

Molecular typing and biological characteristics of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients in Brazil

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ABSTRACT

The present study had as objective to evaluate the genotypic diversity and biological characteristics, such as hemolysin, protease, elastase of 56 clinical strains of *Pseudomonas aeruginosa* isolated from 13 cystic fibrosis (CF) patients attending at the School Hospital of Campinas State University (UNICAMP), Brazil. Genotypic diversity has been determined by Ribotyping (RT) and the pattern of the enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) of each strain. The production of elastase was significantly different only among mucoid and nonmucoid isolates. Joint results obtained by (RT) and ERIC-PCR methods were able to discriminate all strains isolated from both the same and different patients. Additionally, we observed four strain clusters with low diversity. The most infective strains were located in just two clusters. These results suggest that either there is a strong selection towards a specific genotype or that specific isolates could be responsible for the initial and subsequent colonization processes. More studies are necessary to know if these conclusions can be generalized for the general CF population.

Keywords: cystic fibrosis *Pseudomonas aeruginosa* clonal analysis biological characteristics.

[Braz J Infect Dis 2010;14(5):462-467]©Elsevier Editora Ltda.

Submitted on: 3/4/2010
Approved on: 8/4/2010

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This work was supported by grants No. 98/02524-5 and No. 03/0407-0 from Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP, and grant No. 155220/2006-3 from CNPq.

We declare no conflict of interest.

INTRODUCTION

Cystic fibrosis (CF) is the most common single gene disorder in Caucasian populations. It occurs approximately once in every 3,600 live births.¹ CF is chiefly characterized by chronic obstruction and infection of the respiratory tract, exocrine pancreatic insufficiency and its nutritional consequences, and elevated levels of sweat electrolytes.² Most CF patients suffer from chronic and, ultimately fatal, pulmonary infections caused by bacterial strains such as *Staphylococcus aureus*, *Haemophilus influenzae*, and *Pseudomonas aeruginosa*. Initial colonization of airways by *P. aeruginosa* is usually due to nonmucoid isolates that convert themselves to the mucoid phenotype, which is refractory to phagocytosis, resistant to antibiotics,³ and predominates during chronic lung infection. Multiple colonial bacterial morphotypes with different antibiotic resistances and a large number of extracellular virulence factors, which are

tightly regulated by cell-to-cell signalling systems, are often isolated from sputum.^{4,5} The formation of mucoid colonies of *P. aeruginosa* composed of alginates, involving *algD* genes, protects the bacterium from the host's immune response and from antibiotics, and thus contributes to chronic pulmonary inflammation.⁶ Other virulence factors can cause pulmonary damage by different mechanisms such as Exoenzyme S, Exotoxin A, Elastase and Phospholipases. Exoenzyme S is encoded by the *exoS* gene, an ADP-ribosyltransferase that is secreted by a type-III secretion system directly into the cytosol of epithelial cells.⁷ Exotoxin A, is encoded by the *toxA* gene and inhibits protein biosynthesis. LasB elastase, a zinc metalloprotease encoded by the *lasB* gene, has an elastolytic activity on lung tissue⁸ and the phospholipids contained in pulmonary surfactants may be hydrolysed in pulmonary surfactants by two phospholipases encoded by *plcH* and *plcN* (PLC-H and PLC-N, respectively).⁹

P. aeruginosa is intrinsically resistant to several antimicrobial drug classes and can rapidly develop resistance to other drugs during chemotherapy, making medical treatment difficult and ineffective. Once chronic infection is established, *P. aeruginosa* is virtually impossible to eradicate and is associated with increased mortality and morbidity in CF patients.¹⁰

Investigations of the nosocomial epidemiology of *P. aeruginosa* have been hampered by the inadequate discriminatory ability of classical phenotypic methods such as serotyping, phage and pyocin typing, and biotyping.^{11,12} Modern DNA-based techniques, such as enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) and Ribotyping, have been widely used in the epidemiological investigation of many microorganisms, including *P. aeruginosa*.¹³

In the present study, genotypic and phenotypic characteristics of 56 *Pseudomonas aeruginosa* isolates obtained from 13 cystic fibrosis patients have been evaluated. The evaluation of the genotypic characteristics was accomplished by combining ribotyping (RT) and enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) techniques as described by Wolska & Szweida (2008) and Liu *et al.* (1995).^{14,15} These results were compared with the phenotypic characteristics (biological) and all joint results were used to establish the epidemiology of this species in CF patients.

MATERIALS AND METHODS

Bacterial strains and media

This was a retrospective study where all 56 strains (26 mucoid and 30 nonmucoid) were isolated from the sputum during an exacerbation crisis of 13 CF patients attending at the Pediatric Sector of Clinical Hospital of the Campinas State University (UNICAMP), Campinas, SP, Brazil, between April 1996 and January 1998 (Ethical Commission Process number n° 045/98 CEP/FCM from 05/27/98). Data about age, sex and antimicrobial treatment were not available. The isolates were identified by colony pigmentation, grape-like odor, motility, and biochemical tests [carbohydrate fermentation of Glucose, Lactose and Sucrose (-), citrate assimilation (+), lysine decarboxylase (-), indol (-), oxidase (+), beta-hemolysis on blood-agar (+), and DNase (-)].¹⁶

Colony morphology

The colony phenotype classified as mucoid or nonmucoid of each isolate was performed after grown on Luria-Bertani (LB) agar plates (24 h incubation at 37°C).¹⁷

Chromosomal DNA extraction

Chromosomal DNA extraction was performed according to the CTAB method.¹⁸

Ribotyping (RT) analyses

Ribotyping was performed as described as Nociari *et al.*, 1996.¹⁹ Genomic DNA of *Pseudomonas aeruginosa*, purified as previously described,¹⁸ was digested with the restriction enzyme PvuII as specified by the manufacturer (Life Technologies) and runned on a 0.7% submersed agarose gel electrophoresis system. Size-separated restriction fragments were transferred to a 0.45 µm nitrocellulose membrane (Pharmacia) and Southern blot experiments were accomplished using the 16s rDNA marked fragment²⁰ as a molecular probe for the rDNA gene. The rDNA fingerprints were recorded using the 1 Kba DNA standard (Life Technologies) as a migration reference in each gel.

ERIC-PCR conditions and primers

Genomic bacterial DNA (50 ng) was used for the ERIC-PCR-reactions using the sequences ERIC 1 (5'-ATGTAAGCTCCTGGGGATTAC-3') and ERIC 2 (5'-AAGTAAGTgACTGGGGTGAGCG-3') as described by Tosin *et al.* (2003)²¹ in a final volume of 50 µL as follows: an initial denaturation (94°C, 7 min), followed by 30 cycles of denaturation (90°C, 30 sec), annealing (52°C, 1 min), and extension (72°C, 8 min) with a single final extension (72°C, 16 min). A 7 µL volume of amplicon was loaded with 2 µL 2x loadingbuffer (10% glycerol, 2 mM EDTA, 0.1% xylene cyanol, 0.1 % bromophenol blue) into one well of a 15-well 1.2% agarose gel in 1x Tris-Borate-EDTA (TBE) buffer with 0.5 µg/ml ethidium bromide mL⁻¹. A 1 kb DNA ladder (Life Technologies) was placed at both ends and in the middle of each gel, which was runned at 80V for 3h at room temperature. Each ERIC-PCR test was performed in duplicate to ensure conformity of each fingerprint.

Fingerprint analyses and statistical analyses

Fingerprints of DNA fragments either obtained by RT²² or by ERIC-PCR were recorded. The presence of a given band was coded as 1, and the absence of a given band was coded as 0 in a data matrix, which was analyzed by means of the POPGENE software (Version 1.31) with the Unweighted Pair Group Method using Arithmetic averages (UPMGA).²³ A single dendrogram of similarity comprising both techniques (RT and ERIC-PCR) was constructed for all isolates studied. Statistical analyses were accomplished through Chi-square methodology using the Fisher test. The association between rows (groups) and columns (outcomes) was considered statistically significant using one tail (<http://graphpad.com/quickcalcs/Contingency1.cfm>). Cluster was defined as a group of strains sharing either identical or similar characteristics.

Hemolysin production

The method to detect the hemolysin production employed sheep blood agar. The strains were spread out onto the surface of blood agar plates and incubated for 18 h at 37°C. The formation of a clear halo around the colony was indicative of the production of hemolysin.²⁴

Protease production

Protease production was assayed by growing the strains in BHI medium overnight (37°C), followed by inoculation in depth in tubes containing 5 mL of a 12% gelatin solution and incubation at 37°C for 24 h. Protease activity was verified by gelatin liquefaction.²⁵

Elastase activity

Elastase activity was assayed by growing strains in BHI medium overnight (37°C) and dropping 50 µL of this culture onto a Petri dish containing elastase solution agar. The culture was incubated at 37°C overnight and elastase production was verified by the presence of a clear halo around the colony.²⁴

RESULTS

The studied isolate phenotype characteristics (hemolysin, protease, and elastase) are shown in Table 1. The isolates were identified according to the source patient (e.g., P1, P2, P3, etc.) and each patient's isolated strains were described as A, B, C, or D. Production of alginate by the strain was identified as either M (mucoid) or NM (non-mucoid). For example, P1AM means that a mucus producing strain (mucoid) (A) was isolated from patient 1, while P1AMN is a mucus not producing strains (nonmucoid) that was also isolated from patient 1. There was a characteristic association only between colony morphology and elastase expression ($p < 0.05$).

The ribotyping (RT) assay was able to detect fragments in 50 out of the 56 isolates and demonstrated the presence of eight different DNA fragments with molecular weights ranging from 2,151 bp to 12,216 bp. The detection of ERIC sequences by PCR produced 19 DNA fragments ranging from 396 bp to 12,216 bp with fragments of 12,216 bp, 1,205 bp, 674 bp, and 488 bp found in 70.4%, 52.0%, 52.0%, and 100.0% of the isolates, respectively (results not shown). The dendrogram of similarity obtained using the joint results of RT and ERIC-PCR demonstrated the existence of four main clusters (A-D) with a small dissimilarity between clusters; approximately 18% between clusters A and B, 19% between cluster C and A and B, and 22% between cluster D and the other three clusters (Figure 1). Clusters B contained the majority of the isolates ($n = 30$, 53.6%), while cluster A contained 19

Table 1. Biological characteristics of *Pseudomonas aeruginosa* isolates and colony morphology

Exoenzymes (groups)	Mucoid strain (outcome1)	Nonmucoid strain (outcome2)	p value ($p \leq 0.05$)*
Hem (+) (group 1)	53.85% (14/26)	53.33% (16/30)	p = 0.5911
Hem (-) (group 2)	46.15% (12/26)	46.67% (14/30)	
Prt (+) (group 1)	38.46% (10/26)	36.67% (11/30)	p = 0.5542
Prt (-) (group 2)	61.54% (16/26)	63.33% (19/30)	
Ela (+) (group 1)	34.62% (9/26)	63.33% (19/30)	p = 0.0299*
Ela (-) (group 2)	65.38% (17/26)	36.67% (11/30)	
Hem (+) and Prt (+) (group 1)	11.54% (3/26)	13.33% (4/30)	p = 0.2308
Hem (-) and Prt (-) (group 1)	3.85% (1/26)	23.33% (7/30)	
Hem (+) and Ela (+) (group 1)	15.38% (4/26)	20.00% (6/30)	p = 0.2797
Hem (-) and Ela (-) (group 2)	11.54% (3/26)	33.33% (1/30)	
Prt (+) and Ela (+) (group 1)	3.85% (1/26)	3.33% (1/30)	p = 0.5238
Prt (-) and Ela (-) (group 2)	15.38% (4/26)	3.33% (1/30)	
Hem (+) Prt (+) and Ela (+) [†] (group1)	15.54% (3/26)	16.67% (5/30)	p = 0.3250
Hem (-) Prt (-) and Ela (-) [‡] (group 2)	26.92% (7/26)	16.67% (5/30)	

Hem, Hemolysin; Prt, Proteinase; Ela, Elastase. *Statistically significant (Fisher test) results. [†]Strains positive for all exoenzyme factors. [‡]Strains negative for all exoenzymes factors.

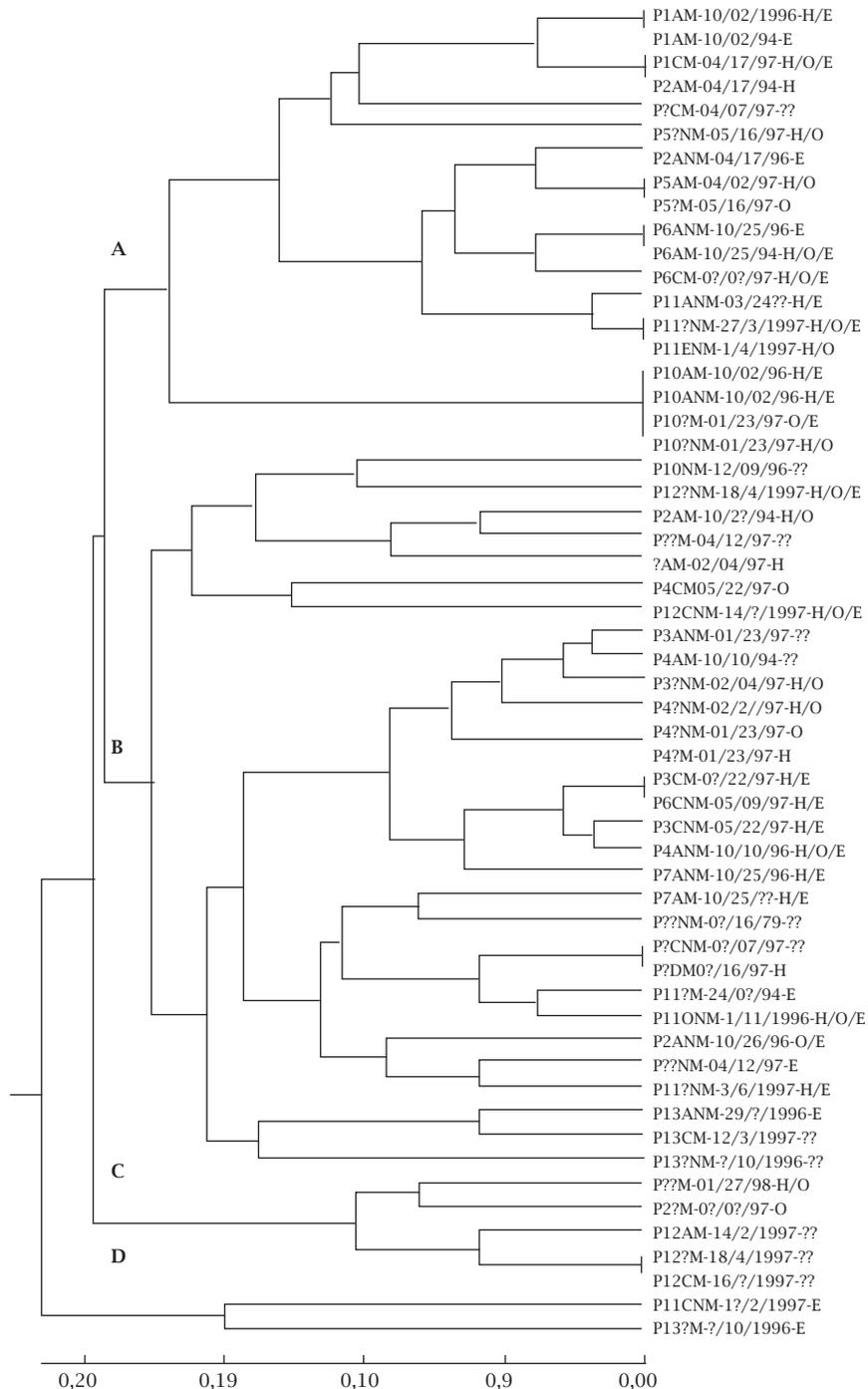
(33.9%), cluster C contained 5 (8.9%), and cluster D contained 2 (3.6%) of the isolates. In these clusters, some isolates were allocated in groups of higher or lower similarity and most strains were discriminated. In cluster A, 14 of the isolates had 100% similarity, forming six real clones; in cluster B, 6 of the isolates had 100%

similarity, forming three real clones. Cluster C had only one real clone, which was formed by two isolates. From these 10 clones, 8 had strains that were isolated from the same patients and 2 had strains isolated from different patients; P1CM and P2AM (cluster A) and P3CM and P6CNM (cluster B). On the other hand, we also verified that the same patient could either have genomically

distinct isolates located in either the same cluster (P2AM and P2ANM, P6ANM, and P6CM) or different clusters (P1, P6, P11, P13).

It was observed that although some colonies were genomically identical according to their location in the dendrogram, they could express different exoenzymes (P1AM and P1ANM; Figure 1).

Figure 1: Dendrogram of dissimilarity (%) of *Pseudomonas aeruginosa* strains including date of isolation and virulence factors.



DISCUSSION

This work was carried out with the prime objective of assessing the genomic variability of *Pseudomonas aeruginosa* strains isolated from patients suffering from cystic fibrosis, combining two different molecular techniques in order to increase the methodology sensitivity.

To date, several techniques have been used to assess the genomic variability of *P. aeruginosa*, including primed PCR,¹¹ pulsed field gel electrophoresis,^{1,11} ribotyping,^{26,27} multi-locus sequence typing (MLST),²⁸ and enterobacterial repetitive intergenic consensus-based PCR (ERIC-PCR).²⁹ Although pulsed field gel electrophoresis (PFGE) is considered to be the gold standard for the determination of genotype characteristics and is largely used throughout the world,^{21,30,31} it is time consuming, requires relatively large DNA quantities, and depends on expensive equipment. For these reasons, although occurring the possibility of losing a better accuracy of the analysed data, we have chosen to combine the results of RT and ERIC-PCR methods to determine genomic variability and discriminate bacterial isolates in different cystic fibrosis patients.^{14,15} All PCR-based approaches represent useful tools for the epidemiological typing of nosocomial bacteria because of their simplicity and speed compared with those of PFGE. In the work of Liu *et al.* (1995), it was demonstrated that discriminatory power of ERIC-PCR was equivalent to that of PFGE.

Additionally, we determined the production of some pathogenicity-related exoenzymes (hemolysin, gelatinase, and elastase) to better characterize these isolates in an attempt to correlate exoenzyme production with colony morphology.

The dendrogram of similarity obtained using the joint results of RT and ERIC-PCR demonstrated the existence of four clusters (A-D), with two of them (A and B) containing most of the studied isolates (n = 49, 87.5%). Additionally, 10 real clones (100% similarity) were detected. Strains were isolated from the same patients in eight of the clones, and in two of the clones, the strains were isolated from different patients. Patients could also be colonized by strains classified as belonging to different clusters (A and B, B and C). These observations suggest that some of the patients were colonized by either the same or different isolates along the time, as previously described by Sener *et al.* (2001).³²

The most relevant result of our study is the finding that four main bacterium clusters, with the predominance of two clusters, are responsible for colonizing CF patients. This suggests that either there is a strong selection towards a specific genotype, which could originate by chromosome rearrangements,^{33,34} or that primarily specific isolates containing pathogenic gene islands may be responsible for the initial and subsequent colonization processes. As this study was carried out using a small number of patients and strains this statement cannot be generalized for all

cystic fibrosis population and further studies must be accomplished to confirm it.

Regarding the biological characteristics studied, a significant difference between mucoid and nonmucoid isolates have been observed only for the production of elastase. The results obtained in this work are similar to those published by Berka *et al.* (1981).³⁵ Hemolysin production correlated with isolate morphotypes; 80% of mucoid and 20% of nonmucoid isolates were positive for this characteristic, which also confirms the results obtained in the previous work of Stehling *et al.* (2008)³⁶ in which nonmucoid isolates presented a statistically significant result regarding elastase production when compared with mucoid isolates. However, this result does not agree with those published by Storey *et al.* (1992)³⁷ and Jagger *et al.* (1983)³⁸ that demonstrated that elastase production in mucoid and nonmucoid isolates of *P. aeruginosa* was not statistically significant.

Finally, the observation that colonies with identical genomic backgrounds are able to express different exoenzymes would reflect either their ability to respond to different physiological moments or different tissues of either the same or different hosts, as suggested by Stehling *et al.* (2008).

ACKNOWLEDGMENTS

This work was supported by grants No. 98/02524-5 and No. 03/0407-0 from Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP, and grant No. 155220/2006-3 from CNPq.

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