Typing of Pseudomonas aeruginosa in cystic fibrosis patients: comparison of genotype and antimicrobial susceptibility

Tipizacija Pseudomonas aeruginosa pri bolnikih s cistično fibrozo: primerjava genotipa in občutljivost za protimikrobna zdravila

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Abstract

Background: The aim of our study was to determine the genotypes and antimicrobial susceptibility profiles of *Pseudomonas aeruginosa* isolates of cystic fibrosis (CF) patients.

Methods: We included 18 *P. aeruginosa* isolates from 10 patients treated as in-patients or out-patients at University Clinic of Respiratory and Allergic Diseases Golnik between 1 January 2006 and 31 December 2008.

Results: We determined 8 pulsotypes of *P. aeruginosa*. Pulsotypes A and C had 4 subtypes each. Different antimicrobial susceptibility patterns were observed in isolates with closely related macrorestriction profiles.

Conclusions: We conclude that at a given time, patients with CF attending our clinic were colonized or infected with phenotypically and genotypically distinct strains of *P. aeruginosa*.

Izvleček

Izhodišče: Namen naše raziskave je bil ugotoviti genotipe in občutljivost za izbrane antibiotike izolatov bakterije *Pseudomonas aeruginosa*, ki smo jih osamili pri bolnikih s cistično fibrozo (CF).

Metode: V raziskavo smo vključili 18 izolatov bakterije *P. aeruginosa*, ki smo jih osamili pri 10 bolnikih, zdravljenih ambulantno ali v Bolnišnici Golnik – Klinični oddelek za pljučne bolezni in alergijo – v obdobju 1. 1. 2006–31. 12. 2008.

Rezultati: Ugotovili smo 8 pulsotipov bakterije *P. aeruginosa*. Pulsotipa A in C sta vsak imela 4 podtipe. Pri izolatih s tesno sorodnimi pulsotipi smo ugotovili različne profile občutljivosti na antibiotike.

Zaključki: V preučevanem obdobju so bili bolniki s CF kolonizirani ali okuženi s fenotipsko in genotipsko raznolikimi sevi bakterije *P. aeruginosa*.

Introduction

Cystic fibrosis (CF) is a hereditary multisystem disorder characterized by chronic pulmonary infection, pancreatic insufficiency, hepatobiliary disease, impaired fertility and increased sweat chloride content. The disorder is due to mutations in cystic fibrosis transmembrane conductance regulator (CFTR). Chronic pulmonary infection is the major cause of morbidity and mortality in CF. One of the first pathogens infecting the airways of CF patients for extended periods is *Staphylococcus aureus*.[1] Later in their teens and through adult life *Pseudomonas aeruginosa* becomes the predominant pathogen chronically colonizing airways of CF patients.[2,3] The organism is a Gram-negative, non-fermentative, aerobic bacillus belonging to the family Pseudomonadaceae. *P. aeruginosa* is a facultative anaerobe which can survive within the relatively hypoxic lungs of CF patients. During early stages of *P. aeruginosa* colonization / infection in patients with CF the bacteria are usually not mucoid, but when the condition becomes chronic the bacteria change to a mucoid phenotype and form biofilms. One interesting property of the *P. aeruginosa* strains found in the lungs of CF patients, which differentiates them from strains found in other clinical conditions, is that they are unusually hypermutable.[4,5]

P. aeruginosa is widespread in the natural environment and it is believed to be the most common source of colonization / in-

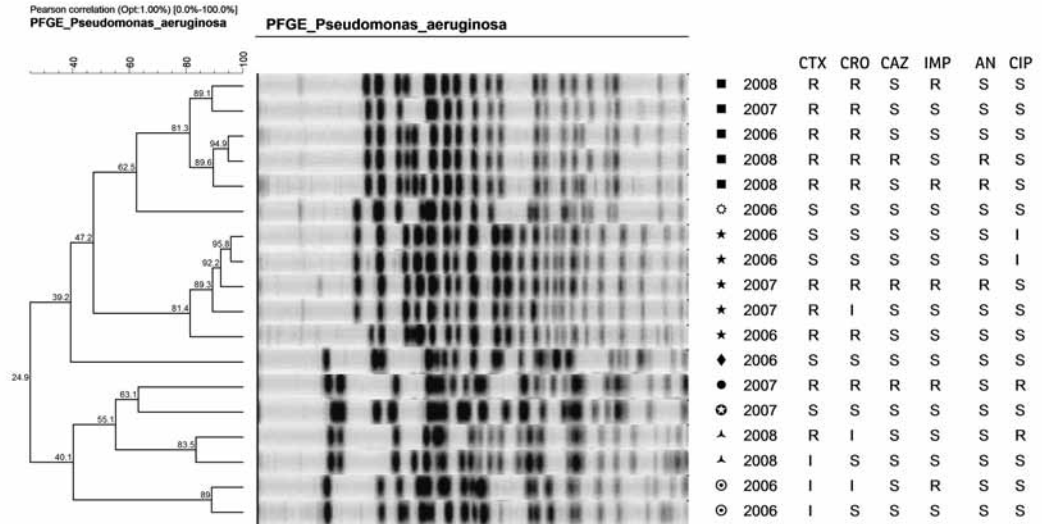

Figure 1: Digitized PFGE patterns and dendrogram of *P. aeruginosa* isolates cut with *SPe*I showing percent similarities of patterns and antimicrobial susceptibility to cefotaxime (CTX), ceftriaxone (CRO), ceftazidime (CAZ), imipenem (IMP), amikacin (AN) and ciprofloxacine (CIP). Susceptibility: S – susceptible, I – intermediately resistant, R – resistant. Pulsotypes: ■ A – A4, ☼ B, ★ C – C4, ♦ D, ● E, ✪ F, ⅄ G – G1, ⊙ H – H1

fection in CF patients. However, cross-infection is possible between related as well as unrelated individuals and has been reported with increased frequency.[6-11]

In the study presented here, we set out to establish the genotypes of *P. aeruginosa* strains colonizing / infecting the CF patients treated at our institution. For this purpose we employed pulsed-field gel electrophoresis (PFGE), which achieved widespread recognition as the »gold standard« for *P. aeruginosa* DNA typing.[12,13]

Methods

The 18 isolates of *P. aeruginosa* were isolated from expectorations of 10 patients treated as in-patients or out-patients at the University Clinic of Respiratory and Allergic Diseases Golnik between 1 January 2006 and 31 December 2008. All phenotypically different isolates from each patient were tested. Any difference in the colour of colonies (green, yellow-green, blue-green, brown), mucoid / non-mucoid and presence / absence of beta-haemolysis was regarded as phenotypical difference. The group of patients comprised 1 male and 9 females, their ages ranging from 13 to 41 years (mean age 21.9 years). Sputa were plated onto enriched blood agar, BD BBL CHROMagar™ Orientation (BD, Sparks, USA), and Burkholderia cepacia agar (Oxoid, Basingstoke, GB) and the plates were incubated at 37 °C for 24 hours. Identification was achieved by API GN (bioMerieux, Marcy l'Etoile, France) or BD BBL Crystal™ E/NF ID system (BD, Sparks, USA). From laboratory records we retrieved susceptibility data of bacterial isolates to the following 6 antimicrobial agents: cefotaxime, ceftriaxone, ceftazidime, imipenem, amikacin and ciprofloxacin. Routine susceptibility testing was performed according to CLSI standards.[14] Only inhibition zones indicating full resistance were taken into account to define antimicrobial resistance profile. Isolates were stored in Cryobank tubes (Mast Diagnostica, Reinfeld, Germany) at -70 °C. For PFGE analysis bacterial cells were grown on a nutrient agar overnight at 37 °C. The plugs were made by suspending organisms in 0.5 mL SE buffer (75 mM NaCl, 25 mM EDTA) to a turbidity of 2.5 McFarland and mixing the suspension with 2 % low-melting-point agarose. The plugs were incubated overnight at 37 °C with Gram-positive lysis buffer (6 mM Tris-HCl, 100 mM EDTA Na_2, 1 M NaCl, 0.5 %(w/v) Brij 58, 0.2 % (w/v) sodium deoxycholate, 0.5 % lauroyl sarcosine, pH 7.5) with 0.5 mg/mL lysozyme added. After removal of Gram-positive lysis buffer the plugs were incubated with Gram-negative lysis buffer (1 % (w/v) lauroyl sarcosine, 500 mM EDTA Na_2 pH 9.5) with 500 µg/mL proteinase K at 56°C. The plugs were washed three times with TE buffer (10 mM Tris-HCl, 10 mM EDTA Na_2, pH 7.5) for 30 minutes at 4 °C. Genomic DNA was digested with *Spe*I (MBI Fermentas, Vilnius, Lithuania) and macrorestric-

Table 1: Demografic data of CF patients with pulsotypes and antimicrobial susceptibility profiles

Patient	Sex / age	PFGE pulsotype	Antimicrobial susceptibility pattern					
			cefotaxime	ceftriaxone	ceftazidime	imipenem	amikacin	ciprofloxacin
1	F / 27	A A1	R R	R R	S S	R S	S S	S S
2	F / 15	A2 A3 A4	R R R	R R R	S R S	S S R	S R R	S S S
3	F / 16	B	S	S	S	S	S	S
4	F / 41	C C1	S S	S S	S S	S S	S S	I S
5	F / 21	C2 C3	R R	R I	R S	R S	R S	S S
6	F / 20	C4	R	R	S	S	S	S
7	M / 13	D	S	S	S	S	S	S
8	F / 33	E F	R S	R S	R S	R S	S S	R S
9	F / 20	G G1	R I	I S	S S	S S	S S	R S
10	F / 14	H H1	I I	I S	S S	R S	S S	S S

Legend: S – susceptible, I – intermediately resistant, R – resistant

tion fragments were separated by PFGE on a CHEF Mapper apparatus (Bio-Rad Laboratories) in a 1.2 % agarose gel run in 0.5 X TBE buffer. The gel run was performed at 6Vcm^{-1} for 30 hours at 12 °C with initial time pulse of 1 s and final pulse time of 59 s. The Lambda Ladder PFGE marker was used as a molecular weight marker (New England BioLabs, Inc., Ipswich, USA). The gel was stained with ethidium bromide, visualized in transiluminator, and photographed. A TIFF image of gel was created and entered into a database in BioNumerics 5.10 software (Applied Maths, Sint-Martens-Latem, Belgium). The percent relatedness was calculated by use of the Dice coefficient, and the unweighted pair group method with arithmetic averages was used for clustering to produce a dendrogram with band optimisation setting of 4 % and a band position tolerance of 1 % and optimization of 1 %. A visual analysis by the method of Tenover et al. was also performed.[15] Strains with up to three band differences were considered closely related, strains with four to six band differences were considered possibly related, and strains with greater then six band differences were considered unrelated.

Results

PFGE analysis carried out with 18 isolates from 10 patients resulted in 17 to 23 bands. The dendrogram produced with BioNumerics 5.10 software (Fig. 1) showed rates of genomic similarity ranging from 24.9 to 95.8 %. There were no two identical strains found. Greater then 80 % similarity was observed between 10 isolates (55.5 %) of 5 patients (between isolates of patients 1 and 2, and between isolates of patients 4, 5 and 6), which corresponds to fewer then six fragment difference in restriction patterns. More then 90 % similarity was observed between two isolates of patient 2 as well as two isolates of patient 4, which also showed more then 90 % similarity with one isolate of patient 5. The isolates from the rest of the patients showed only 24.9 to 63.1 % similarity of macrorestriction patterns. Patient 8 was colonized / infected with two unrelated strains. The isolates showed resistance pro-

files ranging between none and 5 antimicrobial agents. The percentage of resistance to cefotaxime, ceftriaxone, ceftazidime, imipenem, amikacin and ciprofloxacin was 55.5 %, 44.4 %, 16.6 %, 27.7 %, 16.6 %, and 11.1 %, respectively. Demographic data, PFGE pulsotypes and antimicrobial susceptibility profiles are shown in Table 1.

Discussion

Many schemes for the typing of *P. aeruginosa* have been developed. These include PFGE, ribotyping, PCR-based fingerprinting, and MLST.[16,17] In this study we determined PFGE macrorestriction patterns and susceptibility profiles for 6 antimicrobial agents of *P. aeruginosa* isolates in patients with CF. Although there are some publications available on typing of *P. aeruginosa* isolates recovered in different hospital settings, this study provides the first published data on the genetic relatedness of *P. aeruginosa* isolates from CF patients in Slovenia determined by PFGE.[18-20] In agreement with other studies, there was substantial diversity among the isolates.[8,9,21] Patients with CF rarely share genotypes, unless they are siblings or close friends, which suggests that patient-to-patient transmission is rare and requires close contact of longer duration.[6] We also did not detect any fully identical isolates. However, there are several reports of cross-contamination in camps, clinics, and hospitals.[9,22,23] We detected 2 groups of patients with 10 isolates showing greater then 80 % similarity of macrorestriction patterns. Pulsotype A with 4 subtypes was detected in 2 patients and pulsotype C with 4 subtypes was detected in 3 patients. Cross-colonization, common-source contamination, and independent acquisition of genotypes that are more widespread in the local environment are possible explanation for these findings. Any of the three aforementioned ways of bacterial acquisition could be the source of *P. aeruginosa* in 4 out of 5 patients from groups A and C since they reside in the same town or in immediate vicinity. Since these isolates, which could be classified as clusters, had very heterogeneous antimicrobial susceptibility profiles one could argue that the theory of cross-colonization is less likely, but we think the value of antimicrobial susceptibility profiles in determining relatedness between different isolates is low. The rest of the patients were colonized / infected with distinct pulsotypes showing between 24.9 % and 63.1 % similarity. Each of them resides in different part of Slovenia so macrorestriction patterns of their *P. aeruginosa* isolates could represent the local predominant environmental strain.

The results of our study suggest that cross-colonization was probably not the source of acquisition of *P. aeruginosa* in our group of patients. Nevertheless, evidence of patient-to-patient transmission exists and should not be dismissed. Infection-control recommendations, which stress hand hygiene, transmission-based precautions and care for respiratory equipment should be meticulously executed. Further studies of molecular characteristics of *P. aeruginosa* in CF patients in Slovenia are required to give us a more comprehensive insight into actual epidemiology and state of infection-control.

Reference

1. Mainz JG, Naehrlich L, Schien M, Kading M, Schiller I, Mayr S, idr. Concordant genotype of upper and lower airways P aeruginosa and S aureus isolates in cystic fibrosis. Thorax 2009; 64: 535–40.
2. Rajan S, Saiman L. Pulmonary infections in patients with cystic fibrosis. Semin Respir Infect 2002; 17: 47–56.
3. Lyczak JB, Cannon CL, Pier GB. Lung infections associated with cystic fibrosis. Clin Microbiol Rev 2002; 15: 194–222.
4. Oliver A, Canton R, Campo P, Baquero F, Blazquez J. High frequency of hypermutable Pseudomonas aeruginosa in cystic fibrosislung infection. Science 2000; 288: 1251–4.
5. Mena A, Smith EE, Burns JL, Speert DP, Moskowitz SM, Perez JL, idr. Genetic adaptation of Pseudomonas aeruginosa to the airways of cystic fibrosis patients is catalyzed by hypermutation. J Bacteriol 2008; 190: 7910–7.
6. Grothues D, Koopmann U, von der Hardt, Tummler B. Genome fingerprinting of Pseudomonas aeruginosa indicates colonization of cystic fibrosis siblings with closely related strains. J Clin Microbiol 1988; 26: 1973–7.
7. Saiman L, Siegel J. Infection control in cystic fibrosis. Clin Microbiol Rev 2004; 17: 57–71.
8. Anthony M, Rose B, Pegler MB, Elkins M, Service H, Thamotharampillai K, idr. Genetic analysis of Pseudomonas aeruginosa isolates from the sputa of Australian adult cystic fibrosis patients. J Clin Microbiol 2002; 40: 2772–8.

9. Ojeniyi B, Frederiksen B, Hoiby N. Pseudomonas aeruginosa cross-infection among patients with cystic fibrosis during a winter camp. Pediatr Pulmonol 2000; 29: 177–81.
10. Armstrong DS, Nixon GM, Carzino R, Bigham A, Carlin JB, Robins-Browne RM, idr. Detection of a wide-spread clone of Pseudomonas aeruginosa in a pediatric cystic fibrosis clinic. Am J Respir Crit Care Med 2002; 166: 983–7.
11. Van Daele SG, Franckx H, Verhelst R, Schelstraete P, Haerynck F, Van Simaey, idr. Epidemiology of Pseudomonas aeruginosa in a cystic fibrosis rehabilitation centre. Eur Respir J 2005; 25: 474–81.
12. Johnson JK, Arduino SM, Colin Stine O, Johnson JA, Harris AD. Multilocus sequence typing compared to pulsed-field gel electrophoresis for molecular typing of Pseudomonas aeruginosa. J Clin Microbiol 2007; 45: 3707–12.
13. Scott FW, Pitt TL. Identification and characterization of transmissible Pseudomonas aeruginosa strains in cystic fibrosis patients in England and Wales. J Med Microbiol 2004; 53: 609–15.
14. CLSI. Performance standards for antimicrobial susceptibility testing: Eighteenth informational supplement. 2008; M100-S18: 40–41.
15. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BA, Persing DS, idr. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 1995; 33: 2233–9.
16. Syrmis MW, O'Carroll MR, Sloots TP, Coulter C, Wainwright CE, Bell SC, idr. Rapid genotyping of Pseudomonas aeruginosa isolates harboured by adult and paediatric patients with cystic fibrosis using repetitive-element-based PCR assays. J Med Microbiol 2004; 53: 1089–96.
17. Doleans-Jordheim A, Cournoyer B, Bergeron E, Croize J, Salord H, Andre J, idr. Reliability of Pseudomonas aeruginosa semi-automated rep-PCR genotyping in various epidemiological situations. Eur J Clin Microbiol Infect Dis 2009; 28: 1105–1111.
18. Mueller-Premru M, Gubina M, Strazisar T. Tipizacija bakterije Pseudomonas aeruginosa s pulzno elektroforezo. V: Bole-Hribovšek V, Ocepek M, Klun N, ur. Zbornik s programom. 2. kongres slovenskih mikrobiologov z mednarodno udeležbo; 27.- 30. september 1998; Portorož, Ljubljana: Slovensko mikrobiološko društvo, 1998. Str. 503–6.
19. Mueller-Premru M, Gubina M. Serotype, antimicrobial susceptibility and clone distribution of Pseudomonas aeruginosa in a University hospital. Zentralbl akteriol. 1999; 289: 857–67.
20. Mueller-Premru M, Lejko-Zupanc T. Epidemiological typing of imipenem-resistant Pseudomonas aeruginosa. Int J Antimicrob Agents 2002; 20: 380–3.
21. Greenberg D, Yagupsky P, Peled N, Goldbart A, Porat N, Tal A. Lack of evidence of transmission of Pseudomonas aeruginosa among cystic fibrosis patients attending health camps at the Dead Sea, Israel. Isr Med Assoc J 2004; 6: 531–4.
22. McCallum SJ, Corkill J, Gallagher M, Ledson MJ, Hart CA, Walshaw MJ. Superinfection with a transmissible strain of Pseudomonas aeruginosa in adults with cystic fibrosis chronically colonised by P aeruginosa. Lancet 2001; 358: 558–60.
23. Cheng K, Smyth RL, Govan JR, Doherty C, Winstanley C, Denning N, et al. Spread of beta-lactam-resistant Pseudomonas aeruginosa in a cystic fibrosis clinic. Lancet 1996; 348: 639–42.