* or *Ps (Burkholderia) cepacia* directly in sputum.

The nucleotide sequence of 16s ribosomal RNA (16s rRNA) has a unique structure with conserved regions common to all bacteria, as well as highly variable regions which are species specific[10,11] and provide an ideal basis for the synthesis of species specific oligonucleotide probes.[12] rRNA probes have proved useful in diagnosing infections with organisms that are difficult to culture.[13] rRNA probes, however, are not especially sensitive as they only detect about 10^3 cells/ml[14] even though there are about 10 000 copies of rRNA per cell.[15] This lack of sensitivity can be overcome by using PCR to amplify the amount of DNA and thereby increase the sensitivity of any subsequent hybridisations. Hybridisation of a PCR product with a ^{32}P-labelled oligonucleotide can increase sensitivity by a factor of 100 when compared with visualisation of a PCR band on agarose gel electrophoresis. Previous studies have shown that combining the amplification procedure with a slot blot hybridisation resulted in a detection limit of fewer than 10 mycobacterial cells in a sample.[12,16] Other approaches have used PCR primers from conserved regions of the 16s rRNA to amplify the entire gene for 16s rRNA (16s rDNA) from all bacteria.[17]

We describe here the use of both the

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conserved and the variable regions of the 16s rRNA from *Ps aeruginosa* and *Ps (Burkholderia) cepacia* to develop a system of species specific PCR and oligonucleotide probe hybridisation targeted at the 16s rDNA. This species specific system was then applied directly to clinical specimens to identify infection with either *Ps aeruginosa* or *Ps (Burkholderia) cepacia.*

Methods

The type strains *Ps aeruginosa* NCTC 10332, *Ps aeruginosa* NCTC 10662, *Ps (Burkholderia) cepacia* NCTC 10661, and *Ps (Burkholderia) cepacia* NCTC 10744 were obtained from the National Type Culture Collection (Colindale, London). Two other strains of *Ps aeruginosa* (PA 01 and PA 01161) were used as well as one strain of *Ps putida* (P2440).

EXTRACTION OF rRNA

Cells were grown overnight in 100 ml of L-broth at 37°C and then centrifuged at 5000 × *g* at 4°C for 15 minutes. The resulting pellet was resuspended in 1 ml of TE buffer (pH 8·0) (TE buffer is 10 mM TRIS-HCl, 1 mM EDTA, pH 8·0). Lysis buffer[18] (5M guanidium thiocyanate, 10 mM EDTA, 50 mM TRIS-HCl (pH 8·0), 0·5% sarkosyl, 8% mercaptoethanol) was added to give a final volume of 10 ml and the mixture was then vortexed and left at room temperature for 10 minutes to complete cell lysis.

Two chloroform extractions were performed. Nucleic acids in the aqueous phase were precipitated on ice for 15 minutes with an equal volume of isopropanol and 2·5M ammonium acetate. After centrifugation at 6000 × *g* at 4°C for 20 minutes, the pellet was dried and then resuspended in 700 μl of TRIS-HCl (pH 7·6). DNase 1 (5 μl) and 5 μl of 1M magnesium–chloride were added and incubated at 37°C for 15 minutes.

One phenol and two chloroform extractions were performed. The RNA in the aqueous phase was precipitated on ice for 15 minutes with an equal volume of isopropanol and 2·5M ammonium acetate. After centrifugation for 5 minutes at 8000 × *g* the pellet was dried in vacuo and then redissolved in water at a concentration of 3 mg/ml and stored at −20°C.

SEQUENCING REACTION

The sequencing protocol was a modification of the dideoxynucleotide terminated chain elongation method.[19] The primers targeted at the conserved regions of the 16s rRNA were those which have been described before.[19 20]

Oligodeoxynucleotide probes and PCR primers were synthesised on an Applied Biosystems 380B synthesiser using phosphoramidite chemistry. No further purification was performed.

AMPLIFICATION PROCEDURE

A single bacterial colony was suspended in 1 ml of phosphate buffered saline (pH 7·0) and centrifuged at 8000 × *g* for 10 minutes. The resulting pellet was resuspended in 20 μl of lysis buffer (1 × PCR buffer, 0·5% Tween 20, 200 μg/ml proteinase K). The mixture was incubated at 55°C for 1 hour and then heated to 95°C for 10 minutes to inactivate the proteinase K. This reaction (10 μl) was used for PCR without further purification. This was mixed with 9 μl of 10 × PCR buffer (200 mM TRIS-HCl (pH 8·3), 20 mM $MgCl_2$, 250 mM KCl, 0·5% Tween 20, 1 mg/ml nuclease free bovine serum albumin), PCR primers (final concentration 0·25 μmol each), deoxynucleoside triphosphates (final concentration 200 μmol each), and 2·5 units *Taq* polymerase (New Biologicals Limited, Hatfield, UK) per 100 μl reaction. All the reagents except the cell lysis product and the *Taq* polymerase were mixed together and exposed to an ultraviolet transilluminator for 15 minutes to destroy any contaminating DNA.[21] The cell lysis product was then added and cycling started. The mixture was initially denatured at 96°C for 5 minutes and then the *Taq* polymerase was added. Twenty-five cycles were performed as follows: denature 96°C for 15 seconds; anneal 52°C for 30 seconds; extend 70°C for 1·5 minutes. A final extension of 5 minutes at 70°C was performed. Following PCR, 10 μl of the product was electrophoresed on a 1% agarose gel stained with ethidium bromide and viewed on an ultraviolet transilluminator.

DOT BLOT HYBRIDISATIONS

Species specific oligonucleotide probes were labelled at the 5′ ends with [^{32}P]ATP using T4 polynucleotide kinase (BRL).[22] The PCR product (10 μl) was blotted on to a nylon membrane (Hybond-N+, Amersham) using a slot blotter (Biorad). The DNA was denatured and alkali fixed according to the manufacturers' instructions. The membrane was then prehybridised for 1 hour at 42°C in 6 × SSC (20 × SSC is 3M sodium chloride and 0·3M sodium citrate), 5 × Denhardt's solution (100 × Denhardt's solution contains 2% Ficoll, 2% polyvinyl pyrrolidone, and 2% bovine serum albumin), 0·5% sodium dodecyl sulphate (SDS), and 100 μg/ml salmon sperm DNA. Labelled probe with an activity of 10^8-10^9 cpm/μg was added to the reaction mixture and incubated with shaking at 42°C for 4 hours. The membrane was washed twice in 2 × SSC/0·1% SDS for 20 minutes each at 42°C and then in 0·1 × SSC/0·1% SDS for 20 minutes also at 42°C. The slot blot was autoradiographed on X-Omat S film (Kodak) at −70°C for 24 hours.

COLONY BLOT HYBRIDISATIONS

These were performed on laboratory cultures of *Ps aeruginosa, Ps (Burkholderia) cepacia,* and *Ps putida.* The organisms were grown on L-agar at 37°C for 6 hours and then transferred on to a nylon membrane (Gene Screen; Dupont, NEN Research Products). The membrane was treated with sodium hydroxide and then neutralised according to the manufacturer's instructions, and the

DNA fixed by drying at room temperature. Hybridisation was performed as described above.

CLINICAL SAMPLES

Sputum samples from patients with a range of respiratory disorders, including cystic fibrosis, were collected directly from the Public Health Laboratory (PHLS) at Leicester Royal Infirmary. Sputum (0·5 ml) was removed into a separate container before the sample underwent any processing. All the samples were screened by the PHLS for *Ps aeruginosa* and *Ps (Burkholderia) cepacia* using selective media.[8] The sputum specimens for PCR were solubilised by adding an equal volume of dithiothreitol 100 μg/ml in sodium phosphate buffer (pH 7·0). This mixture (100 μl) was centrifuged for 5 minutes, the resulting pellet resuspended in 200 μl of cell lysis buffer, and lysed as described above. This cell lysis product (20 μl) was used for each PCR reaction without further purification. Dot blot hybridisation of sputum PCR products was performed as described above.

Results

SEQUENCE ANALYSIS

About 1000 bases of sequence were obtained for the strains of *Ps aeruginosa*, *Ps (Burkholderia) cepacia*, and *Ps putida*. These sequences were aligned with published 16s rRNA sequences for *Ps aeruginosa*[23] and *Escherichia coli*, allowing the identification of variable regions interspersed among conserved regions. At one site (640) the sequence of *Ps aeruginosa* differed from that of *Ps (Burkholderia) cepacia* by 10 bases (table 1). A computer search of Gen Bank and the European data base (European Molecular Biology Laboratory) showed that the 17 base segment containing this variable region did not match any previously reported rRNA sequence. Oligonucleotide probes specific to either *Ps aeruginosa* (PA 640) or *Ps (Burkholderia) cepacia* (PC 640) were synthesised to complement this variable sequence (table 2). Two other variable sites on the 16s rRNA sequences were chosen for the synthesis of complementary PCR primers specific for either *Ps aeruginosa* or *Ps (Burkholderia) cepacia*. Primers PA 1250 and PC 1250 were 17 bases long with three bases difference between *Ps aeruginosa* and *Ps (Burkholderia) cepacia* (table 3), whereas primers PA 480 and PC 480 had four out of 17 bases which differed (table 4). The PCR primers were matched for G+C content and dissociation temperature.

AMPLIFICATION

Initially amplification was performed using one "universal" primer and one "species specific" primer. The universal primer (P536: 5'CAGCA GCCGC GGTAA TAC3') was synthesised from a sequencing primer complementary to a universal region of the 16s rRNA and was therefore appropriate for the amplification of 16s rRNA from all bacteria.[19] The second primer was 714 bases downstream from the first primer and was specific for either *Ps aeruginosa* or *Ps (Burkholderia) cepacia* as described above (PA 1250 and PC 1250). PCR was also subsequently performed using paired species specific primers (PA 480 and PA 1250, PC 480 and PC 1250) to amplify a segment of 16s rDNA 770 bases long.

When the PCR products from cultures of *Ps aeruginosa*, *Ps (Burkholderia) cepacia*, and *Ps putida* were visualised on agarose gels, there were no amplification products visible when *Ps (Burkholderia) cepacia* and *Ps putida* were amplified with the *Ps aeruginosa* primer and vice versa (fig 1). PCR was also

Table 2 Sequences of oligonucleotide probes and primers used in species specific PCR and DNA hybridisation

Oligonucleotide	*5'*	*Sequence*		*3'*
PA 640	AGCtc	agtAG	cTTtt	ggA
PC 640	AGCct	gccAG	tTTac	ctA
PA 1240	tTGTa	CCGAC	CATTG	TAG
PC 1240	cTGTt	CCGAC	CATTG	TAt
PA 480	GtTAC	CaacA	GAATA	AGC
PC 480	GgTAC	CggaA	GAATA	AGC

Sites where sequences differ between *Ps aeruginosa* and *Ps (Burkholderia) cepacia* are indicated by lower case letters.

Table 3 Alignment of 16s rRNA sequences at PCR primer site 1250

Organism	*3'*	*Sequence*		*5'*
E coli	CUACA	AUGGC	GCAUA	CAA
Parn16S	CUACA	AUGGU	CGGUA	CAA
P2440	CUACA	AUNGU	CGGUA	CAG
PA 01	cUACA	AUNGU	CGGuA	CAa
PL1017	cUACA	AUNGU	CGGuA	CAa
PA01161	cUACA	AUNGU	CGGuA	CAa
PA10332	cUACN	AUNGU	CGGuA	CNa
PC10661	aUACA	AUGGA	CGGaA	CAg
PC10744	aUACA	AUGGU	CGGaA	CAg

Sites where sequences differ between *Ps aeruginosa* and *Ps (Burkholderia) cepacia* are indicated by lower case letters.

Table 1 Alignment of 16s rRNA sequences at the probe site (640)

Organism	*3'*	*Sequence*		*5'*
E coli	AUCUG	AUACU	GGCAA	GCU
Parn16s	UCCAA	AAGCU	ACUGA	GCU
P2440	UCCAA	AANCU	GGCAA	GCU
PA 01	Uccaa	AANCU	acuga	GCU
PL1017	UccNa	AANCU	acuNa	GCN
PA01161	Uccaa	AANCU	acuga	GCU
PA10332	Uccaa	AANCU	acuga	GCU
PC10661	Uaggu	AAACU	ggcag	GCU
PC10744	Uaggu	AAACU	ggcag	GCU

Sites where sequences differ between *Ps aeruginosa* and *Ps (Burkholderia) cepacia* are indicated by lower case letters.

Table 4 Alignment of 16s rRNA sequences at PCR primer site 480

Organism	*3'*	*Sequence*		*5'*
E coli	GTTAC	CCGCA	GAAGA	AGC
Parn16s	GTTAC	CAACA	GAATA	AGC
P2440	GUUAC	CNGCN	GAAUA	AGC
PA01	GuUAC	CaacA	GAAUA	AGC
PL1017	GuUAC	CaacA	GAAUA	AGC
PA01161	GuUAC	CaacA	GAAUA	AGC
PA10332	GuUAC	CaacA	GAAUA	AGC
PC10661	GgUAC	CggaA	GAAUA	AGC
PC10744	GgUAC	CggaA	GAAUA	AGC

Sites where sequences differ between *Ps aeruginosa* and *Ps (Burkholderia) cepacia* are indicated by lower case letters.

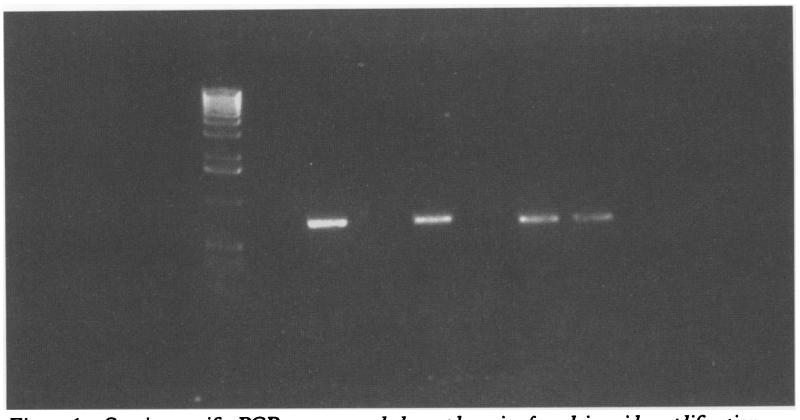

Figure 1 Species specific PCR: agarose gel electrophoresis of nucleic acid amplification products from cultures of Ps aeruginosa and Ps (Burkholderia) cepacia after amplification using primers as described. Lane 1: 1 kilobase DNA ladder (Bethesda Research Laboratories); lane 2: negative control; lane 3: Ps aeruginosa amplified with P536 and PA 1250; lane 4: Ps (Burkholderia) cepacia amplified with P536 and PA 1250; lane 5: Ps (Burkholderia) cepacia amplified with P536 and PC 1250; lane 6: Ps aeruginosa amplified with P536 and PC 1250; lane 7: Ps aeriginosa positive control; lane 8: Ps (Burkholderia) cepacia positive control.

performed on pure cultures of *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Escherichia coli*, *Proteus vulgaris*, and *Klebsiella pneumoniae*, using all sets of PCR primers described. No PCR products were visible on agarose gels.

SPECIES SPECIFIC HYBRIDISATION

The sequence target for the species specific probes PA 640 and PC 640 was included within the region amplified by the PCR reaction. The specificity of the probes was confirmed by colony blot hybridisations with *Ps aeruginosa*, *Ps (Burkholderia) cepacia*, and *Ps putida* using the conditions described. The probes were species specific when hybridised with both the colony blots and the PCR products from the amplification of *Ps aeruginosa* and *Ps (Burkholderia) cepacia*. The presence of a large amount of high copy number DNA, following PCR, results in a strong hybridisation signal and therefore short autoradiography times as well as easy differentiation of positive and negative reactions (fig 2). When 10 μl of PCR product were loaded on to an agarose gel an amplification band was just visible for 10^2 cells/ml, but hybridisation of 10 μl of PCR product gave a positive signal down to a concentration of 10 cells/ml in the original sample.

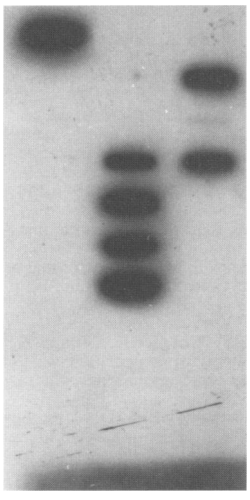

Figure 2 Hybridisation of PCR products: autoradiograph of sputum PCR products amplified with primers P536 and PA 1250 after hybridisation with the ^{32}P-labelled oligonucleotide specific for Ps aeruginosa (PA 640). Lane 1: positive and negative controls; lanes 2 and 3 each contain seven sputum PCR products.

DETECTION OF *pseudomonas* SPP IN CLINICAL SAMPLES

The system of species specific PCR and DNA hybridisation described was applied to sputum samples from patients with a range of respiratory conditions, including cystic fibrosis, and the results compared with conventional culture. Consecutive samples (n = 100) from the Public Health Laboratory at Leicester Royal Infirmary were tested. None was culture positive for *Ps (Burkholderia) cepacia*, but 10 were culture positive for *Ps aeruginosa*. Sixty samples from patients with cystic fibrosis were tested for the presence of *Ps aeruginosa* and 60 for the presence of *Ps (Burkholderia) cepacia*. There was full concordance of the results obtained from conventional culture with those from combined PCR and DNA hybridisation, with 100% specificity for both *Ps aeruginosa* and *Ps (Burkholderia) cepacia* (table 5).

Table 5 Results of species specific PCR and DNA hybridisation applied to clinical samples

	PHLS samples	*Patients with cystic fibrosis*
Ps aeruginosa culture + ve	10	27
Ps aeruginosa PCR/probe + ve	10	27
Ps aeruginosa culture –ve	90	33
Ps aeruginosa PCR/probe –ve	90	33
Ps cepacia culture + ve	0	16
Ps cepacia PCR/probe + ve	0	16
Ps cepacia culture –ve	100	44
Ps cepacia PCR/probe –ve	100	44

When PCR was performed directly on sputum samples and the results analysed on an agarose gel, an amplification product of the correct size was sometimes visible even when conventional culture had not revealed any infection with *Ps aeruginosa* or *Ps (Burkholderia) cepacia* (fig 3). The hybridisation reaction was accurate and species specific as these non-specific PCR products did not hybridise with the species specific probes confirming the absence of both *Ps aeruginosa* and *Ps (Burkholderia) cepacia* in the original sputum sample. These inappropriate PCR products occurred even when the "universal" primer was replaced by a second species specific primer (PA 480 or PC 480), and they were not affected by manipulating the PCR reaction to increase its specificity—that is, by changing the magnesium concentration of the PCR buffer or the annealing temperature of the PCR primers. There was no association between the appearance of these amplification products and a specific bacterial species on conventional culture. These observations suggest that inappropriate priming of bacterial or human DNA was occurring in the PCR reaction which may have been eliminated by further purification of the sputum samples prior to PCR, but this would increase laboratory time and involve the use of potentially hazardous chemicals such as phenol.

Discussion

These results show that a species specific system of PCR and DNA hybridisation based on 16s RNA sequences is applicable in clinical practice. Although it would be possible to amplify DNA from all bacteria in a clinical sample using PCR primers from universal regions of the 16sRNA, a species specific system improves the relative strength of the positive signal in the subsequent hybridisation reaction. The whole process of sputum preparation, PCR, and DNA hybridisation can be performed within a day, with the autoradiography result ready the next day.

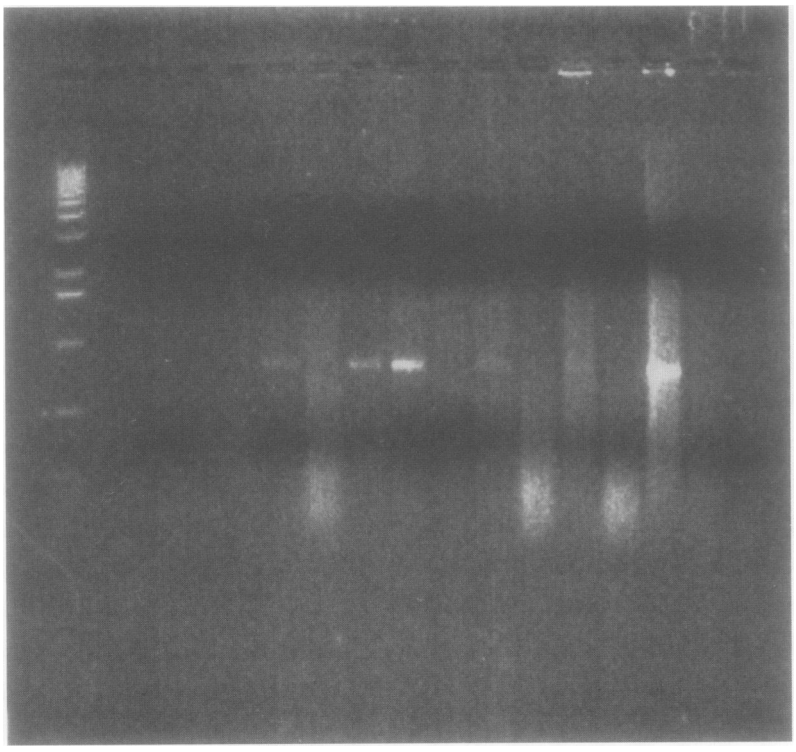

Figure 3 Non-specific sputum PCR products: agarose gel electrophoresis of nucleic acid amplification products from sputum after PCR amplification with primers PA 480 and PA 1250. Lane 1: 1 kilobase DNA ladder (Bethesda Research Laboratories); lanes 2 to 14: sputum PCR products; lane 15: positive control; lane 16: negative control. Only lanes 7, 8, and 15 had positive results after hybridisation with the Ps aeruginosa specific oligonucleotide probe.

To improve the application of this system the labelling of the oligonucleotides should ideally be non-radioactive. Biotin labelling was unsuccessful because of false positive reactions due to non-specific binding of the streptavidin molecules to cellular debris in colony blots as well as in clinical samples after PCR. Previous studies have also shown that the clarity of the positive signal obtained with radiolabelled probes is superior to that from biotinylated probes.[24]

In summary, we have developed a system of PCR and DNA hybridisation for the identification of infection with *Ps aeruginosa* and *Ps (Burkholderia) cepacia* in sputum. Further studies are needed to combine this technique with the detection of serum IgA antibodies specific for *Ps aeruginosa* to determine whether increased antibody titres are a sign of early infection with small numbers of organisms in the respiratory tract of patients with cystic fibrosis.

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