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Original article

Genetic Profiling of *Pseudomonas aeruginosa* Isolates from Iranian Patients with Cystic Fibrosis Using RAPD-PCR and PFGE

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Abstract

Objective(s)
Pseudomonas aeruginosa is the most important cause of chronic lung infections and death in patients with cystic fibrosis. Determining the distribution of specific strains within patient populations is important in order to examine the epidemiology of the disease and the possibility of cross infection among patients.
Materials and Methods
Forty six Iranian patients with cystic fibrosis were studied for colonization with *P. aeruginosa*. Colony phenotype was recorded and antibiotic susceptibility to 11 antibiotics was determined using the disc diffusion method. Genetic fingerprinting was carried out by RAPD–PCR and by PFGE.
Results
Forty five *P. aeruginosa* isolates were recovered from 31 patients including sequential cultures from 9 subjects. The rate of colonization increased with age. All isolates were susceptible to tobramycin and ciprofloxacin, 97.8% were sensitive to amikacin and piperacillin, 93.3% to gentamycin, 91.1% to ticarcillin, 86.7% to colistin, 80% to carbenicillin, 48.9% to cefotaxime, 26.7% to imipenem and 11.1% to ceftazidime. Genetic fingerprinting showed similar distribution profiles for RAPD-PCR and PFGE and the majority of the isolates had unique fingerprints.
Conclusion
No relationship was observed between the obtained genotypes and antibiotic susceptibility profiles and common predominant virulent clones were not found among the isolates.

Keywords: Cystic fibrosis, Genetic fingerprinting, PFGE, *Psuedomonas aeruginosa*, RAPD-PCR

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Introduction

Pseudomonas aeruginosa is believed to be the leading cause of chronic lung infections in patients with cystic fibrosis (CF) (1-3). It is believed that lung colonization usually starts with classical non-mucoid organisms and mucoid phenotype almost exclusive to the CF lung, develops later resulting in chronic infections (4-6). Virulence of *P. aeruginosa* in the CF lung is complex and it has not been elucidated if individual strains are more virulent than others (7). Determining the distribution of specific *Pseudomonas* strains within CF populations may be essential in order to examine the epidemiology of the disease and the clinical impact of chronic infections with individual *P. aeruginosa*. Due to the diverse phenotypic nature of *P. aeruginosa*, traditional typing methods are not discriminatory enough to identify individual clonal clusters (8, 9). Genetic fingerprinting methods have been shown to be discriminatory for DNA fingerprinting of *P.* aeruginosa isolated from CF patients (7, 10-15). We studied forty six Iranian subjects who were diagnosed as patients with cystic fibrosis for colonization with *P. aeruginosa.* The antibiotic susceptibility profiles were determined to 11 antibiotics using disc diffusion. Genetic typing was carried out by random amplification polymorphic DNA (RAPD-PCR) and pulse field gel electrophoresis (PFGE). Genotypes were compared with colony morphology and antibiotic resistance profiles of the clinical *P. aeruginosa* isolates afterwards.

Materials and Methods

Forty six patients, with the age range of 3 months to 23 years from 2 different health centers were studied during a one year period (2004-5). Diagnosis was based on the results of the sweat test. Clinical symptoms were also recorded for each patient (gastrointestinal disorder, GID or respiratory disease, RD). The human study was approved by a local Ethics Committee.

Sputum samples or throat swabs were cultured directly on Brain Heart Infusion (BHI, Difco) agar plates or in Malachite green broth (as transport enrichment medium for *P. aeruginosa*) prior to being cultured on BHI plates. Consecutive specimens obtained from 15 subjects between one to six months, were compared for colony morphology (mucoid vs. non-mucoid), antibiotic susceptibility and genetic fingerprints. Isolated colonies were tested for oxidase reaction, pyocin production on *Pseudomonas* agar plates and other standard biochemical tests required for identification of *P. aeruginosa*.

Using the disc diffusion method (16), susceptibility of *P. aeruginosa* isolates to 11 antibiotics was determined. Amikacin (AN, 30 µg), carbenicillin (CB, 100 µg), ceftazidime (CAZ, 30 µg), ciprofloxacin (CP, 5 µg), gentamycin (GM, 10 µg) and tobramycin (TOB, 10 µg) were purchased from Padtan Teb pharmaceuticals (Tehran, Iran). Imipenem (IPM, 10 µg) and cefotaxime (CTX, 30 µg) were obtained from Iran Darou Co. (Tehran, Iran). Piperacillin (PIP, 100 µg) and ticarcillin (TC, 75 µg) were from Mast Co. (England) and colistin (CO, 10 µg) was obtained from Biomeriux (France). *P. aeruginosa* ATCC 27853 was used as the laboratory standard for susceptibility tests.

For RAPD–PCR analysis, DNA extraction was carried out by a freeze-thaw method (17). Overnight grown cultures of bacteria in BHI broth (1.5 ml) were centrifuged at 13000×g for 5 min and the pellets were re-suspended in 0.1 M NaCl and washed twice in the same buffer. The final pellet was re-suspended in 750 µl of Tris-EDTA buffer (10:1 mM, pH 8) and placed at -20 ^{o}C for 30-45 min followed by thawing at 65 ^{o}C. The freeze-thaw steps were repeated 4 times and the cell lysates were stored at -20 ^{o}C until use. Genetic fingerprinting of *P. aeruginosa* isolates was carried out using primers 272 (AGCGGGCCAA) and 208 (ACGGCCGACC) previously shown to be discriminatory for the CF isolates of *P. aeruginosa* (14). RAPD–PCR reaction mixtures (25 µl) were made optimum for *P. aeruginosa* and contained 1 µl of crude genomic DNA, 0.6 µM oligonucleotide primer, 1 U Smar Taq polymerase (Cinnagen, Iran), 1.25 µM of each of the deoxynucleoside

triphosphates (Cinnagen, Tehran), 10% DMSO (v/v), 0.5 µM BSA (w/v), 3 mM $MgCl_2$, 10 mM Tris-HCl (pH, 8), and 1mM EDTA. Amplification was carried out in a Techne DNA thermal cycler (Genius Model) as follows: 1 cycle of 5 min at 94 ºC, 35 cycles of 1 min at 94 ºC, 1 min at the 39 ºC and 2 min at 72 ºC. A final extension step was carried out at 72 ºC for 10 min. Fifteen µl of each RAPD product and a 1 Kb ladder DNA standard (Fermentas, Cinnagen, Tehran) were run on a 1.5% agarose gel (11 x 16 mm) in Tris-Borate EDTA buffer, pH 8 (TBE) at 80 mV for 2.5 hr. The gels were stained with 1 µM ethidium bromide for 10 min and photographed. Each reaction was repeated at least three times for reproducibility and the fingerprints were analyzed visually using a computer.

Procedures for PFGE have been already described (18, 19). Briefly, *P. aeruginosa* isolates were grown in Lauria Bertani (LB) broth overnight at 37 ^{o}C to late exponential phase. Bacteria were harvested by centrifugation at 1400×g, 10 min and the pellets were resuspended in 500 µl of SE buffer (75 mM NaCl, 25 mM EDTA, pH 7.4). Bacterial density was adjusted to an optical density of 0.8 at 620 nm using a Beckman (DU 520) spectrophotometer and 200 µl of bacterial cells were mixed with an equal volume of 2% low melting point agarose (BioRad) for making the agarose plugs. Plugs were then incubated in PEN buffer (0.5 M EDTA, 1% N-laurylsarcosine, pH 9.6) containing 1 mg/ml pronase (Calbiochem, EMD Biosciences Inc., CA) and incubated rotating at 37 ^{o}C, overnight. Agarose plugs were washed (6 x), 40-60 min/wash in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored in TE at 4 ^{o}C until use. A 2-3 mm portion of each plug was cut and digested overnight with 10 U of *Spe*I restriction endonuclease (New England Biolabs) in the reaction buffer recommended by the manufacturer. Agarose blocks were then inserted in 1.2% agarose gels and sealed with 1.2% low melting temperature agarose in 0.5×TBE. Electrophoresis was carried out in 0.5×TBE at 14 ^{o}C at an electric field of 5 V/cm using a linear increase of pulse intervals for 38-40 hr in a CHEF-DR2 cell system (BioRad). Agarose blocks containing 1 Kb λ DNA ladders (BioRad) were used to estimate the size of DNA fragments. The gels were stained with ethidium bromide and visualized using a gel documentation system (BioRad Doc 1000).

Results

Of the 46 CF patients, 27 were male (M) and 19 were female (F). Figure 1a shows the distribution of the patients into four age groups. Group 1 consisted of patients under 1 year of age (11 patients, 5 M, 6 F), group 2, patients 1–9 years old (11 M, 8 F), group 3, patients 10–18 years old (6 M, 5 F) and group 4, patients over 18 years old (5 M). Colonization of CF patients with *P. aeruginosa* occurred in 31 patients (67.4 %) of all age groups. Colonization rate increased with age and was 80 % in age group 4, 72.7% in age group 3, 66.6% in age group 2 and 58.3% in age group 1 (Figure1b). Mucoid colonies were isolated from 15 patients, 12 carried non-mucoid *P. aeruginosa* and 4 harbored both colony types. Mucoid phenotype was found in 33.3% of age group 1, 85.7% of group 2, 71.4% of group 3 and finally 50% of group 4. There was no significant difference between bacterial colonization and the patients' sex. Of the 15 patients from whom consecutive specimens were obtained, 9 were colonized and the rest did not yield *P. aeruginosa*.

Antibiotic susceptibility results are shown in Figure 2. All isolates were sensitive to tobramycin and ciprofloxacin. Susceptibility to other antibiotics was: 97.8% sensitivity to amikacin and piperacillin, 93.3% to gentamycin, 91.1% to ticarcillin, 86.7% to colistin, 80% to carbenicillin, 48.9 % to cefotaxim, 26.7% to imipenem and 11.1% to ceftazidime. Overall, majority of the isolates were sensitive to most of the antibiotics which are commonly used to treat CF patients in Iran. There was no significant difference in antibiotic sensitivity between mucoid and non-mucoid strains or consecutive isolates during the course of study.

a.

b.

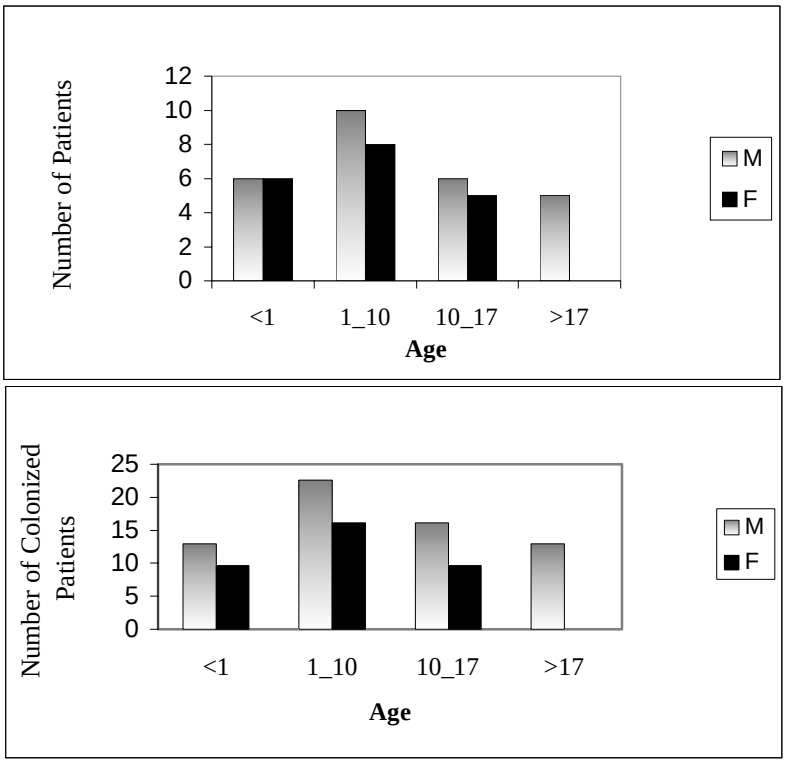

Figure 1. Age and sex distribution of 46 Iranian patients with cystic fibrosis (a), and colonization rate with *P. aeruginosa* (b). M, male; F, female.

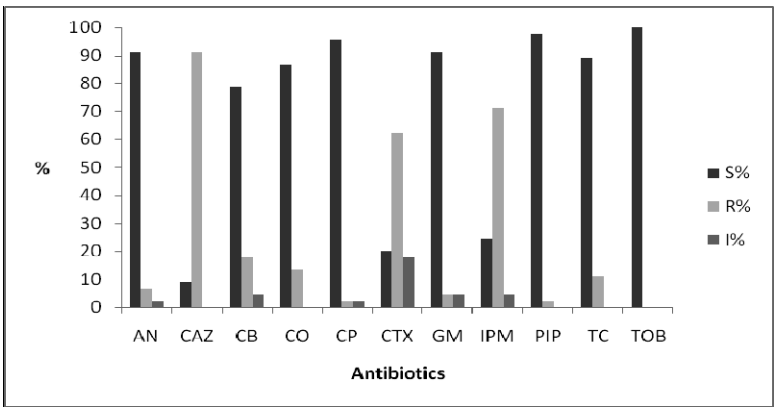

Figure 2 Antibiotic susceptibility of *P. aeruginosa* isolates from Iranian patients with cystic fibrosis. Amikacin (AN), carbenicillin (CB), ceftazidime (CAZ), cefotaxime (CTX), ciprofloxacin (CP), colistin (CO), gentamycin (GM), piperacillin (PIP), tobramycin (TOB), ticarcillin (TC), imipenem (IMP).

RAPD fingerprinting results of *P. aeruginosa* isolates showed that both primers produced reproducible profiles. However, primer 272 generated larger numbers of bands within the fingerprints and was shown to be more discriminating compared to primer 208 as has been shown previously (14, 20). Figure 3 presents some of the fingerprints using primers 208 and 272. Of the 31 patients infected with *P. aeruginosa*, three had identical fingerprints which were also observed in their sequential cultures (patients 2, 8 and 11). The rest of the isolates had unique fingerprints each and were placed in separate genotypes and no predominant fingerprint was found among the isolates. PFGE fingerprinting results agreed with RAPD-PCR in producing similar distribution profiles. There was no relation between the genetic profiles, colony morphology and antibiotic susceptibility of the isolates. These results suggest that patients were colonized with unique *P. aeruginosa* strains as has been suggested by many other investigators (1, 7, 14, 15, 24).

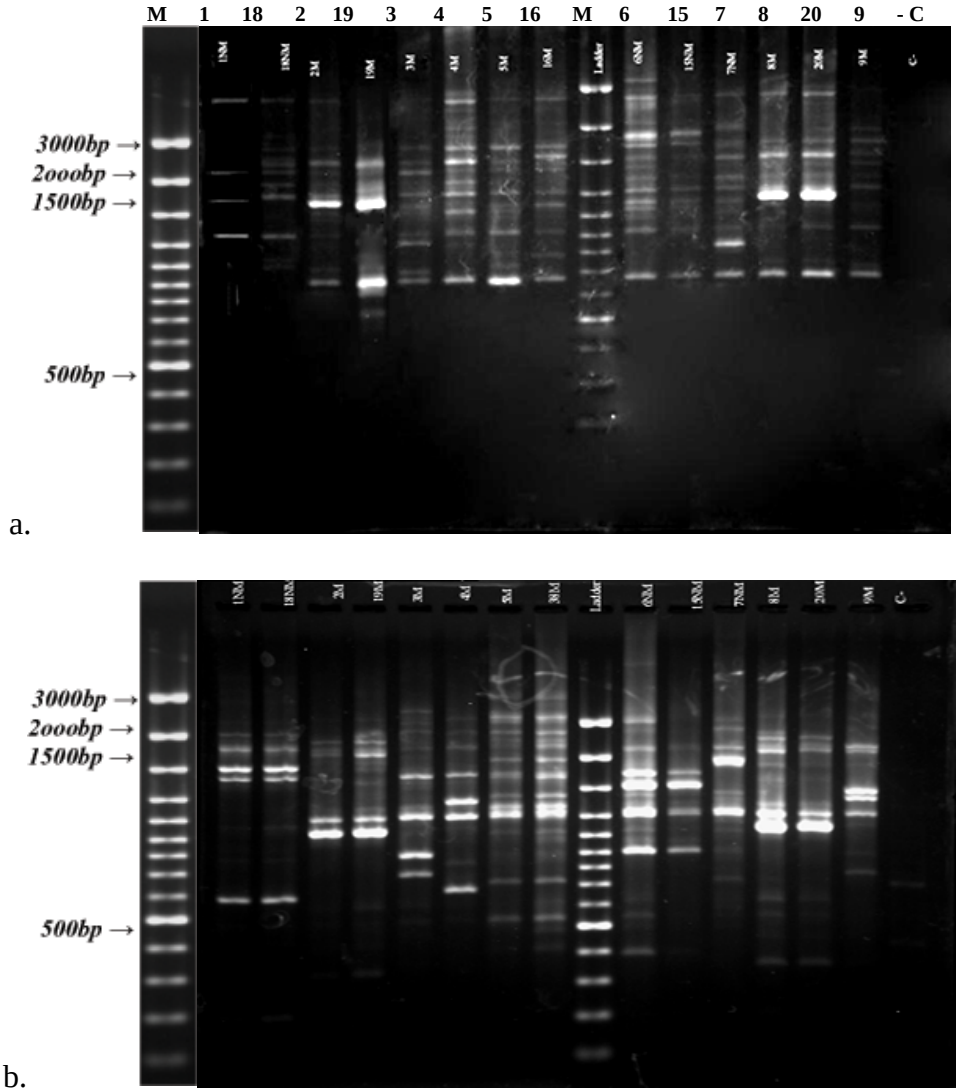

Figure 3. RAPD-PCR fingerprints of some *P. aeruginosa* isolates from Iranian patients with cystic fibrosis using primer 208 (a) and primer 272 (b). Strains 1 & 18, 2 & 19, 5 & 38, 6 & 15 and 8 & 20 were isolated from patients 1, 2, 5, 6 and 8 respectively. M, DNA size markers, C, negative control.

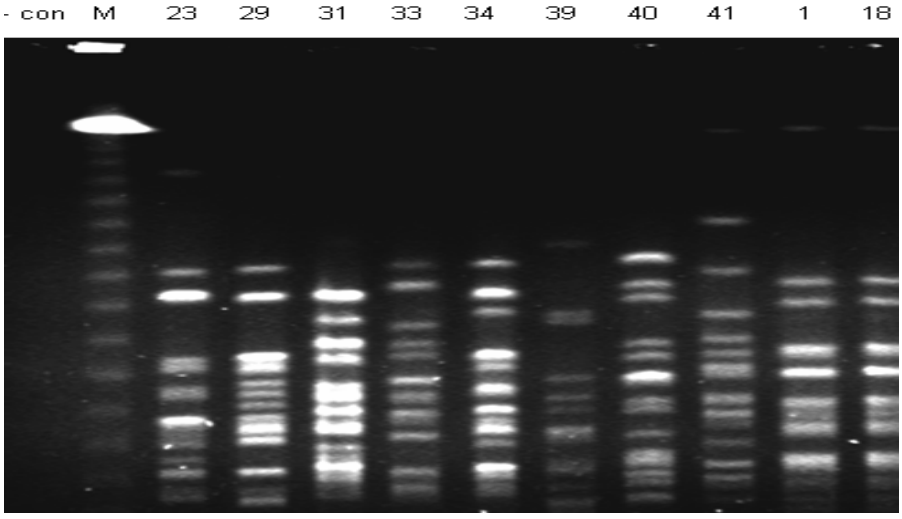

Figure 4 PFGE fingerprints of some *P. aeruginosa* isolates from Iranian patients with cystic fibrosis. M, DNA size markers ranging from 48.5 - 1 Kb, - C, negative control.

Discussion

In this research, colonization of CF patients with *P. aeruginosa* occurred in all age groups. As expected, colonization rate increased with age. Surprisingly, two thirds of the patients who were negative for *P. aeruginosa* (10/15) were between 3 to 10 years old. This may have been due to the fact that these patients received antibiotic therapy and that we were unable to find complete records on the subjects. All *P. aeruginosa* isolates were sensitive to tobramycin and ciprofloxacin, the two antibiotics which are frequently used to treat *P. aeruginosa* infections in Iranian CF patients. On the other hand, 88.9 % were resistant to ceftazidime which is also commonly used singly to treat CF infections in Iran. Interestingly, 73.3 % resistance was found to imipenem which is not used as a therapeutic agent. In a clinical investigation in Spain, imipenem resistant *P. aeruginosa* strains were recovered from patients who did not receive imipenem therapy. The main mechanism of resistance to imipenem was suggested to be diminished activity or lack of an outer membrane protein (21). Unstable imipenem resistant subpopulations of *P. aeruginosa* lacking a 50 kD outer membrane protein were also detected *in vitro*, in which sensitivity was recoverable after sub cultivations in the laboratory (22). Increasing prevalence of imipenem resistance in *P. aeruginosa* has been recently reported (23).

For PCR reactions, bacterial lysates generated by freeze-thawing were directly used as the DNA source in reaction mixtures. Despite the fact that DNA yields are not the same, the method proved to be fast and reproducible. Fingerprints obtained by PFGE provided a similar distribution profile as in RAPD-PCR (Figure 4). These results agree with other reports that RAPD-PCR could be reliable in bacterial genotyping compared to the time consuming and cumbersome PFGE method which is considered as the gold standard (14, 15).

The majority of infections among CF patients are believed to be clonal and individual CF patients acquire unique *P. aeruginosa* strains from diverse environmental sources (1, 2, 14). It is also believed that cross infection among CF patients is rare (1, 7, 15, 24-26). However, using molecular fingerprinting, highly transmissible strains of *P. aeruginosa* have been reported in Europe. It is speculated that mutator strains may evolve from the initially infecting *P. aeruginosa* strains to produce genetic variants (26-28). Interestingly, comparison of clonal and non-clonal CF isolates showed that of a number of virulence factors, protease activity may contribute to transmissibility (29). We found that the majority of our subjects were colonized with unique strains of *P. aeruginosa* and no common bacterial clone was found among the Iranian CF patients. Future studies with larger numbers of bacteria are needed to explore the presence of transmissible isolates among the Iranian patients. Rapid molecular methods such as RAPD-PCR have been shown to be useful for genetic fingerprinting of clinical isolates in epidemiological studies. Similar fingerprints found in different patients would be an indication of transmissible and/or virulent bacterial strains. On the other hand, different genetic fingerprints of the isolates obtained from the same patient over a period of time may indicate infection with a new bacterial strain or a mutation which could determine the course of antibiotic therapy.

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