flora, regardless of colonial morphology and appearance were enumerated and the total viable count (TVC) was expressed as $\log_{10}$ colony forming units (cfu) per gram (cfu/g) of original sputum.

Results demonstrated that there was no statistical difference ($P>0.05$; 5%) between sputum counts within a dosing regimen over time, with the exception of the placebo control at visit 1 and visit 6 ($P=0.0104$), or between dosing regimens at any visit (Table 1). The overall sputum loading associated with all patients and all dosing regimens was $\log_{10}$ 8.45 cfu/g sputum.

Recruitment of large numbers of neutrophils to the lungs of CF patients in response to the chronic presence of mainly Gram-negative organisms, specifically *P. aeruginosa*, establishes a chronic cycle of infection/inflammation that leads eventually to irreversible matrix tissue damage as a result of the presence of neutrophil elastase. Hence, any therapeutic intervention that can minimise the effect of elastase on lung tissue would appear to be a prudent approach to maintaining adequate lung function. However, neutrophil elastase is an important agent of non-oxidative killing of bacteria, along with lysozyme, defensins, bacterial/permeability-increasing (BPI) protein, cathepsin G and proteinase 3 activities.[5] Thus, any reduction in the efficacy of bactericidal activity due to therapeutically altered neutrophil elastase should be examined carefully.

In the present study, treatment of the patient population with up to 500 mg recAAT did not change the quantitative loading of bacteria in the lung, suggesting that the bactericidal function of elastase was not significantly altered.

Overall, this study serves to illustrate the importance of monitoring potential changes in the existing quantitative microbial ecology whenever the stringency of a microbiological hurdle is lowered, particularly if an antimicrobial agent is involved. In conclusion, treatment of adult male CF patients with recAAT in this study did not alter the bacterial loading of sputum in this patient population. □

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References

1 McKone EF, Emerson SS, Edwards KL, Aitken ML. Effect of genotype on phenotype and mortality in cystic fibrosis: a retrospective cohort study. *Lancet* 2003; **361**:1671–6.

2 Conway SP, Brownlee KG, Denton M, Peckham DG. Antibiotic treatment of multidrug-resistant organisms in cystic fibrosis. *Am J Respir Med* 2003; **2**: 321–32.

3 Mariani-Kurkdjian P, Bingen E. Pathogenic bacteria in cystic fibrosis. *Archives de pediatrie* 2003; **10**(S2): 342s–6s.

4 Tebbutt SJ. Technology evaluation: transgenic alpha-1-antitrypsin (AAT), PPL therapeutics. *Curr Opin Mol Ther* 2000; **2**: 199–204.

5 Thomas EL, Lehrer RI, Rest RF. Human neutrophil antimicrobial activity. *Rev Infect Dis* 1988; **10**(S2): S450–6.

Molecular (PCR) detection of *Pseudomonas* spp. other than *P. aeruginosa* directly from the sputum of adults and children with cystic fibrosis

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Cystic fibrosis (CF) is the most common genetic disease in Caucasians, with an incidence of 1 in 2500 live births and a carriage rate of 1 in 20 individuals. Patients with CF suffer from recurrent and chronic respiratory tract infections and the majority of the associated morbidity and mortality is due to such infections.

These infections are usually dominated by Gram-negative organisms, especially the pseudomonads, particularly *Pseudomonas aeruginosa*. However, although various other species within the genus *Pseudomonas* have been identified as clinically significant in such patients,[2–4] relatively few studies have examined prevalence, either by conventional culture or by molecular techniques, of such species in patients' sputa.

The aim of this study is to use a multiplex polymerase chain reaction (PCR) assay to determine the presence of genomic DNA from *Pseudomonas* spp. and *P. aeruginosa* directly from the sputa of CF patients.

Sputum (1 mL, minimum) specimens were collected from 116 children and 57 adult patients who had a well-characterised history of CF and placed in sterile (100 mL) plastic disposable containers. Sputum was collected immediately after a standardised session of physiotherapy, stored at ambient temperature and processed within 4 h of collection. Fresh sputum (1 mL, minimum) was mixed with an equal mass of Sputasol (Oxoid SR089A, Oxoid, Poole, England) and incubated in a water bath at 37°C for 15 min, before further qualitative processing for the molecular detection of *P. aeruginosa* and *Pseudomonas* spp.

All DNA isolation procedures were carried out in a class II biological safety cabinet (MicroFlow, England) in a room physically separate from that used to set up nucleic acid amplification reaction mixes and also from the 'post-PCR' room, in accordance with the good molecular diagnostic procedures (GMDP) guidelines proposed by Millar *et al.*,[5] in order to minimise contamination and hence the possibility of false-positive results.

Bacterial genomic DNA was extracted directly from patients' sputa, as well as from the *P. aeruginosa* reference strain ATCC 27853 (Schroeter; Migula), using the Roche High Purity PCR Template Preparation Kit (Roche, England), following the manufacturer's instructions. Extracted DNA was stored at –80°C prior to PCR amplification. With each batch of extractions, a negative extraction control containing all reagents but no sputum was performed, as well as an

Table 1. Oligonucleotoides and empirically optimised experimental PCR amplification conditions.

Target	Gene locus	Primer 5'----------3'	Optimum $MgCl_2$ concentration (mmol/L)	Annealing temperature (˚C)	Size of Amplicon (bp)	Ref.
Eubacteria	16S rRNA	f: 5'- AGG ATT AGA TAC CCT GGT AGT CCA-3' r: 5'- ACT TAA CCC AAC ATC TCA CGA CAC -3'	2.5	55	312	(6)
Pseudomonas aeruginosa	*opr*L*	f: 5'-ATG GAA ATG CTG AAA TTC GGC-3' r: 5'-CTT CTT CAG CTC GAC GCG ACG-3'	2.5	55	504	(7)
Pseudomonas spp.	*opr*I*	f: 5'-GAC AAC GCC CTC AGC ATC ACC AGC-3' r: 5'-CGC TGG CCC ATT CGC TCC AGC GCT-3'	2.5	55	249	(7)

* outer membrane lipoprotein

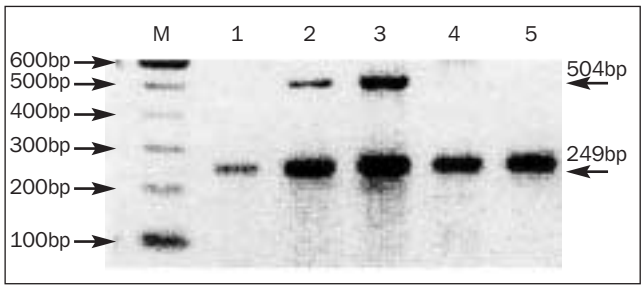

Fig. 1. Multiplex (duplex) PCR assay for the detection of *Pseudomonas aeruginosa* (*opr*L locus; 504 bp) and *Pseudomonas* spp. (*oprI*; 249 bp) directly from the sputum of adults and children with cystic fibrosis. Lane M: molecular weight marker (100 bp; Gibco Life Technologies, Paisley, Scotland); lanes 1,4 and 5: *oprI* +ve (*Pseudomonas* spp.); lanes 2 and 3: *opr*L +ve (*P. aeruginosa*).

extraction positive control with *P. aeruginosa*.

Initially, PCR amplification conditions were optimised by varying $MgCl_2$ concentration, annealing temperature, primer concentration and DNA template concentration. Following optimisation, reaction mixes (100 µL) were set up in a multiplex (duplex) PCR assay as follows: 10 mmol/L Tris-HCl (pH 8.3); 50 mmol/L KCl; 200 µmol/L (each) dATP, dCTP, dGTP and dTTP; 1.25 U *Thermus aquaticus* (*Taq*) DNA polymerase (Amplitaq; Perkin Elmer); 0.1 µmol/L (each) primer (*opr*I and *opr*L; Table 1) and 4 µL DNA template.

Two previously described PCR assays were employed that targeted the outer membrane lipoprotein (opr) of the pseudomonads, either specifically (in the case of the *opr*L gene locus for *P. aeruginosa*) or the genus *Pseudomonas* (in the case of *opr*I gene locus). Following a 'hot start', the reaction mixtures were subjected to empirically optimised thermal cycling parameters in a Perkin Elmer 2400 thermocycler, as detailed in Table 1. Positive (*P. aeruginosa* ATCC 27853 DNA) and multiple negative (water) amplification controls were included in every set of PCR reactions. In addition, a broad-range or universal 16S rDNA PCR was employed with each sputum to demonstrate successful extraction of bacterial DNA from the specimen, as well as lack of PCR inhibition, using the highly conserved 16S rDNA primers PSL/PSR (Table 1), as previously described.[6] Any sputum which failed to amplify this locus was re-extracted and amplified until a positive signal was obtained.

Following amplification, samples (10 µL) were removed from each reaction mixture and examined by electrophoresis (80 V, 45 min) in gels composed of 2% (w/v) agarose (Gibco, UK) in TAE buffer (40 mmol/L Tris, 20 mmol/L acetic acid, 1 mmol/L EDTA [pH 8.3]), stained with ethidium bromide (5 µg/100 mL). Gels were visualised under ultraviolet (UV) illumination using a gel image analysis system (UVP Products, England) and all images were archived as digital graphic files (*.bmp). Where a band was visualised at the expected size for *opr*I (249 bp) and/or *opr*L (504 bp) loci, the specimen was considered positive for *Pseudomonas* spp. and/or *P. aeruginosa*, respectively.

All patients' specimens were extracted and amplified in accordance with the negative and positive extraction and PCR amplification controls. Figure 1 shows the amplification of *Pseudomonas* spp. and *P. aeruginosa* from five patients' specimens employing the multiplex PCR assay.

Comparison of genomic DNA from *P. aeruginosa* and *Pseudomonas* spp. in children showed that 88 (75.8%) were positive for *P. aeruginosa* with the *opr*L gene locus, and 98 (84.5%) were positive for *Pseudomonas* spp. with the *opr*I gene locus. Among the adults, 33 (63.5%) were positive for *P. aeruginosa* and 42 (80.7%) were positive for *Pseudomonas* spp., using the multiplex PCR assay.

In this study, two PCR assays were performed in multiplex format for the molecular detection of *P. aeruginosa* and *Pseudomonas* spp. directly from the sputa of patients with CF. Two gene loci were targeted: the *opr*L gene locus as a specific marker of *P. aeruginosa* and the *opr*I gene locus as a marker for *Pseudomonas* spp. As sputa containing *P. aeruginosa* would be positive for both loci, it was not possible to detect the presence of other pseudomonads when *P. aeruginosa* was present. Identification of *Pseudomonas* spp. that were positive for the *opr*I locus was attempted to the species level by sequencing the amplified *opr*I locus. At present, however, the genus *Pseudomonas* consists of approximately 100 recognised species (www.bacterio.cict.fr) and only 17 (including *P. mendocina, P. oleovorans, P. pseudoalcaligenes, P. alcaligenes, P. putida, P. cichorii, P. syringae, P. caricapapayae, P. fluorescens, P. corrugata, P. tolaasii, P. chlororaphis aureofaciens, P. marginalis, P. agarici, P. taetrolens, P. asplenii,* and *P. aeruginosa*) have *opr*I sequences deposited in GenBank.[8] Hence, species identification by sequencing of the *opr*I PCR product may be

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impossible and it may be necessary to use an alternative gene locus, such as the 16S rDNA or 23S rDNA locus, where phylogenetic data is available for all recognised species within the genus.

In the present study, other species within the genus *Pseudomonas* accounted for 10.2% and 21.4% of the total *Pseudomonas* spp. detected in children and adults, respectively. These values may be clinically significant for two reasons: (i) there may be significant under-reporting of the occurrence and significance of other *Pseudomonas* spp. that have not been described to date as being well-established pathogens of CF, and (ii) other species within the genus *Pseudomonas* may play a role as an early ecological coloniser/successor, thereby preparing a favourable physiological/ecological niche for the colonisation and eventual chronic infection with *P. aeruginosa*.

As not all laboratories employ molecular detection methods for *P. aeruginosa* or other *Pseudomonas* spp., either from culture plates or patients' sputa, small numbers of colonies (n=1–2) may be missed when present in the early stages of colonisation preceding infection of a patient's airway, particularly where single colonies are mixed with other phenotypically similar genera on the primary culture plate.[4]

Pragmatic, practical and cost implications make it impossible to identify the total bacterial microflora qualitatively from sputa on non-selective primary plates. Therefore, any rapid molecular screening method should be encouraged in an effort to detect low copy numbers of organisms in the early stages of colonisation/infection when the main value of the diagnostic assay is the rapid screening of patients with no (or only intermittent) history of colonisation with *Pseudomonas* spp.

Although such assays are not generally available in most clinical diagnostic laboratories, access to such technology is generally available at regional specialist microbiology centres, and therefore it may be prudent to establish routine analysis of CF sputum at annual review. In conclusion, employment of this multiplex PCR assay may permit the rapid detection of other *Pseudomonas* spp. in addition to *P. aeruginosa* directly from the sputa of CF patients. □

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References

1 Høiby N. Cystic fibrosis: infection. *Schweiz Med Wochenschr* 1991; **121**: 105–9.

2 Garske L, Moore JE, Crowe MJ, Elborn JS. Cystic fibrosis and guidelines to assess significance of new colonisers? *Irish J Med Sci* 2002; **171**: 116–7.

3 Klinger JD, Thomassen MJ. Occurrence and antimicrobial susceptibility of Gram-negative non-fermentative bacilli in cystic fibrosis patients. *Diagn Microbiol Infect Dis* 1985; **3**: 149–58.

4 Clarke L, Moore JE, Millar BC *et al*. Development of a diagnostic PCR assay that targets a heat-shock protein gene (*gro*ES) for detection of *Pseudomonas* spp. in cystic fibrosis patients. *J Med Microbiol* 2003; **52**: 759–63.

5 Millar BC, Xu J, Moore JE. Risk assessment models and contamination management: implications for broad-range ribosomal DNA PCR as a diagnostic tool in medical bacteriology. *J Clin Microbiol* 2002; **40**: 1575–80.

6 Campbell PW, Phillips JA, Heidecker GJ, Krishnamani MR, Zahorchak R, Stull TL. Detection of *Pseudomonas* (*Burkholderia*) *cepacia* using PCR. *Pediatr Pulmonol* 1995; **20**: 44–9.

7 De Vos D, Lim A Jr, Pirnay JP *et al*. Direct detection and identification of *Pseudomonas aeruginosa* in clinical samples such as skin biopsy specimens and expectorations by multiplex PCR based on two outer membrane lipoprotein genes, *opr*I and *opr*L. *J Clin Microbiol* 1997; **35**: 1295–9.

8 De Vos D, Bouton C, Sarniguet A, De Vos P, Vauterin M, Cornelis P. Sequence diversity of the *opr*I gene, coding for major outer membrane lipoprotein I, among rRNA group I pseudomonads. *J Bacteriol* 1998; **180**: 6551–6.

Life-threatening post-partum hypercalcaemia

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A 23-year-old woman presented as an acute medical admission six weeks following the delivery of her second child. She presented with polyuria, polydipsia and lethargy. Blood analysis had excluded hyperglycaemia (random glucose 5.7 mmol/L) but her corrected serum calcium was extremely high at 5.29 mmol/L.[1] The 24-hour urinary calcium was high at 33.3 mmol/day, creatinine clearance 76 mL/h and urine output 5.2 L/day. Parathyroid hormone (PTH) was strikingly high at 1286 pg/L, confirming the diagnosis of primary hyperparathyroidism (PHPT).

Interestingly, her corrected serum calcium had been checked 24-hours post-partum and was found to be elevated at 2.94 mmol/L, but this was not followed up until her presentation as an emergency. She was treated initially with vigorous rehydration and intravenous disodium pamidronate (90 mg over 90 minutes) and after 10 days the serum calcium had fallen to 2.7 mmol/L.

An ultrasound of the neck confirmed a large adenoma (2.5 cm diameter) at the right lower pole of the thyroid. X-ray examination of the hands showed osteopaenia, sub-periosteal bone resorption and acro-osteolysis; and a bone scan showed extensive, generalised increased uptake throughout the skeleton.

A pre-operative vocal cord check was normal. She underwent an open parathyroidectomy two weeks after her emergency presentation. Surgery revealed one large parathyroid adenoma (3.2 x 2.6 x 2 cm), which was resected along with half of one other identifiable gland. Of the remaining glands, one was unidentifiable and the other

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