Molecular epidemiology of *Pseudomonas aeruginosa* in adult patients with cystic fibrosis in Northern Ireland

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ABSTRACT

Isolates (n=51) of *Pseudomonas aeruginosa* obtained from the sputa of 29 adult patients attending the Regional Cystic Fibrosis Centre in Northern Ireland were compared using an enterobacterial repetitive intergenic consensus sequence (ERIC2) primer in a random amplification of polymorphic DNA (RAPD) polymerase chain reaction (PCR) method. Resulting banding patterns showed a high degree of genetic heterogeneity among all isolates from the patients examined, suggesting a non-clonal relationship between isolates from these patients, when employing this genotyping technique.

KEY WORDS: Cystic fibrosis.
Genotyping.
Pseudomonas aeruginosa.

Introduction

The most common complication of cystic fibrosis (CF) is the recurrence of chronic chest infection, usually caused by bacterial pathogens.[1,2] Patients with CF suffer from recurrent and chronic respiratory tract infections and most morbidity and mortality is due to such infections.[3] These infections are usually dominated by Gram-negative organisms, especially by the pseudomonads including *Pseudomonas aeruginosa*, *Burkholderia cenocepacia* and *Stenotrophomonas maltophilia*, where *P. aeruginosa* is the single most important pathogen in this patient population, leading to increased morbidity and mortality.

Molecular genotyping of *P. aeruginosa* is of major importance in the elucidation of transmission routes. Genetic variability of isolates from CF patients may be compared by examination of phylogenetic distances, which can give an important insight into the interrelationship of bacterial isolates. Detailed genetic analysis at the subspecies (strain) level gives insights into the variability within a bacterial population and generates evidence on genome evolution, which leads in turn to bacterial adaptation to various environmental conditions. This information can be used in a clinical setting to identify pan-resistant strains of *P. aeruginosa*, thus preventing cross-infection by separating those patients who have the pan-resistant strain from those who do not have the pan-resistant strain.

This group previously has not reported the molecular epidemiology of adult CF patients from Northern Ireland. Therefore, the aim of this study is to examine the genetic relatedness of *P. aeruginosa* isolated from the sputa of adult CF patients attending the Northern Ireland Regional Adult Cystic Fibrosis Unit at Belfast City Hospital through employment of an enterobacterial repetitive intergenic consensus sequence (ERIC2) primer in a random amplification of polymorphic DNA (RAPD) polymerase chain reaction (PCR) method.

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Materials and methods

Source of P. aeruginosa *isolates*

Fresh sputum (minimum of 1 mL) specimens were collected in sterile (100 mL) plastic disposable containers from 29 adult patients (19 male, 10 female) with a well-characterised history of CF. Patients ranged in age from 17 to 30 years, and one was 70 years old. Sputum was collected immediately after a standardised session of physiotherapy and then stored at ambient temperature until processed within 4 h of collection. Fresh sputum was mixed with an equal mass of Sputasol (Oxoid SR089A, Oxoid, Poole, England) and was incubated in a water bath at 37°C for 15 min, before further qualitative processing for the detection of *P. aeruginosa*.

Processed sputa (10 µL) were inoculated and incubated on several selective media for the isolation of *P. aeruginosa*, including Columbia blood agar (CBA; Oxoid CM0331) supplemented with 5% (v/v) defibrinated horse blood, MacConkey agar (Oxoid CM0007) and *Pseudomonas* isolation agar (PIA; Oxoid CM0559 + SR0102). All media were incubated aerobically at 37°C for 48 h unless otherwise stated. The PIA plates were incubated at room temperature

for a further three days following the initial 48-h incubation. The majority of patients had sputum collected at two different time intervals for the isolation of *P. aeruginosa*. For each patient at each sampling time, the predominant morphotype was picked from the presumptive colonies that phenotypically resembled *P. aeruginosa* on the PIA selective plate.

***Phenotypic and genotypic confirmation of* P. aeruginosa**

All *P. aeruginosa* isolates were identified phenotypically by a combination of conventional identification methods (e.g., oxidase) and the API identification scheme (API 20NE; bioMérieux, Les Halles, France). In addition, all isolates were confirmed by PCR for the detection of the *groES/groER* gene locus[4] and the *opr* gene locus.[5]

DNA extraction

Genomic DNA was extracted from confirmed isolates of *P. aeruginosa*. Isolates were cultured on CBA (Oxoid CM0331) supplemented with 5% (v/v) defibrinated horse blood for 24 h at 37˚C. All DNA isolation procedures were carried out in a class II biological safety cabinet (MicroFlow, England) 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 procedure (GMDP) guidelines of Millar *et al*.,[6] in order to minimise contamination and hence the possibility of false-positive results.

Bacterial genomic DNA was extracted from a single colony of *P. aeruginosa* from each isolate under standard conditions using the Roche High Purity PCR Template Preparation Kit (Roche, England), in accordance with the manufacturer's instructions. Extracted DNA was stored at –80˚C prior to PCR amplification. A negative extraction control containing all reagents but no organism was performed for each batch, along with a positive control with *P. aeruginosa*.

RAPD PCR amplification

All reaction mixes were set up in a PCR hood in a room separate from that used to extract DNA, and also from the amplification and post-PCR room, in order to minimise contamination. Initially, PCR amplification conditions were optimised by separately varying magnesium chloride concentration, annealing temperature, primer concentration and DNA template concentration. Following optimisation, reaction mixes (25 µL) were set up as follows: 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 200 µmol (each) dATP, dCTP, dGTP and dTTP, 1.25 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Amplitaq, Perkin Elmer), 0.1 µmol of the RAPD primer ERIC2 (5′ – AAG TAA GTG ACT GGG GTG AGC G – 3′) and 1 µL DNA template.

Following a 'hot start', the reaction mixtures were subjected to the following empirically optimised thermal cycling parameters in a Perkin Elmer 2400 thermocycler: 94˚C for 5 min then 40 cycles of 94˚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. Multiple negative (water) amplification controls were included in every set of PCR reactions.

Detection of amplicons

Following amplification, aliquot 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). In order to improve resolution of the images, resulting PCR amplicons were resolved on 2% (w/v) Metaphor agarose (Flowgen, UK), as described above.

For improved resolution of bands, ERIC2 PCR products were run on a polyacrylamide (PAGE) gel using the ExcelGel DNA analysis kit (Amersham Pharmacia, UK), in accordance with the manufacturer's instructions, and was performed on a Multiphor II electrophoresis unit (Amersham Pharmacia, UK) at 600 V/50 mA/30 watts for 85 minutes, with the thermostatic circulator set to 14˚C.

Following electrophoresis, the bands were visualised by silver staining, employing the PlusOne DNA silver staining kit (Amersham Pharmacia, UK). Ethidium bromide-stained agarose 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). Silver-stained PAGE gels were scanned on an AGFA Snapscan 310 scanner and the images saved as *.tif format files.

Interpretation of banding patterns

All ERIC2 RAPD *.bmp images were converted to *.tif format files for importation into the GelCompare software (Applied Maths, Belgium). The GelCompare software normalised data from separate PAGE gels, according to sets of molecular size standards run at regular intervals through each gel. Subsequent alignment by the software allowed for inter- and intra-gel inconsistencies and variations in electrophoresis conditions to be corrected. A dendogram for cluster analysis was produced, based on similarity matrices calculated from the Pearson correlation UPGMA algorithm.

Results and discussion

All *P. aeruginosa* isolates examined in this study generated an ERIC2 RAPD banding pattern, ranging in size, from approximately 250 bp to 2000 bp, with between three and 15 bands per isolate, and a mean band number of 9.2 bands per isolate (Fig. 1). Overall, there was a high degree of clonal heterogeneity between all the isolates examined. No two isolates examined shared a similar banding profile, with the most closely related isolates (isolates 47 and 48, and isolates 18 and 19) showing two and three band shifts, respectively.

Performing molecular epidemiological studies with isolate collections such as those studied here is important to help understand acquisition risk factors for *P. aeruginosa* and to help trace epidemic strains within such patient populations, as well as to help identify common virulent and/or transmissible strains. Previously, several molecular typing schemes have been applied to help elucidate the molecular epidemiology of populations of *P. aeruginosa* in CF units, and these methodologies have been reviewed comprehensively by Speert.[7] The present study employed an RAPD method, with ERIC2 as the arbitrary primer.

The choice of method and primer was driven largely by a previous publication by Renders *et al*.,[8] who reported positively on the value of this method against pulsed-field gel electrophoresis (PFGE). In their study, these workers

demonstrated that RAPD using the ERIC2 primer could expect full concordance with typing data obtained by PFGE when single band differences are neglected. In addition, they showed a higher degree of genetic relatedness among the 19 CF patients examined, as demonstrated by highly similar banding profiles, which was not the case in the present study.

As there were no single band differences between any two isolates in the current study, the closest relationships between strains started with a minimum of two and three band shifts, equating to 0.95 (95%) genetic relatedness For example, isolates 47 and 48 were from the same CF patient, but taken one year apart, while isolates 18 and 19 were from the same sputum on the same day. In the absence of robust and well-tested interpretation criteria for *P. aeruginosa* using the RAPD method in conjunction with the ERIC2 primer, we set stringent criteria arbitrarily, namely that two or more band shifts represented a different clonal type of *P. aeruginosa*. Using such criteria, we were not able to identify any clonally related clusters in the Belfast CF centre.

Unlike the Renders *et al*. study,[8] which compared RAPD with PFGE typing techniques, the current study employed RAPD alone as the genotyping tool of choice. This was based on the demonstrated concordance of typing results between RAPD and PFGE by Renders *et al*.,[8] who employed the same arbitrary primer as used in the current study, as well as taking account of the relative expensive, slow throughput and complexity of the PFGE technique as factors that would favour the use of RAPD in the clinical microbiology setting.

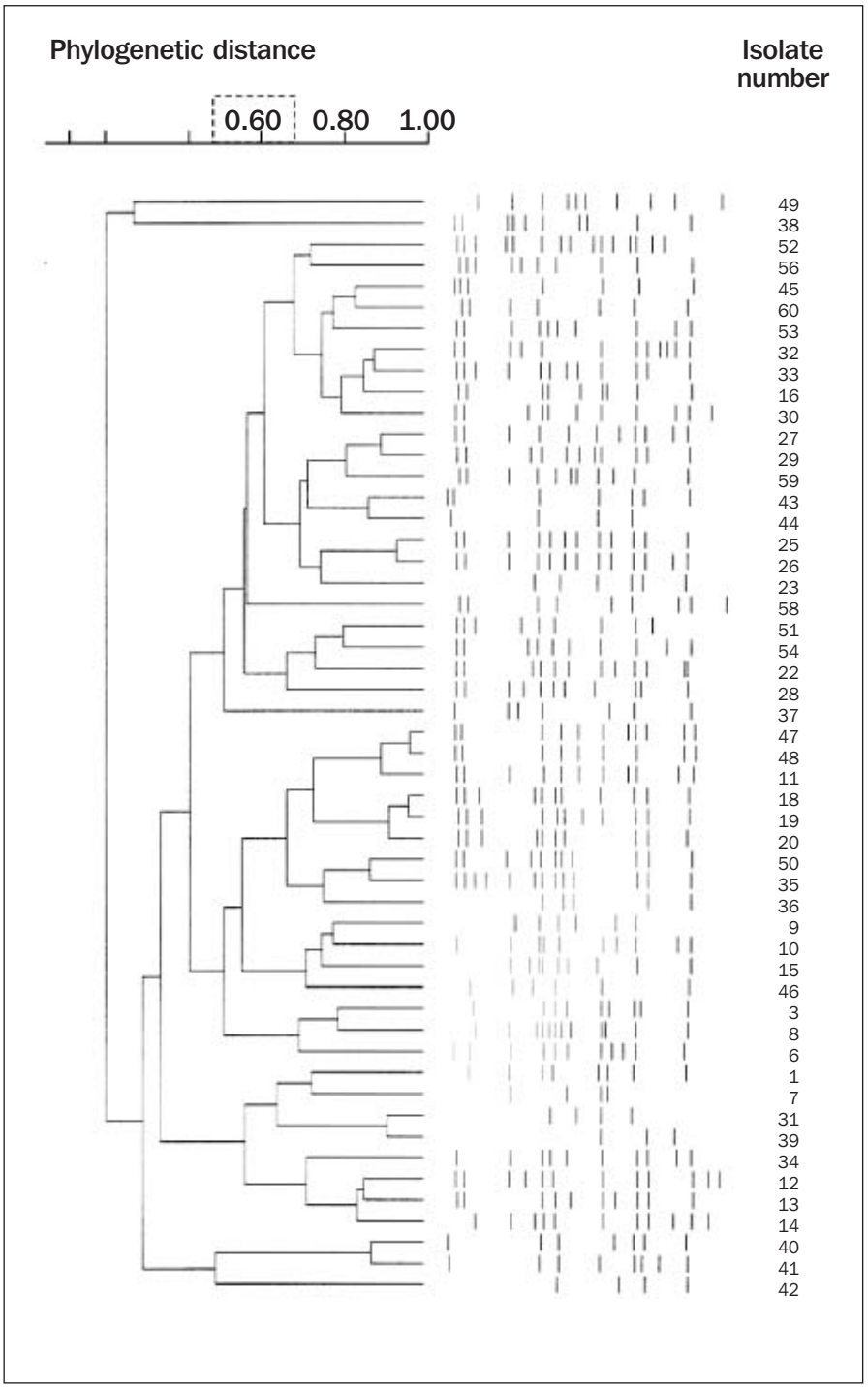

Fig. 1. Dendogram based on the phylogenetic relatedness of 51 isolates of *Pseudomonas aeruginosa* examined by ERIC2-RAPD-PCR.

Employment of ERIC2 RAPD PCR in the current study demonstrated greater clonal diversity among the populations of *P. aeruginosa* examined than was described by Renders *et al*.,[8] in their study of *P. aeruginosa* from CF patients in Belgium.

The reasons for these differences merit further exploration and discussion. Firstly, one must consider the reproducibility of the RAPD technique. Previously, this technique has been described generically as being hard to reproduce and may suffer from methodological artefacts (e.g., concentration of bacterial genomic DNA, concentrations of PCR components and PCR cycling conditions). However, in a previous study by Campbell *et al*., using RAPD with 200 *P. aeruginosa* isolates, identical typing results were obtained for 197/200 isolates when tested in triplicate, resulting in the identification of 131 distinct RAPD types.[9]

In order to address this widely held view of RAPD as a genotyping technique, the authors were acutely aware of such potential pitfalls in the methodology and thus were conscious of the need for complete standardisation of the technique. This was accomplished by using reagents from a single batch, employing a PCR technique that had undergone rigorous optimisation of annealing temperature and magnesium concentration.

In the authors' experience, on a day-to-day basis, a single band shift may have occurred when employing this technique, although this did not alter the clustering of the isolates and hence did not affect the overall relationship between the isolates. However, such differences may be significant when comparing data sets between laboratories, even when similar PCR reagents and protocols are used. Therefore, it would be unwise to make direct banding comparison between the current study and that undertaken by Renders *et al.*,[8] except to comment on the relative degree of clonal heterogeneity/homology described by both studies.

Furthermore, by accepting the relatively greater diversity shown by the Northern Ireland *P. aeruginosa* isolates, in comparison to those in the Belgian study, this would indicate that the Northern Ireland patients were acquiring these isolates from a much wider (and less related) environmental pool, which requires further investigation.

In conclusion, the present study demonstrates a high degree of clonal diversity between isolates in the collection examined, wherein no isolates shared an identical ERIC2 banding pattern. Importantly, ERIC2 RAPD PCR proved to be a highly discriminatory molecular typing tool for *P. aeruginosa* isolates recovered from patients with CF. □

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