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.

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 tissue[8] and the phospholipids contained in pulmonary surfactants may be hydrolysed 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 & Szweda (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 Comission 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 peformed 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 fragment[20] 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´-ATGTAAGCTCCTGGGGATTCAC-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 µgethidium 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 RT[22] 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 (nonmucoid) 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*

Exoenzimes (groups)	Mucoid strain (outcome1)	Nonmucoid strain (outcome2)	p value (p ≤ 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.
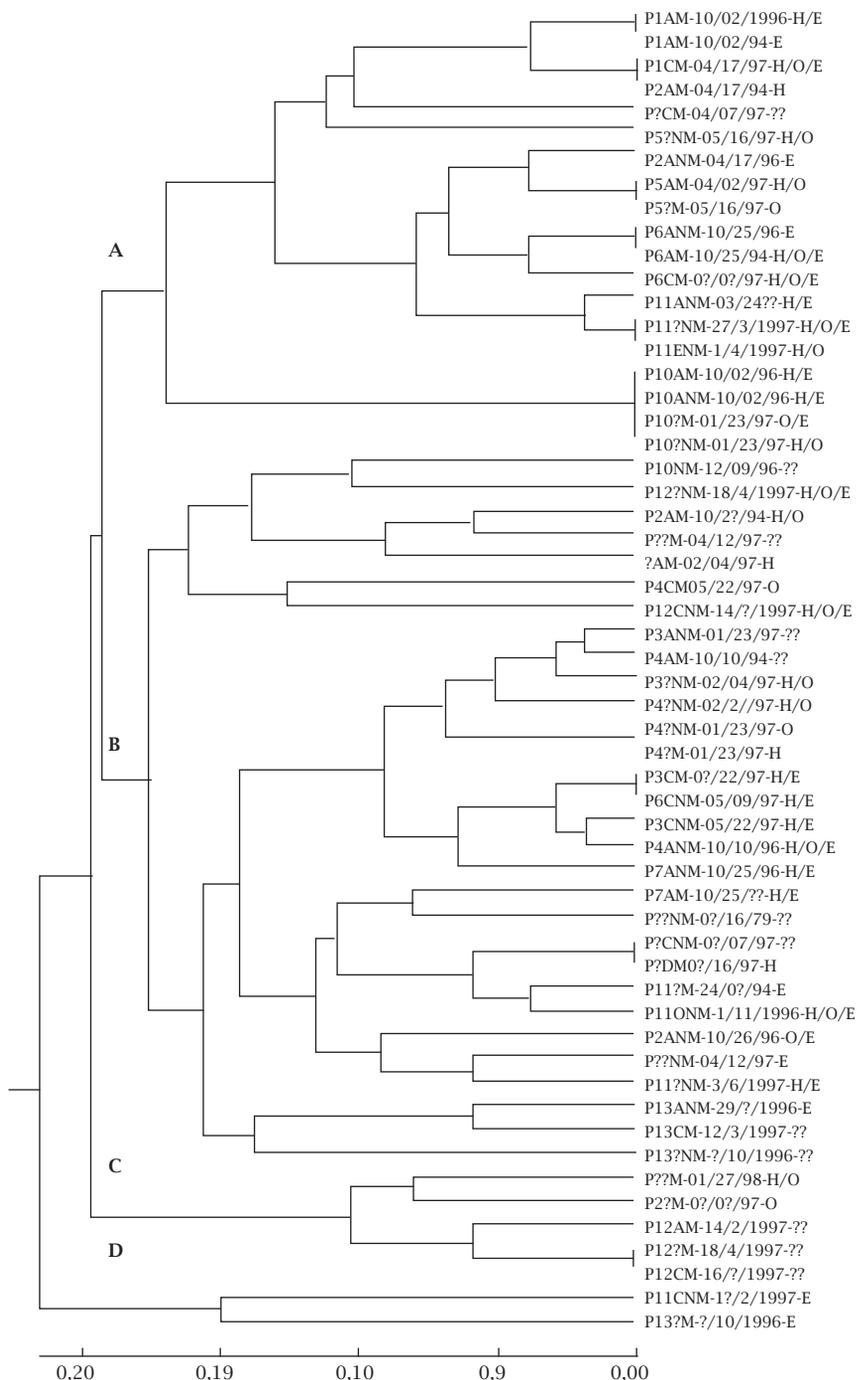
DISCUSSION

This work was carried out with the prime objetive of assessing the genomic variability of *Pseudomonas aeruginosa* strains isolated from patientes 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 loosing 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 patientes 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).

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REFERENCES

1. Renders N, Römling U, Verbrug H, Van Belkum A. Comparative typing of *Pseudomonas aeruginosa*, by random amplification of polymorphic DNA or pulsed field gel electrophoresis of DNA macrorestriction fragments. J Clin Microbiol 2001; 34:3190-5.

2. Boat TF, Welch M, Beaudet AL. In the Metabolic Basis of Inherited Diseases. Scriver CL, Beaudet AL, Sly WS & Valle D (eds). McGraw-Hill Inc, New York, 1995. pp. 3799-876.

3. Yoon SS, Coakley R, La GW *et al*. Anaerobic killing of mucoid *Pseudomonas aeruginosa* by acidified nitrite derivatives under cystic fibrosis airway conditions. J Clin Invest 2006; 116:436-46.

4. Mereghetti L, Marquet-van der Mee N, Loulergue J *et al*. *Pseudomonas aeruginosa* from cystic fibrosis patients: study using whole cell RAPD and antibiotic susceptibility. Path Biol (Paris) 1998; 46:319-24.

5. Van Delden C, and Iglewski BH. Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. Emerg Infect Dis 1998; 4:551-60.

6. Govan JR, Deretic V. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and Burkholderia cepacia. Microbiol Rev 1996; 60:539-74.

7. Yahr TL, Hovey AK, Kulich SM, Frank DW. Transcriptional analysis of the *Pseudomonas aeruginosa* exoenzyme S structural gene. J Bacteriol 1995; 177:1169-78.

8. Jaffar-Bandjee MC, Lazdunski A, Bally M *et al*. Production of elastase, exotoxin A, and alkaline protease in sputa during pulmonary exacerbation of cystic fibrosis in patients chronically infected by *Pseudomonas aeruginosa*. J Clin Microbiol 1995; 33:924-29.

9. Konig B, Vasil ML, Konig W. Role of haemolytic and non-haemolytic phospholipase C from *Pseudomonas aeruginosa* in interleukin-8 release from human monocytes. J Med Microbiol 1997; 46:471-8.

10. Swiatecka-Urban A, Moreau-Marquis S, Maceachran DP *et al*. *Pseudomonas aeruginosa* inhibits endocytic recycling of CFTR in polarized human airway epithelial cells. Amer J Physiol Cell Physiol 2006; 290:C862-C872.

11. Kersulyte D, Struelens MJ, Deplano A, Berg DE. Comparison of arbitrarily primed PCR and macrorestriction (pulsed-field gel electrophoresis) typing of *Pseudomonas aeruginosa* strains from cystic fibrosis patients. J Clin Microbiol 1995; 33:2216-9.

12. Liu Y, Davin-Regli A, Bosi C *et al*. Epidemiological investigation of *Pseudomonas aeruginosa* nosocomial bacteraemia isolates by PCR-based DNA fingerprinting analysis. J Med Microbiol 1996; 45:359-65.

13. Speijer H, Savelkoul PH, Bonten MJ *et al*. Application of different genotyping methods for *Pseudomonas aeruginosa* in a setting of endemicity in an intensive care unit. J Clin Microbiol 1999; 37:3654-61.

14. Wolska K, Szweda P. A comparative evaluation of PCR ribotyping and ERIC-PCR for determining the diversity of clinical *Pseudomonas aeruginosa* isolates. Pol J Microbiol 2008; 57:157-63.

15. Liu PY, Shi ZY, Lau YJ *et al*. Comparison of different PCR approaches for characterization of Burkholderia (Pseudomonas) cepacia isolates. J Clin Microbiol 1995; 33:3304-7.

16. Giraldi GL. 1990. Pseudomonas and related genera, pp. 429-441. In A Balows WJ, Hausler Jr KL *et al*. (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.

17. Lee B, Haagensen JA, Ciofu O, Andersen JB, Hoiby N, Molin S. Heterogeneity of biofilms formed by nonmucoid *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. J Clin Microbiol 2005; 43:5247-55.

18. Ausubel FM, Brent R, Kingston RE *et al*. Curr prot in molecul biol. New York, Wiley Interscience 1987.

19. Nociari MM, Catalano M, Centron Garcia D *et al*. Comparative usefulness of ribotyping, exotoxin A genotyping, and SalI restriction fragment length polymorphism analysis for *Pseudomonas aeruginosa* lineage assessment. Diagn. Microbiol. Infect. Dis 1996; 24:179-90.

20. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. New York, Cold Spring Harbour 1989.

21. Tosin I, Silbert S, Sader HS. The use of molecular typing to evaluate the dissemination of antimicrobial resistance among Gram-negative rods in Brazilian hospitals. Braz J Infect Dis 2003; 7:360-9.

22. Ojeniyi B, Petersen US, Hoiby N. Comparison of genome fingerprinting with conventional typing methods used on *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. Apmis 1993; 101:168-75.

23. Yeh WC, Yang RC, Boyle. Popgene version 1.31 Microsoft Windows-Based freeware for population genetic analysis. Department of Renewable Resources, University of Alberta, Edmonton, AB Canada 1999.

24. Morihara K. Production of elastase and proteinase by *Pseudomonas aeruginosa*. J Bacteriol 1964; 88:745-57.

25. Wilson ED. Studies in bacterial protease I. the Relation of protease production to the culture medium. J Bacteriol 1930; 20:41-59.

26. Agodi A, Sciacca A, Campanile F *et al*. Molecular epidemiology of *Pseudomonas aeruginosa* from cystic fibrosis in Sicily: genome macrorestriction analysis and rapid PCR-ribotyping. New Microbiol 2000; 23:319-27.

27. Bennekov T, Colding H, Ojeniyi B *et al*. Comparison of ribotyping and genome fingerprinting of *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. J Clin Microbiol 1996; 34:202-4.

28. Curran B, Jonas D, Grundmann H *et al*. Development of a multilocus sequence typing scheme for the opportunistic pathogen *Pseudomonas aeruginosa*. J Clin Microbiol 2004; 42:5644-9.

29. Yan W, Shi L, Jia WX *et al*. Evaluation of the biofilm-forming ability and genetic typing for clinical isolates of *Pseudomonas aeruginosa* by enterobacterial repetitive intergenic consensubased PCR. Microbiol Immunol 2005; 49:1057-61.

30. Spencer D. Clinical outcome in relation to care in centres specializing in cystic fibrosis. Cross infection with *Pseudomonas aeruginosa* is unusual. Brit Med 1999; 318:58.

31. Spencker FB, Haupt S, Claros MC *et al*. Epidemiologic characterization of *Pseudomonas aeruginosa* in patients with cystic fibrosis. Clin Microbiol Infect 2000; 6:600-7.

32. Sener B, Koseoglu O, Ozcelik U *et al*. Epidemiology of chronic *Pseudomonas aeruginosa* infections in cystic fibrosis. Int J Med Microbiol 2001; 291:387-93.

33. Mahenthiralingam E, Campbell ME, Foster J *et al*. Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. J Clin Microbiol 1996; 34:1129-35.

34. Romling U, Fiedler B, Bosshammer J *et al*. Epidemiology of chronic *Pseudomonas aeruginosa* infections in cystic fibrosis. J Infect Dis 1994; 170:1616-21.

35. Berka RM, Gray GL, Vasil ML. Studies of phospholipase C (heat-labile hemolysin) in *Pseudomonas aeruginosa*. Infect Immun1981; 34:1071-4.

36. Stehling EG, Silveira WD, Leite DS. Study of biological characteristics of *Pseudomonas aeruginosa* strains isolated from patients with cystic fibrosis and patients with non-pulmonary infections. Braz J Infect Dis 2008; 12:86-8.

37. Storey DG, Ujack EE, Rabin HR. Population transcript accumulation of *Pseudomonas aeruginosa* exotoxin A and elastase in sputa from patients with cystic fibrosis. Infect Immun 1992; 60:4687-94.

38. Jagger KS, Bahner DR, Warren RL. Protease phenotypes of *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis. J Clin Microbiol 1983; 17:55-9.