RESEARCH ARTICLE

Molecular identification and genotyping of Pseudomonas aeruginosa isolated from cystic fibrosis and non-cystic fibrosis patients with bronchiectasis

Nadia Eusebio¹,², Adelina A. Amorim³, Fernanda Gamboa⁴ and Ricardo Araujo¹,²,*

¹IPATIMUP, Institute of Molecular Pathology and Immunology of the University of Porto, Rua Dr. Roberto Frias s/n, 4200-465 Porto, Portugal, ²Faculty of Sciences, University of Porto, Rua do Campo Alegre s/n, 4169-007 Porto, Portugal, ³Department of Pneumology, Faculty of Medicine of the University of Porto and Hospital S. João, Alameda Prof. Hernani Monteiro, 4200-319, Porto, Portugal and ⁴Serviço de Pneumologia, Hospital Universidade Coimbra-CHUC, EPE, Rua Fonseca Pinto, 3000-075 Coimbra, Portugal

*Corresponding author: IPATIMUP, Institute of Molecular Pathology and Immunology, University of Porto, Rua Dr. Roberto Frias, s/n; 4200-465 Porto, Portugal. Tel: +351 225570700; Fax: +351 225570799; E-mail: ricjparaujo@yahoo.com

ABSTRACT

There is no standard methodology for the molecular identification and genotyping of Pseudomonas aeruginosa which are frequently isolated in bronchiectasis patients. Hence, the main goal of this work was to propose a methodology capable to simultaneously identify and genotype, in less than 6 h, clinical P. aeruginosa collected from cystic fibrosis (CF) and non-CF patients with bronchiectasis. Molecular analyses were conducted in clinical isolates by testing the newly colony-PCR strategy and SNaPaer assay. A total of 207 isolates of P. aeruginosa were collected from clinical samples. To assess the assay specificity, other Gram-negative non-aeruginosa bacteria, namely Pseudomonas and Burkholderia, were tested. The complete group of 23 markers included in the SNaPaer panel was observed exclusively in P. aeruginosa; more than 18 markers failed in other bacteria. A total of 43 SnaP profiles were obtained for clinical P. aeruginosa, being the profiles highly patient-specific. Six CF patients were colonized with P. aeruginosa isolates with very distinct SnaP profiles, particularly following adjustments on antibiotic therapy, thus suggesting changes on the dynamics and dominance of these bacteria. SnaPaer proved to be a good and reliable tool for identification and genotyping of clinical P. aeruginosa in a single-tube multiplex PCR. Combined with the proposed colony-PCR strategy, SnaPaer assay facilitates the molecular analysis of P. aeruginosa.

Key words: bacterial identification; bronchiectasis; genetic diversity; MLST; multiplex primer extension assay; SNP; SNaPaer

INTRODUCTION

Bronchiectasis is an abnormal and irreversible dilation of the bronchi, resulting from a vicious cycle of inflammation, recurrent infection and bronchial impairment (Goeminne and Dupont 2010). The diagnosis of lung condition is frequently achieved late and is established by the characteristic features of the high-resolution computed tomography (HRCT) of the
thorax (Fuschillo, De Felice and Balzano 2008; White et al., 2012). Bronchiectasis is presently reported in more than 50% of cystic fibrosis (CF) patients and is associated with increased morbidity and mortality (Corbyn 2012). The classic symptoms of bronchiectasis are chronic cough and daily mucopurulent sputum production associated with recurrent acute exacerbations (Reid et al., 2013).

*Pseudomonas aeruginosa* is frequently isolated in bronchiectasis patients, particularly CF patients (Vonberg et al., 2005). Nevertheless, several studies suggest that *P. aeruginosa* infection can be underdiagnosed particularly in patients with idiopathic bronchiectasis (King et al., 2007; Fuschillo et al., 2008). The occurrence of *P. aeruginosa* is associated with a decrease of pulmonary function and frequent exacerbations (Davies et al., 2006; O’Donnell 2008; Reid et al., 2013). The first stage of *P. aeruginosa* colonization can be controlled by antibiotic treatment that eradicates rough and smooth initial populations of *P. aeruginosa* (Burns et al., 2001).

Early identification and genotyping of bacteria allow the administration of more directed and intense antibiotic therapy that prevents *P. aeruginosa* chronic infection. A standard molecular methodology for the identification and genotyping of *Pseudomonas* species still needs to be achieved. Multilocus sequence analysis and multilocus sequence typing (MLST) have been suggested as excellent alternatives for identification and separation of closely related bacterial species (Maiden 2006; Martens et al., 2008; Almeida and Araujo 2013). In a previous work, we have standardized a practical and sensitive SNaPser assay for *P. aeruginosa* genotyping revealing a discriminatory power of 0.9993 compared with MLST (Eusebio et al., 2013b). Clinical *P. aeruginosa* may greatly differ in growth, morphology or pigmentation but phenotypically distinct isolates may have the same genotype when few loci are affected (Breitenstein et al., 1997; Winstanley et al., 2009).

The present work proposes a molecular strategy that allows identification and genotyping of clinical *P. aeruginosa* in bronchiectasis patients in less than 6 h. Therefore, the study was designed accordingly: (1) to develop a practical and less time-consuming procedure using colony-PCR to obtain DNA for molecular studies; (2) to confirm the ability of MLST and SNaPser assays to identify *P. aeruginosa* by testing a group of Gram-negative non-*aeruginosa* bacteria; (3) to apply the proposed molecular strategy in clinical samples, testing its ability to identify and genotype *P. aeruginosa* collected from bronchial secretions and (4) to characterize the dynamics and dominance of the different bacterial genotypes in patients with CF and non-CF bronchiectasis.

**MATERIALS AND METHODS**

**Subjects and clinical data**

After obtaining institutional and ethics committees approval, biological secretions (sputa) from a group of 19 patients with bronchiectasis admitted to the ‘Centro Hospitalar de São João’ (Porto, Portugal) and ‘Hospitais da Universidade de Coimbra’ (Coimbra, Portugal) were collected consecutively between 2009 and 2012. The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. A written informed consent was obtained from all participating patients. Key inclusion criterion included evidence of bronchiectasis, diagnosed by HRCT. The severity of the disease was characterized by the presence of symptoms such as chronic cough, dyspnea and sputum production. Another key inclusion criterion was the evidence of CF, whose diagnosis was based on two positive sweat (chloride) tests and/or the identification of the mutations in cystic fibrosis transmembrane conductance regulator (CFTR) gene. Using these methodologies, 13 patients were diagnosed with CF, while the remaining 6 patients were diagnosed with non-CF bronchiectasis (history of tuberculosis and HIV was described in a single patient; five other patients presented idiopathic bronchiectasis). Personal information and clinical history for each patient were registered, including history of exacerbation and antibiotic treatments, bacterial colonization, pulmonary function, forced expiratory volume on the first second, radiographic abnormalities, hemoptysis and other associated diseases.

All patients included in this study presented at least 50% of positive samples for *P. aeruginosa* in the preceding 12 months, being characterized as chronically colonized (Pressler et al., 2011). Generally, the bronchiectasis patients showed a stable clinical picture punctuated by exacerbation episodes (at least one exacerbation episode during the observational period). Exacerbation was defined as an increase of the bronchiectasis symptoms and requiring course of oral or intravenous antibiotics (White et al., 2012).

**Control and clinical bacteria and sample collection**

A group of Gram-negative non-*aeruginosa* bacteria were included as controls of bacterial identification: 10 *Pseudomonas* (*P. fluorescens* Po–1, *P. putida* KT2440, *P. savastanoi* pv. *glycinea* 5066, *P. savastanoi* pv. *phaseolicola* 2245, *P. syringae* pv. *helianthi* 5067, *P. syringae* pv. *maculicola* 5071, *P. syringae* pv. *oryzae* 10912, *P. syringae* pv. *syringae* 10604, *P. syringae* pv. *tabaci* 5393 and *P. syringae* pv. *tomato* DC 3000, kindly provided by Prof. Fernando Tavares, Faculty of Science of the University of Porto); 2 *Xanthomonas* (X. *axonopodis* pv. *citri* 9322 and X. *campestris* pv. *campestris* 558, kindly provided by Prof. Fernando Tavares) and 4 clinical isolates of *Burkholderia cenocepacia*.

Single colonies of each isolate were suspended in 5 mL of Lysogeny broth medium and grown overnight with 180 rpm agitation at 37°C. DNA was extracted from cells as previously described (Cheng and Jiang 2006). At the end, two cycles of DNA purification with 70% ethanol were performed. Bacterial DNA was resuspended in 50 μL of ultrapure water and stored at −20°C.

Patient specimens were cultured on Cetrimide Agar plates for culture of *P. aeruginosa*. The plates were incubated at 37°C for a period of 48 to 72 h. Isolated bacterial colonies were recultured in new Cetrimide Agar plates for further molecular assays (a maximum of eight random colonies were tested per clinical sample whenever possible to evaluate intra-patient bacterial genetic diversity). A total of 46 clinical samples were collected from the patients and a total of 207 isolates of *P. aeruginosa* were obtained for further confirmation of identification and genotyping analysis.

**Colonies-PCR preparation**

For colony-PCR, bacteria were cultured as described above and used straight away skipping the bacterial DNA extraction steps. This procedure was used in order to simplify the molecular tests with *P. aeruginosa* in clinical and other laboratories. A total of 207 colonies of clinical *P. aeruginosa* collected from patients with bronchiectasis were processed. Briefly, small amount of bacteria was suspended in 20 μL ultrapure water, vortexed and heated at 95°C for 60 min. Then, this mixture was incubated at 56°C for 60 min with 2 μL of Proteinase K (at 2 mg mL⁻¹; Finnzymes).
Proteinase K was inactivated by vortexing and heating the mixture at 100°C for 8 min. Subsequent centrifugation was performed at 14,000 rpm for 4 min and the final mixture (supernatant) collected and stored at −20 °C.

**SNaPaer assay**

The amplification of MLST genes was performed as previously described (Eusebio et al., 2013b). SNaPaer assay targets single nucleotide polymorphisms (SNPs) critical for characterization of *P. aeruginosa* isolates following multiplex PCR (Eusebio et al., 2013b). Amplicons sizes were evaluated after separation by polyacrylamide gel electrophoresis and standard silver-staining detection (Qu et al., 2005). SNaPaer automated electrophoretic assays were carried out in a final volume of 5 μL, containing 1.5 μL of PCR product (purified with ExoSap-IT, as described above), 1 μL of SNaPaer primer mix (each one at 1 μM), 1 μL of ABI Prism SNaPshot® Multiplex Kit (Applied Biosystems) and 1.5 μL of ultrapure water (Eusebio et al., 2013b). Unincorporated ddNTPs were removed with 1 U of SAP (shrimp alkaline phosphatase; USB Corporation) following incubation for 1 h at 37 °C and 15 min at 85 °C, as suggested by the manufacturer. The products (0.5 μL) were mixed with 9.0 μL of HiDi™ formamide (Applied Biosystems) and 0.5 μL of GeneScan-120 LIZ size standard (Applied Biosystems). Electrophoresis was performed on a 3130xl Genetic Analyzer (Applied Biosystems) using filter set E5 and the data analyzed with the software GeneMapper version 4.0 (Applied Biosystems).

**Data and statistical analyses**

The capability of the MLST for identification of *P. aeruginosa* was tested in silico. A set of 45 *Pseudomonas* species complete genomes available at NCBI database were downloaded to an in-house record; 12 were complete genomes of *P. aeruginosa*. The sequences of the genes *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA* and *trpE* were identified in each genome using Geneious Pro 6.0.6 software (Biomatters Ltd). Concatenated MLST data were obtained by extracting the information from the complete genome of these strains. MLST data were aligned employing Clustal W (Thompson, Higgins and Gibson 1994). Phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei 1987) and the genetic distances were computed using the Maximum Composite Likelihood model; bootstrap values were calculated for tree nodes. Similar procedure was followed with SNaPaer data by testing a set of SNaP profiles obtained from the 45 genomes described before for extraction of MLST data. MLST and SNaP methodologies are interrelated and the sequencing results can be easily converted for both systems and compared (Soares and Araujo 2014). More details of these phylogenetic analyses can be consulted in (Figs S1 and S2, Supporting Information).

The Network software v4.6.1.0 (www.fluxus-engineering.com/sharenet.htm) (Polzin and Daneschmand 2003) was used to better visualize the distribution of bacterial haplotypes. Additional MLST data were retrieved from the database available online (http://pubmlst.org/paeruginosa/).

**RESULTS**

**Testing SNaPaer in non-aeruginosa *Pseudomonas* bacteria**

In order to evaluate the suitability of the SNaPaer assay for *P. aeruginosa* identification, this assay was tested in a group of non-*aeruginosa* bacteria. Atypical gel MLST profiles were observed in non-*aeruginosa* bacteria (Fig. 1), which in some strains barred the amplification of some MLST genes and in others led to the appearance of unspecific bands. Automated electrophoresis involved in SNaPaer assay showed the absence of several markers in the electropherograms of non-*aeruginosa* bacteria. Accurate and reliable analysis of the SNaPaer electropherograms included the exclusion of peaks with height below 50; the lengths of the fragments were carefully evaluated and displacements exceeding in 2 bp the theoretical position of *P. aeruginosa* peaks were eliminated. The markers G264, P268, N255, G6, T331 and A491 were found in non-*aeruginosa* tested bacteria; the markers Ac78, N162, A98, M9, G49, Ac7, T205 and Ac387 were...
occasionally found in this group of bacteria. The markers A264, G219, M204, Ac339, M36, P100, M228, N288 and T349 were never observed in non-
P. aeruginosa bacteria and clearly represented specific markers for P. aeruginosa (Table 1).

Genotyping of clinical P. aeruginosa

A collection of 207 P. aeruginosa isolates (109 from CF patients and 98 from non-CF patients with bronchiectasis) was genotyped using colony-PCR for amplification of MLST genes and further by applying SNaP assay (Table S1, Supporting Information). The complete group of markers was amplified in these isolates giving a final SNaP profile with 23 SNPs for each isolate. SNaP genotyping revealed 43 distinct SNaP profiles (defined by difference in at least one marker) among the 207 isolates; 35 SNaP profiles were observed in the CF patients (n = 13) and 9 SNaP profiles in the non-CF patients with bronchiectasis (n = 6). The SNaP profile analysis revealed that 20 out of the 23 SNP markers were polymorphic in this set of clinical isolates confirming the panel practicability. Most SNaP profiles were patient-specific (Fig. S3, Supporting Information) with the exception of three SNaP profiles which were found in different CF patients.

Non-CF bronchiectasis patients were predominantly colonized by isolates with single SNaP profiles (67% of patients), while most CF patients (75%) were colonized by multiple SNaP profiles. The probability for the observation of multiple SNaP profiles was higher in CF patients versus patients with idiopathic bronchiectasis (Table S2, Supporting Information). Pseudomonas aeruginosa colonies that were phenotypically distinct also showed a distinct SNaP profile, but few isolates collected from the same patient showed different phenotype and similar SNaP profile.

Several cases of microvariation or ‘variants’ (difference in a single marker when considering the group of isolates collected from the same patient) were observed in CF and non-CF, whether under antibiotic therapy or not. The ‘variants’ were more common in CF patients than in patients with idiopathic bronchiectasis (60% versus 30% of the patients; Table S1, Supporting Information). For six CF patients, very distinct SNaP profiles (six to eleven markers were distinct) were detected in consecutive samples, but a correlation between antibiotic therapies and the modification of SNaP profile could not be established, as the patients were subjected to different antibiotic regimes and the study considered a short period (2–3 yr) of patient follow-up (Fig. 2 and Table S2, Supporting Information). Pseudomonas aeruginosa isolates collected from exacerbation periods revealed SNaP profiles similar to the stable phases on the disease.

Identification of clinical isolates of P. aeruginosa by SNaP

To date, some MLST schemes have shown to be useful for identification of closely related species (Maiden 2006; Martens et al., 2008; Almeida and Araujo 2013). With the phylogenetic analyses of the MLST data obtained from complete genome sequences of 45 Pseudomonas species (details in Fig. S1, Supporting Information), we showed that MLST was capable to group the 12 P. aeruginosa strains apart from the remaining species, including two strains of P. mendocina, a taxonomic closely related P. aeruginosa species (Anzai et al., 2000). Similar in silico phylogenetic tests were conducted using the information of the 23 SNPs included in the SNaP assay (details in Fig. S2, Supporting Information). The first in silico analyses of SNaP data proved that the method was capable to identify typical isolates of P. aeruginosa, although
Eusebio et al.

Figure 2. Examples of cystic fibrosis (CF) patients with multiple SNaP profiles: (A) SNaP profile and isolation dates; (B) Antibiotic therapy received by four CF patients (all these patients received azithromycin during the entire described period).

DISCUSSION

In the previous work performed, SNaPaer assay has been standardized for P. aeruginosa genotyping and a discriminatory power similar to MLST was observed (Eusebio et al., 2013b). This genotyping method showed high reproducibility and in this study it could also be used for identification of clinical P. aeruginosa isolates following a colony-PCR approach. MLST gene amplification was conducted employing bacterial cells and pre-treatment with proteinase K. This modification to the standard protocol allowed a quicker and more practical molecular test of clinical P. aeruginosa. The removal of DNA extraction steps saves several hours of routine and laboratorial work. The successful application of this simplified approach to 207 isolates confirmed its usefulness in clinical laboratories.

Clinical isolates of P. aeruginosa have been described as being highly diverse by several research groups worldwide (van Mansfeld et al., 2010; Cramer et al., 2011; Eusebio et al., 2013b; Hogardt and Heesemann 2013). SNaPaer assay was used here for genotyping of P. aeruginosa obtained from bronchial samples of chronic CF and non-CF patients with bronchiectasis collected consecutively. Both CF and non-CF patients presented bacteria with the same genotype in sequential samples. Patients were colonized with isolates with closely related SNaP profiles (variants), mainly the CF patients. This is a consequence of the well-known microevolution in patients with chronic colonization due to the plasticity of P. aeruginosa genome to accumulate changes and adapt to a stressful pulmonary environment (van Mansfeld et al., 2010; Cramer et al., 2011; Eusebio et al., 2013b; Hogardt and Heesemann 2013).

Portuguese bronchiectasis patients (CF and non-CF) were generally colonized by specific P. aeruginosa strains, each patient colonized with P. aeruginosa presenting a very distinct SNaP profile from the other. These results concur with previously reported data where patient-specific SNaP profiles have been observed (van Mansfeld et al., 2010). Independently acquired P. aeruginosa by CF and non-CF patients is very frequent; cross-transmission or cross-infection in clinical units cases is in fact only occasionally reported (Vonberg et al., 2005). Once acquired, chronic P. aeruginosa is very difficult to eradicate (White et al., 2012, Wiehlmann et al., 2012, Reid et al., 2013). The patients included in this study were chronically colonized by P. aeruginosa for short (1 yr) or long (more than 10 yr) periods confirming the discriminatory capacity of the SNaPaer assay as a fast and reliable genotyping method for both groups of patients.

Bronchiectasis patients with chronic P. aeruginosa selected for this study showed a stable clinical state punctuated by exacerbation episodes which were frequently controlled by ciprofloxacin and/or levofloxacin. Occasionally other antibiotics and more intensive regimens were administered. Isolates collected from exacerbation periods revealed P. aeruginosa with similar SNaP profile to those collected from clinically stable phases, as
previously reported for CF patients (Aaron et al., 2004; Tunney et al., 2013). Six CF patients were found colonized by multiple P. aeruginosa with very distinct profile, particularly following changes of antibiotic therapy. It is known that antibiotics alter the dynamics and dominance of P. aeruginosa genotypes found in the patient lungs and similar observations were found on our patients. This fact highlights the importance of characterizing completely the bacteria in patients with bronchiectasis as it can be critical for definition of the accurate timing for antibiotic therapies. The emergence of new P. aeruginosa genotypes in the presence of ciprofloxacin was recently reported, revealing forms with 32- to 192-fold greater minimal inhibitory concentrations (Wong, Rodrigue and Kassen, 2012). Therefore, it remains critical to understand the consequence of certain antibiotic regimens to the dynamics of microbial communities and to the emergence of new genotypes in the lungs of bronchiectasis patients.

The presence of multiple strains of P. aeruginosa is more common in CF patients and has additional implications. As a standard practice in clinical microbiology laboratories, a single bacterial colony per patient is used for identification or susceptibility testing, unless the bacterial isolates show different phenotypes. This practice loses essential information to guide antimicrobial therapy in patients colonized by multiple strains as these colonies can be phenotypically similar, though with a very distinct genetic profile. Hence, the value of genotyping data for complete characterization of patient colonizers is unquestionable.

The ability of SNaPPaer assay to identify of P. aeruginosa in the laboratory was also evaluated. First, the phylogenetic analysis of the MLST and SNaP profiles from clinical isolates of CF and non-CF patients was shown to be clearly distinct from the profiles of other Pseudomonas species. Furthermore, MLST gene amplification was tested in a group of non-aeruginosa bacteria and typical gel banding patterns were only observed for P. aeruginosa. The analysis of the 23 SNPs demonstrated that nine markers (A264, G219, M204, Ac339, M36, P100, M228, N288 and T349) were clearly specific of P. aeruginosa. Therefore, P. aeruginosa isolates could be easily distinguished from other species by typical gel bands and SNaPPaer electropherogram with 23 peaks. The primers included in this kind of SNP based panels are very specific as recently shown to B. cenocepacia (Eusebio et al., 2013a). Nevertheless, it cannot be excluded that atypical P. aeruginosa can be neglected by SNaPPaer assay and some markers fail the amplification. When necessary, it can be considered testing other markers in atypical isolates and include the pertinent SNP markers in the SNaPPaer panel as the methodology allows such addition of extra markers. Hypermutable variants have been reported in CF and non-CF patients concerning isolates with reduced motility, deficiencies in DNA repair systems, increased genetic mutations, drug resistance and reduced production of type II and III secretion effector determinants (Oliver et al., 2000; Maciá et al., 2005). It would be useful in a near future to test the feasibility of the proposed molecular methodology for identification and genotyping of hypermutable isolates and other collections of isolates, mainly testing non-clinical P. aeruginosa.

An important achievement of this work was the successful establishment of a colony-PCR strategy that avoids DNA extraction for molecular tests and facilitates the molecular analyses of bacterial populations. Molecular identification of P. aeruginosa is valuable for clinical and environmental laboratories. The ability to perform simultaneous genotyping analyses in a single-tube multiplex PCR can be seen as an added value of molecular tests. Bacterial identification and genotyping can in fact be obtained in a single reaction without increased cost (€10 per sample) and in less than 6 h. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a powerful approach that has been adopted by several clinical laboratories to identify clinical bacteria and more recently tested for P. aeruginosa genotyping (Syrmis et al., 2013). A comparison of SNaPPaer assay versus MALDI-TOF MS can be very interesting, and therefore collaborative studies with more isolates should assess the value of each methodology for testing isolates in the clinical laboratory.

SUPPLEMENTARY DATA
Supplementary data is available at FEMSPD online.

ACKNOWLEDGEMENTS
The authors thank to Fernando Tavares (Faculty of Science, University of Porto) by providing some of the bacterial isolates included in the study.

FUNDING
This work was supported by a grant from Fundação Calouste Gulbenkian (nº. 35-9924-S/2009). RA is supported by Fundação para a Ciência e a Tecnologia (FCT) Ciência 2007 and by the European Social Fund. IPATIMUP is an associate laboratory of the Portuguese ministry of science, technology and higher education and is partially supported by FCT.

Conflict of Interest
The authors declare no conflict of interest. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES
Nucleic Acids Res et al.


