Development of a diagnostic PCR assay that targets a heat-shock protein gene (*groES*) for detection of *Pseudomonas* spp. in cystic fibrosis patients

Lena Clarke,¹ John E. Moore,¹ B. Cherie Millar,¹ Luke Garske,^{2,3} Jiru Xu,¹ Michael W. Heuzenroeder,⁴ Mary Crowe¹ and J. Stuart Elborn^{2,5}

Correspondence

John E. Moore

jemoore@niph.dnet.co.uk

¹Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Belfast BT9 7AD, Northern Ireland, UK

²Northern Ireland Regional Adult Cystic Fibrosis Centre, Belfast City Hospital, Belfast BT9 7AB, Northern Ireland, UK

³Department of Respiratory Medicine, Prince of Wales Hospital, Brisbane, Queensland, Australia

⁴Infectious Diseases Laboratory, Institute of Medical and Veterinary Science, Frome Road, Adelaide, South Australia

⁵Department of Respiratory Medicine, The Queen's University of Belfast, Level 8, Belfast City Hospital, Lisburn Road, Belfast BT9 7AB, Northern Ireland, UK

Laboratory detection of *Pseudomonas* spp., in particular *Pseudomonas aeruginosa*, remains an important assay in the management of patients with cystic fibrosis (CF). As the *groES* and *groEL* genes of *P. aeruginosa* have now been cloned and their nucleotide sequences determined, the aim of this study was to develop a novel PCR assay for the detection of *Pseudomonas* spp. from patients with CF by employing conserved primer regions of the *groE* heat-shock protein domain gene. A PCR assay was designed that targeted a 536 bp region of the *groE* gene to detect *Pseudomonas* spp. PCR amplification of genomic DNA from extracted organisms generated an amplicon of the expected size (approx. 536 bp) for all *P. aeruginosa* ($n = 60$), *Pseudomonas putida*, *Pseudomonas fluorescens* and *Pseudomonas stutzeri* isolates examined, but did not produce a positive amplicon for several other genera and species that are commonly isolated from the sputum of CF patients. RFLP analysis of the amplicons of all *P. aeruginosa* isolates demonstrated a single RFLP type that consisted of three bands at approximately 80, 190 and 250 bp; direct sequencing of the amplicons demonstrated the presence of a single sequence type, indicating the highly conserved nature of this region. In addition, the assay successfully produced a positive signal from primary non-selective plates of three known *P. aeruginosa* culture-positive CF patients, but was unable to generate a signal in a further six CF patients who had no history of infection with *P. aeruginosa* or other *Pseudomonas* spp. This assay is recommended to detect the presence of *Pseudomonas* spp., including *P. aeruginosa*, from primary culture plates that originate from laboratory analysis of CF patients' sputum, particularly at review, in those patients with no previous history of *Pseudomonas* infection or those who appear to be transiently colonized by this organism. Employment of such molecular methodologies, in conjunction with routine clinical sputum cultures, may provide improved information on the microbial status of CF patients, which will aid clinicians in both optimum patient management in terms of antibiotic regimes and CF centre infection-control practices.

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INTRODUCTION

Cystic fibrosis (CF) is the most common genetic disease of Caucasians, with an incidence of 1 in 2500 live births and a

carriage rate of 1 in 20 individuals. CF patients continue to suffer from recurrent and chronic respiratory tract infections and most of the morbidity and mortality of this disease is due to such infections, which occur throughout their life (Hoiby, 1991). These infections are usually dominated by Gram-negative organisms, especially the pseudomonads (particularly *Pseudomonas aeruginosa*). However, various other

Abbreviation: CF, cystic fibrosis.

The GenBank/EMBL/DDBJ accession number for the partial sequence of *groES* of *P. aeruginosa* strain LC99 is AY150814.

species of the genus *Pseudomonas* have been identified as clinically significant in such patients (Klinger & Thomassen, 1985; Garske *et al.*, 2002).

The heat-shock response is an important homeostatic mechanism that enables cells to survive a variety of environmental stresses. A set of heat-shock proteins, also known as chaperonins, are induced when cells are exposed to higher temperatures. This phenomenon has been observed in all organisms, from bacteria and fungi to plants and animals. The chaperonins form a well-characterized subgroup of molecular chaperones; this subgroup includes the GroE subclass, which comprises two proteins: chaperonin 60 and chaperonin 10. The GroEL protein is *Escherichia coli* chaperonin 60 and GroES is the *E. coli* chaperonin 10 protein; these are the most extensively studied chaperones. GroEL is critical for the correct folding of a number of cellular proteins under both normal and stress conditions. Indeed, in *E. coli*, deletion of the *groE* operon is lethal and the GroEL and GroES proteins are required for survival. The chaperonin 60 family have a double-ring structure that comprises 14 subunits; a central cavity is formed, in which the unfolded substrate protein binds by hydrophobic interactions. The GroES subunit protein also binds to the apical domain of GroEL. The process of protein folding requires ATP, which binds to GroEL; when ATP binding occurs, GroES forms a lid on top of the GroEL barrel, causing enlargement of the central cavity and protein folding.

Heat-shock proteins appear to be constituents of the cellular machinery of protein folding, degradation and repair (Feltham & Gierasch, 2000). These bacterial molecular chaperones play an important role in normal growth by mediating the folding and/or assembly of different polypeptides, as well as the transport of some secretory proteins across membranes. For successful reactivation and assembly of some proteins, GroEL requires the presence of another heat-shock protein, GroES. The general properties of the heat-shock response of *P. aeruginosa* have been characterized; synthesis of at least 17 proteins, including DnaK and GroEL, is transiently induced by an increase in cell temperature.

As the *groES* and *groEL* genes of *P. aeruginosa* have now been cloned and their nucleotide sequences determined (Fujita *et al.*, 1998), the aim of this study was to develop a novel PCR assay for detection of *Pseudomonas* spp. from the sputum of patients with CF by employing conserved primer regions of the *groE* heat-shock protein domain gene.

METHODS

Patient population. *P. aeruginosa* isolates ($n = 60$) were obtained from 35 adult patients with a confirmed and well-documented history of CF who attended the Northern Ireland Regional Adult CF Centre, Belfast City Hospital, from January 1996 to June 1998. The age range of the patients was 17–30 years, with one patient aged 70 years. The male:female ratio was 24:11. All *Pseudomonas* isolates were obtained from fresh sputum specimens cultured on Columbia Blood Agar (Oxoid) that contained 5% (v/v) defibrinated horse blood. The identity

of all isolates was confirmed by using an API 20NE biochemical testing kit (bioMérieux).

Design of oligonucleotide primers. DNA sequence data of the heat-shock protein genes (*groES* and *groEL*) were obtained from GenBank; conserved and variable regions were subsequently identified by aligning published sequences of *P. aeruginosa* (accession numbers U17072, U19797, U64101, U64102, U64103, U64104, S77424 and M63957), *Pseudomonas putida* (X78435), *Pseudomonas stutzeri* (Y13828), *Burkholderia vietnamiensis* (AF104908), *Burkholderia cepacia* (AF104907), *Stenotrophomonas maltophilia* (U68778), *Salmonella typhi* (U01039), *Salmonella typhimurium* (AB033231), *Erwinia carotovora* (AB008152) and *E. coli* (M11294, X07850) by the CLUSTAL alignment method in the DNASTAR sequence alignment software package. A novel primer pair was designed, forward [*groES*f 5'-ATGAAGCTTCGTCCTCTGCAT-3' (21-mer)] and reverse [*groES*r 5'-GTCTTTCAGTCGAT-3' (15-mer)], which targeted conserved regions of the heat-shock protein gene for *Pseudomonas* spp. The binding sites of the *groES*f and *groES*r primers in *P. aeruginosa* (GenBank no. M63957) relate to positions 42–63 and 577–563, respectively, yielding a fragment of 536 bp.

DNA extraction. All DNA isolation procedures were carried out in a Class II Biological Safety Cabinet (Astec Microflow) in a room physically separated 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 guidelines of Millar *et al.* (2002), in order to minimize contamination and hence the possibility of false-positive results. Bacterial genomic DNA was extracted from *P. aeruginosa* reference strain ATCC 27853, as well as from 60 well-characterized wild-type *P. aeruginosa* isolates from the sputum of adult CF patients, by employment of the Roche High Pure PCR Template Preparation kit in accordance with the manufacturer's instructions. Genomic DNA was also extracted from well-characterized isolates of *P. putida*, *P. fluorescens* and *P. stutzeri*, as well as from the following 21 non-*Pseudomonas* organisms: *Asaia* sp., *Alcaligenes xylosoxidans* NIPHL 96/01, *Bacillus licheniformis*, *Burkholderia cepacia* genomovar IIIa NIPHL CF/97/58, *Burkholderia cepacia* genomovar IIIa NIPHL CF/96/27, *Burkholderia cepacia* genomovar IIIa NIPHL CF/96/02, *Burkholderia multivorans*, *Campylobacter jejuni*, 'Chromobacter violaceum' NIPHL 96/51, *Curtobacterium* sp., *Haemophilus influenzae*, *Inquilinus limosus*, *Lactobacillus gasseri*, *Mycobacterium malmoeense*, *Pandoraea apista* NIPHL 02/02, *Ralstonia paucula*, *Serratia marcescens* NIPHL CF/97/27, *Sphingomonas paucimobilis* NIPHL CF/97/40, *Staphylococcus aureus*, *Stenotrophomonas maltophilia* NIPHL 97/23 and *Thermoactinomyces* sp. Extracted DNA was stored at -80°C prior to PCR amplification. For each batch of extractions, a negative extraction control was performed that contained all reagents minus any organism, as well as an extraction positive control with *P. aeruginosa*.

PCR amplification. Amplification reactions were set up in accordance with Good Molecular Diagnostic Procedures, as detailed in the guidelines of Millar *et al.* (2002). All reaction mixes were set up in a PCR hood in a room separate from that used to extract DNA and the amplification and 'post-PCR' room, in order to minimize contamination. Initially, PCR amplification conditions were optimized by varying the following conditions separately: magnesium chloride concentration, annealing temperature, primer concentration and DNA template concentration. Following optimization, reaction mixes (100 μl) were set up as follows: 10 mM Tris/HCl, pH 8.3; 50 mM KCl; 2.5 mM MgCl_2 ; 200 μM (each) dATP, dCTP, dGTP and dTTP; 1.25 U *Taq* DNA polymerase (Amplitaq; PerkinElmer); 0.1 μM (each) primer (*groES*f and *groES*r); and 4 μl DNA template. Reaction mixtures, following a 'hot start', were subjected to the following empirically optimized thermal cycling parameters in a PerkinElmer 2400 thermocycler: 96°C for 5 min, followed by 40 cycles of 96°C for 1 min, 50°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 10 min. Positive (*P. aeruginosa* ATCC 27853

DNA) and multiple negative (water) amplification controls were included in every set of PCRs. In addition to all ($n = 60$) *P. aeruginosa* isolates from CF patients, genomic DNA from *P. stutzeri*, *P. fluorescens*, *P. putida* and the non-*Pseudomonas* organisms described above was amplified by using these conditions.

Detection of amplicons. Following amplification, aliquots (10 μ l) were removed from each reaction mixture and examined by electrophoresis (80 V, 45 min) in gels composed of 2 % (w/v) agarose (Gibco) in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3), stained with ethidium bromide (0.05 μ g ml⁻¹). Gels were visualized under UV illumination by using a gel image analysis system (UVP Products) and all images were archived as digital graphic files.

RFLP analysis of PCR amplicons. A restriction-site map was constructed of the theoretical PCR product by using MapDraw Software (DNASTAR) to determine a suitable enzyme to obtain four or five restriction sites. *Hae*III was selected as it yielded five restriction sites, at 204, 413, 428, 438 and 447 bp. All PCR amplicons were restricted with *Hae*III (Pharmacia) by using the following restriction conditions: PCR product, 10.5 μ l; restriction buffer (One-Phor-All $\times 10$; Pharmacia), 3.5 μ l; *Hae*III, 0.7 μ l (12 U); molecular-grade water, 20.3 μ l. Restricted fragments were separated by PAGE, performed by using an ExcelGel DNA Analysis kit (Amersham Biosciences) as per the manufacturer's instructions. Briefly, 4 μ l of each PCR product was analysed; the gel was run at 600 V, 50 mA, 30 W, at 15 °C for 85 min and subsequently stained by using a PlusOne DNA Silver Staining kit (Amersham Biosciences) as per the manufacturer's instructions. Banding patterns were analysed by using GelCompar software (Applied Maths).

Sequencing of PCR amplicons and analysis of sequence data. All *P. aeruginosa* PCR amplicons were purified by using a QIAquick PCR Purification kit (Qiagen) and eluted in Tris/HCl (10 mM, pH 8.5) prior to sequencing to remove dNTPs, polymerases, salts and primers. Amplicons were sequenced in both directions on an ALFexpress II automated sequencer (Amersham Biosciences) by using both the forward and reverse primers, *groESf* and *groESr*, which were labelled with Cy-5 fluorescent dye (Oligosynthesis Unit, The Queen's University of Belfast, UK) and used in conjunction with a Thermo Sequenase Fluorescent Labelled Primer Cycle Sequencing kit (Amersham Biosciences). The resulting sequences were aligned by employing MegAlign software (DNASTAR) and compared with those stored in GenBank by using BLAST alignment software.

Screening of CF patients. Sputum from nine adult CF patients was examined in a small evaluation study to demonstrate proof of principle with regard to the methodology (Table 1). Fresh sputum from these patients, which had been collected for routine microbiological surveillance, was plated onto Columbia Blood Agar with defibrinated horse blood (as described above) and incubated at 37 °C for 48 h. The resulting culture plates were flooded with 1 ml 1 $\times$ Tris (pH 8.0), all colonies were emulsified and total genomic DNA was extracted from 200 μ l, as described above. PCR was subsequently performed on all extracts as described above.

RESULTS AND DISCUSSION

In silico analysis of the primer pair *groESf* and *groESr* demonstrated that, of currently available sequence data, only *Pseudomonas* spp. had 100 % similarity to these primers. PCR amplification of genomic DNA from extracted organisms generated an amplicon of the expected size (approx. 536 bp) for all *P. aeruginosa* ($n = 60$), *P. putida*, *P. fluorescens* and *P. stutzeri* isolates examined, but was not able to produce this amplicon for any of the 21 non-*Pseudomonas* isolates that were challenged (Fig. 1). RFLP analysis of the amplicons of all *P. aeruginosa* isolates demonstrated a single RFLP type that consisted of three bands at approximately 80, 190 and 250 bp (data not shown). Sequence analysis of all *P. aeruginosa* amplicons demonstrated the presence of a single sequence type and direct sequence analysis showed a highly conserved amplified region. This sequence, which encodes a partial region of the heat-shock protein GroES, has subsequently been deposited in GenBank under accession no. AY150814. This sequence demonstrated 98.2 % similarity to that of *P. aeruginosa* (GenBank accession no. S77424), followed by 76.7 % similarity to *P. stutzeri* (Y13828), 72.7 % similarity to *P. putida* (X78435) and 62.5 % similarity to *Enterobacter cloacae* (M88012).

Screening of total-cell harvests from sputum of nine adult CF patients on non-selective plates generated a positive PCR result for the three patients (A–C) with a history of *P.*

Table 1. Examination of supernatant from non-selective culture plates from nine CF patients' sputum by employing *groES* PCR to detect the presence of *Pseudomonas* spp.

Patient	Sex	Age (years)	CF genotype	Chronic flora (semi-quantitative result)	<i>groES</i> PCR
A	M	33	Δ F508 : R553X	<i>Pseudomonas</i> sp. (++)	+
B	F	23	Δ F508 : Δ F508	<i>P. aeruginosa</i>	+
C	F	16	Δ F508 : R1070P	<i>P. aeruginosa</i> (++)	+
D	F	17	Δ F508 : G551D	<i>Staphylococcus aureus</i>	–
E	F	36	UN : UN	<i>Staphylococcus aureus</i>	–
F	F	31	Δ F508 : Δ F508	<i>Staphylococcus aureus</i> (MRSA)	–
G	M	45	UN : UN	<i>Candida albicans</i> and commensals	–
H	F	19	UN : UN	Coliforms	–
I	M	16	Δ F508 : R117H	<i>Staphylococcus aureus</i>	–

UN, Unknown; MRSA, methicillin-resistant *Staphylococcus aureus*.

Fig. 1. PCR amplification of a 536 bp fragment of the *groES* gene of Gram-negative organisms associated with lung infection in patients with cystic fibrosis. Lane M, 100 bp DNA ladder (Invitrogen); lane 1, *P. aeruginosa* ATCC 27853; lanes 2–4, *P. aeruginosa* wild-type isolates from adult patients with CF; lane 5, *Stenotrophomonas maltophilia*; lane 6, negative control (molecular-grade water).

aeruginosa infection, but was unable to generate a product for those patients (D–I) who had no previous history of *P. aeruginosa* or other *Pseudomonas* spp. infection.

Sequence-based typing and identification systems most often employ well-characterized ‘housekeeping’ genes for analysis. These genes are under no strong selective pressure and therefore acquire mutations in their DNA at a slow and constant rate, which can be measured by sequence analysis. The genes chosen should be found in all bacterial species. Genes involved in the heat-shock response seem to be ideally suited to this purpose (Zeilstra-Ryalls *et al.*, 1991).

P. aeruginosa is the single most important bacterial pathogen in patients with CF (Speert, 2002), as demonstrated with high prevalence data in most national CF registries. Chronic *Pseudomonas* colonization of the major airways, which leads to debilitating exacerbation of pulmonary infection, is the major cause of morbidity and mortality in patients with CF. Although *P. aeruginosa* is the most important species that causes pulmonary infection, several other species in this genus have also been reported (Klinger & Thomassen, 1985). Historical review at our CF centre of laboratory reports of 120 adult CF patients since approximately 1997 has demonstrated a small but important presence of three other *Pseudomonas* species, namely *P. putida*, *P. fluorescens* and *P. stutzeri*, which were considered to be clinically significant in eight patients (8/120; 6.7%) (Table 2). Consequently, it is important not only to reliably detect *P. aeruginosa* but also other species within this genus, albeit at a lower incidence. Although there have been several publications that described PCR detection of *P. aeruginosa* (McIntosh *et al.*, 1992; Karpati & Jonasson, 1996), there have been relatively few descriptions of generic *Pseudomonas* PCR assays that are designed to detect *Pseudomonas* spp. besides *P. aeruginosa*.

It is important that primary diagnostic bacteriology laboratories have the ability to detect *Pseudomonas* spp. as early as

Table 2. Prevalence of clinically significant non-*aeruginosa Pseudomonas* spp. in adult CF patients in Northern Ireland

Patient	Sex	Age (years)	CF genotype	Clinical symptoms	Lung function (FEV ₁ , predicted)	Colonizing, non- <i>aeruginosa Pseudomonas</i> spp.	Other chronic flora
1	M	29	G542X:V520F	Respiratory exacerbations	92.0%	<i>P. putida</i>	<i>Burkholderia cepacia</i>
2	F	32	UN:UN	Stable	40.8%	<i>P. putida</i>	<i>Burkholderia cepacia</i>
3	F	29	UN:UN	Admitted with cellulitis	NA	<i>P. putida</i> and <i>P. fluorescens</i>	Occasional <i>P. aeruginosa</i>
4	F	16	UN:UN	Respiratory exacerbation	76.0%	<i>P. putida</i> and <i>P. fluorescens</i>	<i>Burkholderia cepacia</i>
5	M	19	ΔF508:ΔF508	Stable/mild cough	72.0–77.0%	<i>P. stutzeri</i> and <i>P. fluorescens</i>	<i>P. aeruginosa</i>
6	M	18	UN:UN	Stable	15–19%	<i>P. fluorescens</i>	<i>Burkholderia cepacia</i>
7	M	29	G551D:R117H	Stable	107%	<i>P. fluorescens</i>	Commensals
8	M	14	UN:UN	Stable	71.0–83.0%	<i>P. fluorescens</i>	Commensals
9	F	23	R117H:E60X	Dyspnoea and increased sputum	NA	<i>P. putida</i> and <i>P. fluorescens</i>	Commensals

UN, Unknown; NA, not available.

possible so that aggressive antibiotic regimes may be commenced and the patient is managed optimally, in an attempt to avoid early biofilm formation and chronic colonization by *Pseudomonas* spp. More recently, West *et al.* (2002) showed by using a combination of serum IgG, IgA and IgM anti-*P. aeruginosa* antibodies, in conjunction with these authors' Wisconsin Cystic Fibrosis Radiograph score, that *P. aeruginosa* infection occurred approximately 6–12 months before the organism was recovered from respiratory secretions. In addition, this study demonstrated that mixing of a young child with chronically colonized older children was associated with a significantly increased risk of *P. aeruginosa* acquisition.

Given that not all laboratories employ selective media for the detection of *Pseudomonas* spp. from sputum or other respiratory secretions, small numbers of *Pseudomonas* colonies ($n = 1-2$) may be missed when present in the early stages of infection after colonization of the patient's airways, particularly where such single colonies are mixed with other phenotypically similar genera on the primary culture plate. Pragmatic, practical and cost implications render it impossible to qualitatively identify total bacterial microflora present on non-selective primary plates from sputum. Therefore, any rapid screening method that uses these plates should be encouraged to detect low copy numbers of cells in the early stages of infection. The main value of this diagnostic assay is for the rapid screening of patients with no or an intermittent history of *Pseudomonas* colonization. The above PCR assay offers the opportunity to screen for *Pseudomonas* spp. by modifying the above methodology to include an additional stage, whereby total colonies from primary non-selective plates from sputum are harvested in Tris/HCl (pH 8.0) and examined as described.

Given that the *groES* locus is highly conserved in multiple bacterial genera, the current database of available *groES* sequences is not as comprehensive as those for other highly conserved gene loci, such as the 16S rRNA gene, and hence may not contain sequence data for several commonly isolated, as well as newly emerging, CF organisms. Therefore, the proposed use of these primers in diagnostic screening assays, where *Pseudomonas* spp. are detected in a molecular manner, should be confirmed phenotypically and antibiotic susceptibility patterns should be obtained.

Overall, we recommend employment of this assay to detect the presence of *Pseudomonas* spp., including *P. aeruginosa*, from primary culture plates that originate from laboratory analysis of CF patients' sputum, particularly at review, in those patients with no previous history of *Pseudomonas*

infection or in those patients who appear to be transiently colonized by this organism. Employment of molecular methodologies, in conjunction with routine clinical sputum cultures, may provide improved information on the microbial status of CF patients, which will aid clinicians in both optimum management of patients and CF centre infection-control practices.

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