

Isolation and genetic fingerprinting of *Pseudomonas aeruginosa* from Iranian patients with cystic fibrosis using RAPD-PCR

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Abstract

Sixty four Iranian patients with cystic fibrosis (CF) were studied for colonization with *Pseudomonas aeruginosa*. The patient's age ranged between 2 months to 18 years old. Twenty one patients were colonized, 15 with non-mucoid and 6 with mucoid strains of *P. aeruginosa*. The colonization rate increased with age and the mucoid phenotype was only recovered from the older patients. All mucoid strains came from patients with respiratory disease whereas most of the non-mucoid isolates ($n=13$) were from patients with gastrointestinal disorder. The antibiograms of the isolates showed 100% sensitivity to Imipenem and Collistin followed by Ciprofloxacin (90.5%), Ceftazidime, and Tobramycin (85.7%), Amikacin, Piperacillin and Tazobactam-Piperacillin (81%), Ticarcillin (76%), Gentamycin (62%), Mezlocillin (52.4%) and Carbenicillin (43%). The MICs for Ceftazidime, Gentamycin and Tobramycin agreed with the disk test results. However, MIC determination for Amikacin showed a 100% sensitivity compared to the disk test where 81% sensitivity was observed. The discrepancy may be due to the fact that over 20% of the isolates had borderline MIC values for Amikacin. The genomic fingerprinting of the 21 isolates as well as the non-mucoid revertants of

the mucoid strains was carried out by RAPD-PCR using primer 272 which was previously used for typing *P. aeruginosa* isolates from CF patient's. Thirteen genotypes were found among the 21 isolates. One fingerprint (A) was found in 6 patients and another (B) was shared by 2 patients, all from the same health center. The idea of the hospital as environmental source or cross infection between patients cannot be ruled out.

Keywords: *Pseudomonas aeruginosa*, cystic fibrosis, antibiotic susceptibility, RAPD-PCR.

INTRODUCTION

Pseudomonas aeruginosa is the leading cause of lung infection and death in patients with cystic fibrosis (CF) (Govan and Deretic, 1996; Hoiby *et al.*, 1986; Koch and Hoiby, 1993). It is believed that non-mucoid, classical strains of *P. aeruginosa* colonize the CF lungs initially and by producing an extracellular alginate form mucoid colonies in chronic lung infections (Burns and May, 1968; Diaz *et al.*, 1970; Speert *et al.*, 1990). Formation of mucoid phenotype is almost exclusive to the CF lung and gives the organism the advantage of surviving the host defense mechanisms and antibiotic therapy (Govan and Deretic, 1996; Govan and Fyfe, 1978; Simpson *et al.*,

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1988). It is generally accepted that different *P. aeruginosa* strains colonize the lungs of unrelated CF patients. Once colonized, the patient harbors the same strain for long periods (Govan and Deretic, 1996). Cross infection may occur between siblings or under certain environmental conditions. The diverse phenotypic nature of *P. aeruginosa*, has made conventional typing methods such as serotyping, pyocin typing, biotyping and antibiograms not discriminatory enough for identification and epidemiological studies (Brokopp and Farmer 1979; Hancock *et al.*, 1983). Molecular typing methods such as restriction fragment length polymorphism (RFLP) (Ogle *et al.*, 1987, The International *P. aeruginosa* Typing Study Group 1994), genomic fingerprinting using pulse-field gel electrophoresis (PFGE) (Grundmann *et al.*, 1995; Ojeniyi *et al.*, 1993), arbitrarily primed polymerase chain reaction (AP-PCR) and a random amplified polymorphic DNA (RAPD) (Welsh and McClelland 1990; Williams *et al.*, 1989) have been used to discriminate the identity of these organisms. RAPD-PCR has been verified as a sensitive and discriminating method for typing *P. aeruginosa* isolates from CF patients and compared favorably with PFGE method (Mahenthalingam *et al.*, 1996). It was shown that isolates from different patients have distinct genetic fingerprints but sequential isolates taken from the same patients over a long period display the same genotype. So far, a relationship between colony phenotype and genotype has not clearly been established but genotypic studies are more useful in epidemiology and determining the source of infecting microorganisms.

We studied sixty four Iranian patients who were diagnosed as having cystic fibrosis for colonization with *Pseudomonas aeruginosa*. The bacteria were isolated, their antibiotic susceptibility was determined to 12 antibiotics using the disk diffusion method and minimum inhibitory concentrations were measured for 4 antibiotics. A RAPD-PCR method using an arbitrarily chosen primer was also used to discriminate between isolates from different patients and to compare genotype with morphology and antibiograms of the *P. aeruginosa* isolates.

MATERIALS AND METHODS

Patients: Over a period of 15 months, sixty-four patients from 4 different health centers who were diagnosed as having CF were studied. The age range of

the patients was from 2 months to 18 years old. Diagnosis was based on the results of the sweat test and the clinical symptoms were recorded for each patient (gastrointestinal disorder, GID, or respiratory disease, RD).

Isolation of *P. aeruginosa*: Sputum samples or throat swabs were collected from CF patients depending on the patient's age and were plated on blood agar plates. Colony morphology was reported as non-mucoid or mucoid and the isolated colonies were subjected to standard biochemical tests for identification of *P. aeruginosa*. Gram negative, non-lactose fermenting, oxidase positive colonies which oxidized glucose and maltose and grew on ceftrimide agar at 42°C were identified as *P. aeruginosa*. Pigment formation was detected on Pseudomonas agar (P-Agar). Passages of the mucoid colonies were made on nutrient agar to obtain nonmucoid revertants which only differed from the mucoid parents in alginate production. All isolates were kept at -20°C in media containing 8% dimethyl sulfoxide (DMSO) until use.

Antibiotic susceptibility, disk diffusion: The susceptibility of the *P. aeruginosa* isolates was determined to Amikacin (AN, 30 µg), Carbenicillin (CB, 100 µg), Ceftazidime (CAZ, 30 µg), Ciprofloxacin (CP, 5 µg), Collistin (CO, 10 µg), Gentamycin (GM, 10 µg), Imipenem (IPM, 10 µg), Mezlocillin (MZ, 10 µg), Piperacillin (PIP, 100 µg), Tazobactam-Piperacillin (TZP, 100 µg), Ticarcillin (TC, 75 µg) and Tobramycin (TOB, 10 µg) on Mueller Hinton agar using the disk diffusion assay (Bauer *et al.*, 1966). The antibiotic disks were obtained from two different companies (Mast, England and Pasteur Institute, France). *Pseudomonas aeruginosa* ATCC 27853 was used as the laboratory standard for these tests.

Minimum Inhibitory Concentrations (MIC): A tube dilution method was used and doubling dilutions of Amikacin (Darou Pakhsh, Iran), Ceftazidime and Tobramycin (Green Ford, England), and Gentamicin (Alborz Darou, Iran) were prepared in Mueller Hinton broth (64–0.03 µg/ml). A final concentration of 5 X 10⁵ overnight grown bacteria was added to each tube and the MICs were recorded after 18 hrs at 37°C (National Committee for Clinical Standards 1997).

Isolation of *P. aeruginosa* genomic DNA: A single colony was inoculated into 2 ml of LB broth in a snap top tube and was grown over night with end-over-end

rotation at 37°C. The bacteria were harvested by centrifugation at 3000 ×g for 10 min, the pellet resuspended in 0.4 ml of GET buffer [50 mM glucose, 70 mM EDTA, in 50 mM Tris-HCl (pH 8.0), and 0.2 ml] was transferred to a microcentrifuge tube containing 0.5 ml of 0.1 mm diameter glass beads and 0.9 ml of lysis buffer [50 mM Tris-HCl (pH 8.0), 50 mM EDTA, 1% sodium dodecyl sulfate, 30 µg of RNase/ml]. The bacteria were lysed by a 3 min pulse on a mini-bead beater (Biospec Products, USA), and RNA was digested by incubation at 37°C for 1 hr. The lysate was cleared by brief centrifugation and 0.7 ml was transferred to a fresh tube. Saturated ammonium acetate was added (one third of the volume), the contents were mixed vigorously and the tube was centrifuged to remove the protein and polysaccharide precipitate. The genomic DNA was collected from the cleared lysate by ethanol precipitation, washed with 70% ethanol and was dissolved in 100 µl of TE (Tris-EDTA, pH 8.0) (Sambrook *et al.*, 1989). The DNA was quantitated by absorbance at 260 nm.

RAPD Analysis: Primer 272 (AGCGGGCCAA) which was previously shown to be a good discriminatory primer chosen from a collection of 100 primers for *P. aeruginosa* was used for fingerprinting of the Iranian CF isolates (Mahenthiralingam *et al.*, 1996). RAPD-PCR reaction mixtures (25 µl) were made optimum for *P. aeruginosa* and contained 40 ng of genomic DNA, 40 pg oligonucleotide, 1u of Taq polymerase (Gibco-BRL, Germany), 250 µM of each of the deoxynucleoside triphosphates (ultra-pure, Pharmacia, Sweden), 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 0.001% gelatin and 3 mM MgCl₂. Each reaction mixture was overlaid with 25 µl of mineral oil and amplified with a Perkin-Elmer Cetus DNA Thermal Cycler (model TC-1) as follows: (i) 4 cycles each consisting of 5 min at 94°C, 5 min at 36°C, and 5 min at 72°C, and (ii) 30 cycles each consisting of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C, followed by a final extension step at 72°C for 10 min. One third of each RAPD product (reaction mixture) was separated on a 1.5% agarose gel (20 wells, 11 x 14 cm) with 1x TBE running buffer at 9 V/cm for 3 hrs. Molecular size standards were included (1 Kb ladder, Gibco-BRL, Germany). The gels were stained with ethidium bromide and photographed onto film (Tri-X pan, Eastman Kodak). The RAPD fingerprints were analyzed both by eye and also by computer with Gelmanager for windows V1.5 software (Biosystem-atica, USA).

RESULTS

The patients were divided into three age groups: 1; under one year old, 2; 1-10 years and 3; 11-18 years old. Figure 1a shows the age and sex distribution of the Iranian CF patients. The majority of the patients were under 1 year of age (68.75%), followed by age group 2 (18.75%) and age group 3 (12.5%). *Pseudomonas aeruginosa* was recovered from 21 patients (32.8%) of which 15 were non-mucoid and 6 had mucoid phenotype. Figure 1b shows the distribution of the colonized patients in different age groups. Twelve nonmucoid strains were isolated from group 1 followed by 4 (2 mucoid and 2 nonmucoid strains) from group 2 and finally 5 isolates (4 mucoid and 1 nonmucoid) from age group 3. Overall, colonization rate increased with age and 62.5% of the older patients (group 3) were colonized in comparison with 33.3% in age group 2 and 27.3% in age group 1. All of the patients from whom mucoid *P. aeruginosa* was recovered had severe respiratory disease (RD). Thirteen of the 15 non-mucoid isolates came from patients with gastrointesti-

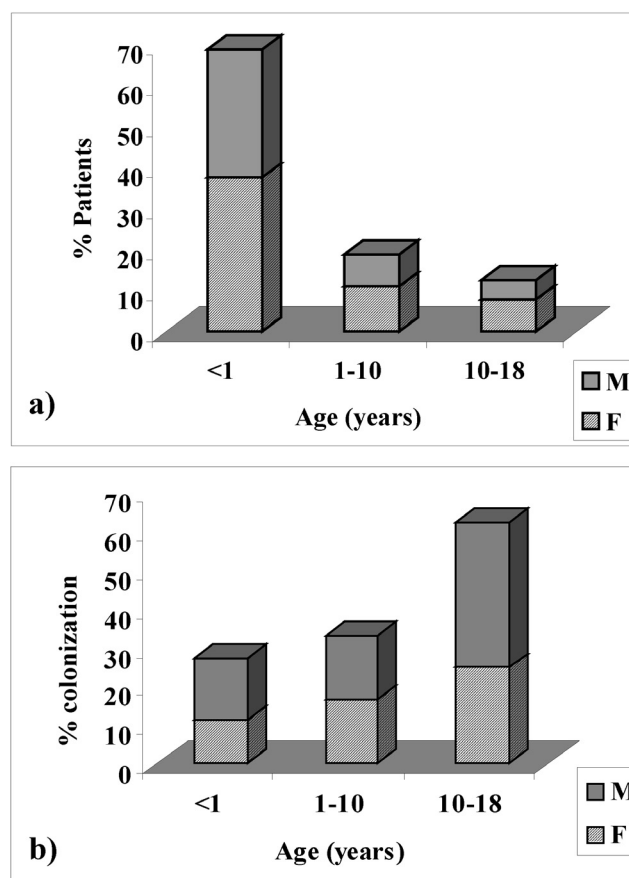


Figure 1. Age and Sex distribution of the Iranian CF patients (1a) and the rate of colonization with *Pseudomonas aeruginosa* (1b). M, male and F, female.

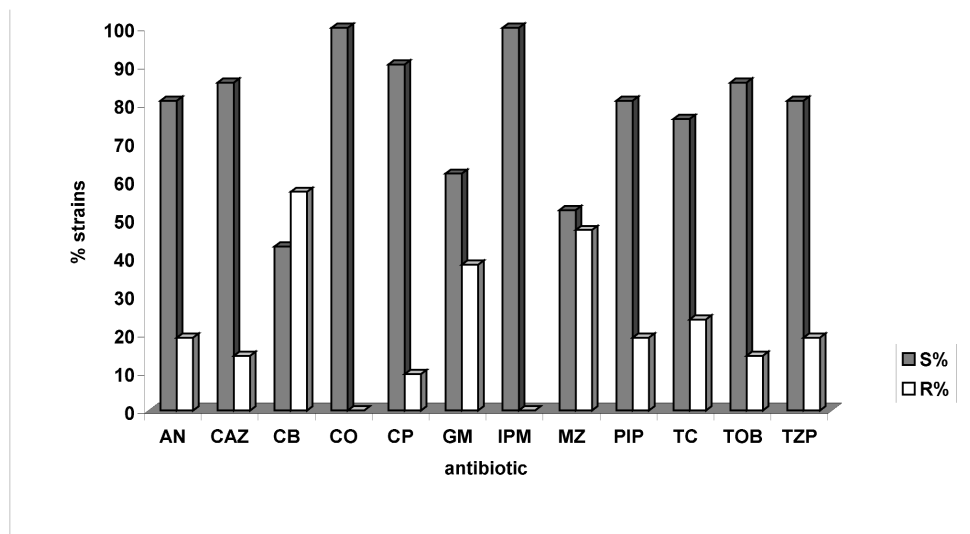


Figure 2. The antibiotic susceptibility of *Pseudomonas aeruginosa* isolates from the Iranian CF patients using the disk diffusion method. Amikacin (AN), Ceftazidime(CAZ), Carbenicillin (CB), Collistin (CO), Ciprofloxacin (CP), Gentamycin (GM), Imipenem (IPM), Mezlocillin (MZ), Piperacillin (PIP), Tetracycline (TC), Tobramycin (TOB), and Tazobactam-Piperacillin (TZP) were used.

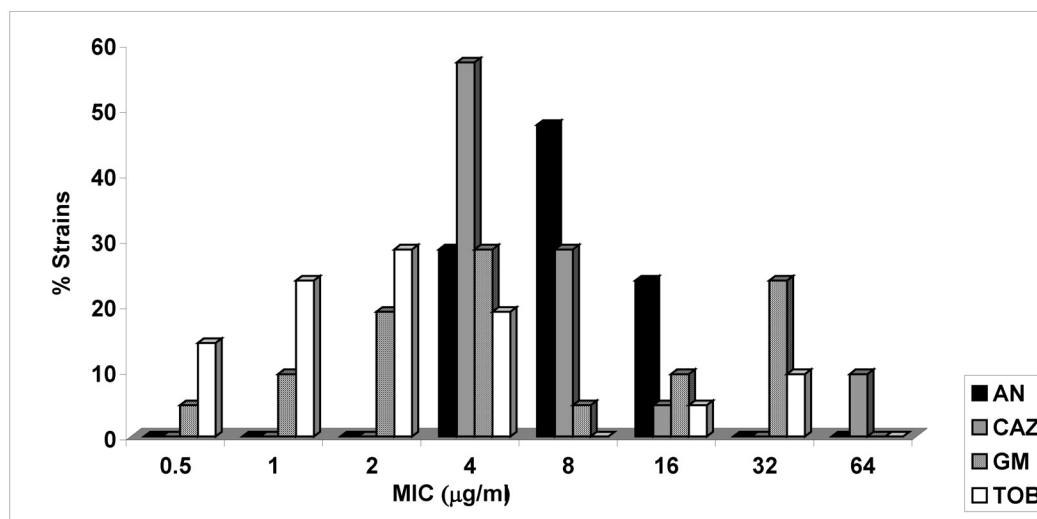


Figure 3. Minimum inhibitory concentrations. MIC of Amikacin (AN), Ceftazidime (CAZ), Gentamycin (GM) and Tobramycin (TOB) measured against *Pseudomonas aeruginosa* isolates from the Iranian CF patients.

nal disorder (GID) and only two were from patients with RD. There was no significant difference between colonization and the patient's sex.

Antibiotic Susceptibility: The results of the antibiotic susceptibility of the isolates using the disk method is shown in figure 2. All isolates were sensitive to Imipenem and Collistin, the two antibiotics that are not used to treat *P. aeruginosa* infections of CF patients in Iran. Susceptibility to the other antibiotics was; 90.5% sensitivity to ciprofloxacin, 85.7% to Ceftazidime and Tobramycin, 81% to Amikacin, Piperacillin and

Tazobactam-Piperacillin, 76% to Ticarcillin, 62% to Gentamycin, 52.4% to Mezlocillin and 43% to Carbenicillin.

Measurement of MICs for the 4 test antibiotics (Fig. 3) showed that all of the isolates were sensitive to Amikacin (MIC≤16 µg/ml), 85.7% were sensitive to Ceftazidime (MIC≤8 µg/ml) and Tobramycin (MIC≤ 4 µg/ml) and 57.1% were sensitive to Gentamycin (MIC≤4 µg/ml). Comparison of these results with those found by the disk method showed identical sensitivity for Ceftazidime and Tobramycin and similar results for Gentamycin. However, the sensitivity to

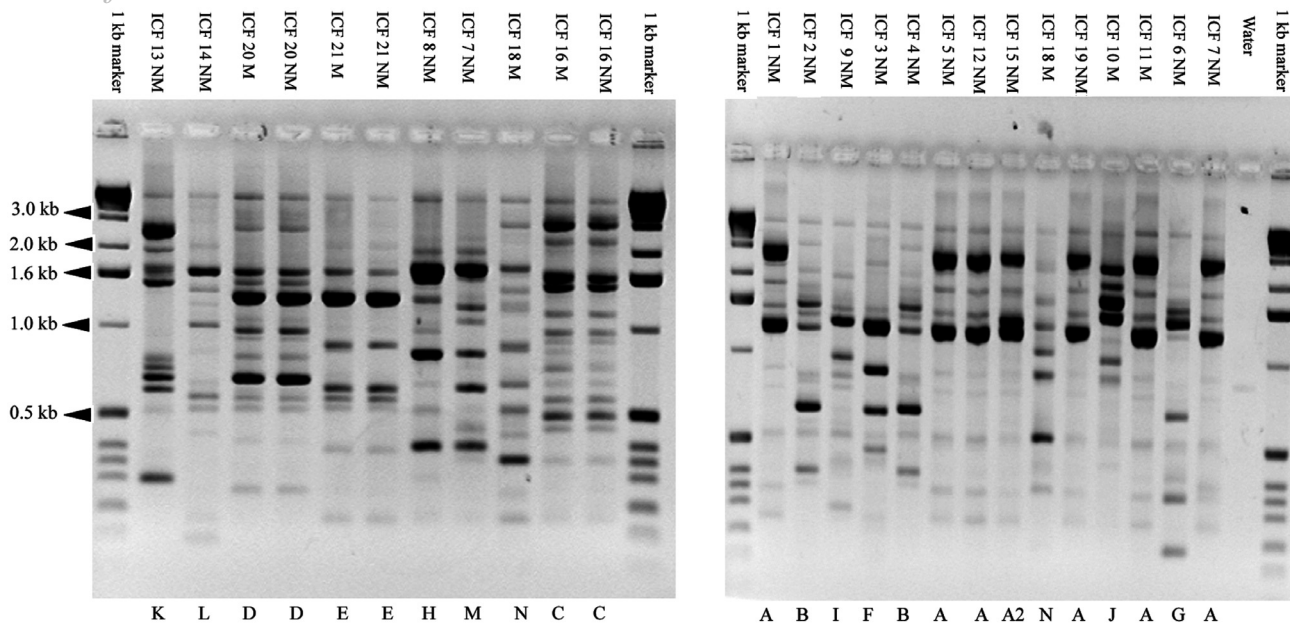


Figure 4. RAPD-PCR fingerprints of *P. aeruginosa* isolates from Iranian patients with cystic fibrosis using primer 272, M, mucoid, NM, non-mucoid.

Amikacin was 100% by the MIC method, whereas 81% sensitivity was obtained by the disk test. As shown in figure 3, over 20% of the isolates had MIC values of 16 µg/ml, which is the borderline value and could account for the discrepancy.

RAPD Fingerprinting: All isolates (15 nonmucoid and 6 mucoid strains) as well as 3 nonmucoid revertants of the mucoid strains (16, 20 and 21) from Iranian CF patients were fingerprinted using primer 272 (AGCGGGCCAA) (Mahenthalingam *et al.*, 1996). The results showed that 13 different fingerprinting patterns were present among the 21 isolates (Fig. 4). Six isolates (1, 5, 7, 11, 12, 19) had identical fingerprints (A). Another isolate (15) had a similar pattern which was placed in A₂ but needs to be further studied by other methods such as PFGE. Two isolates (2 and 4) were placed as type B and the rest of the bacterial strains had unique fingerprints and each was placed in a separate genotype (shown in Fig. 4). Interestingly, each mucoid-nonmucoid pair had a different but identical fingerprint (C, D and E), which shows that the mucoid phenotype does not affect genetic fingerprinting. The two strains with the B fingerprint had identical antibiograms and were both resistant to 6 of the test antibiotics. Strains belonging to genotype A had similar but not identical antibiograms and no correlation could be made between

genotype and antibiograms.

DISCUSSION

In this study, 64 Iranian patients who were diagnosed as having cystic fibrosis were examined for colonization with *Pseudomonas aeruginosa*. Mucoid phenotype was recovered only from the older patients which correlates with the belief that CF patients are primarily colonized with non-mucoid *P. aeruginosa* and mucoid phenotype develops later in the chronic lung infections (Koch and Hoiby, 1993; Pederson, 1992; Speert *et al.*, 1990). The mucoid phenotype was associated with respiratory disease whereas the non-mucoid isolates were mostly recovered from patients with gastrointestinal disorder. It must be stressed that most of the patients usually came to the health centers once and a history of the development of clinical symptoms or previous antibiotic therapy was not available.

There was no significant difference for antibiotic sensitivity between mucoid and non-mucoid strains *In Vitro*. The MIC measurements showed a good correlation with the disk test results but in most cases were close to the borderline cut off values for sensitivity. This is perhaps one of the reasons for using combination therapy in treating *P. aeruginosa* infections in CF patients to avoid selecting for resistant organisms.

The RAPD fingerprinting results showed that there

was no correlation between bacterial phenotype and its genotype and most patients were colonized with unrelated organisms. These findings agree with other studies which found no relation between bacterial phenotype and genotype and also that different CF patients were colonized with different *P. aeruginosa* isolates (Mahenthiralingam *et al.*, 1996; Ogle *et al* 1987; Ojeniyi *et al.*, 1993 and The International *P. aeruginosa* Typing Study Group 1994). Remarkably, genotype A was common in 6 isolates from different patients and genotype B was shared by two. These patients were all hospitalized in the same health center but the information on the date and the length of hospitalization, or if these patients were roommates was not clear. Hence, the idea of the hospital environment as the source of infection, or cross infection between patients cannot be ruled out. Clearly a larger number of patients, better history of antibiotic therapy and hospitalization and sequential isolates from the same patients are needed to help answer some of the questions raised. This is the first report on the microbial colonization of Iranian patients with cystic fibrosis and further extensive work is needed.

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