Original Article

Phenotypic characterization and PCR-Ribotypic profile of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients in Iran

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Abstract

Background: *Pseudomonas aeruginosa*, is the most common pathogen in patients with cystic fibrosis (CF) that shows various resistance to antibiotics, acquires mucoidity and multiple genotypes. This survey was performed to study phenotypic and genotypic variations among *P. aeruginosa* isolates in CF patients at Alzahra Hospital in Isfahan, Iran.

Materials and Methods: The isolates of *Pseudomonas aeruginosa* from CF patients at Alzahra Hospital was identified by appropriate biochemical and microscopic tests, then performed antibiotic resistance tests and mucoid colony morphotyping. The genum of isolates extracted and confirmed on 16S rDNA-based PCR assay and typed on 16S rDNA-23SrDNA spacer, restricted with Hinf1 restriction enzyme.

Results: *P. aeruginosa* was isolated from 21 of the 59 CF patients (35.5%), Out of 21 isolates 9 (42.8%) strains were revealed mucoid morphotype. 81.8% isolates of mucoid strains were resistance to at least one of four antibiotics (GM, AN, PIP and CP). Most of the isolates (86%) showed resistance to ceftazidime. Ribotyping revealed two patterns (P1, P5).

Conclusion: The isolates of *P. aeruginosa* showed meaningful difference between drug resistance to antibiotics. The majority of *P. aeruginosa* isolated from CF patients showed pattern1 of PCR-Ribotyping.

Key Words: Cystic fibrosis, PCR–ribotyping, *Pseudomonas aeruginosa*

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INTRODUCTION

Pseudomonas aeruginosa causes pulmonary infections in cystic fibrosis (CF) patients, also PMN response reduced in CF patients.[1] It is a Gram-negative opportunistic pathogen of recurrent infections in hospitalized or compromised patients and often persists in the respiratory tract patients, despite intensive antibiotics therapy, causing chronic lung infection and pulmonary failure.[1] Eradication of this bacterium is difficult even with combined antibiotic

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treatment and becomes predominant with age and predicts shortened CF survival.[2] It forms biofilm in the sinuses, which constitute an important bacterial reservoir for subsequent lung infection.[1] A period of intermittent colonization with *P. aeruginosa* early in the life of the patient precedes the establishment of persist and chronic infections. Early infection of *P. aeruginosa* strains typically resembles those found in the environment, being non-mucoid, fast growing, and relatively susceptible to antibiotics. Presumably CF patients acquire unique *P. aeruginosa* strains from diverse sources and expanding of *P. aeruginosa* genotype clone occurs in CF patients who develop persistent infection. New *P. aeruginosa* strains can appear after eradication therapies.[3] With chronicity of infection and prolonged administration of drugs, *P. aeruginosa* strains become resistant against antibiotics, diversifies its colony morphology, acquires mucoidity and shows multiple genotypes.[4] There are six different colony morphotypes of *P. aeruginosa*, type 5 (mucoid Type) was found to be the most common morphotype in chronic patients.[4,5] The mucoidity helps an organism to grow as biofilm, these mucoid isolates promotes more resistance to antibiotics and frustrates the immune system responses to clear the organism.[4,6] It is important to monitor the antibiotic susceptibility pattern, different genotypes and morphotypes, especially mucoidity of *P. aeruginosa* isolates to reduce morbidity and mortality in patients with cystic fibrosis.[4,5] It has been observed that a patient with cystic fibrosis can be colonized by genotypically distinct *P. aeruginosa* strains simultaneously.[7-10] PCR-ribotyping based on PCR amplification of the spacer region between 16S-23S rRNA has been widely used for differentiating bacteria up to species and even strain level.[11] According to morphotyping and PCR-ribotyping, there is clonal relatedness between clinical and environmental isolates of *P. aeruginosa.*[12,13] There is not any data about characterization of *P. aeruginosa* isolates in CF patients in Iran. The present research was performed to study phenotypic and genotypic variations among *P. aeruginosa* isolated in CF patients at Alzahra Hospital in Isfahan, Iran.

MATERIALS AND METHODS

Subjects

All 59 patients with CF referred to Azzahra Medical Center in Isfahan between June 2003-March 2008 were included in the study, (median age 6/2 years). The diagnosis of CF was based on both clinical and laboratory parameters.[14] Fifty-nine CF patients were followed up monthly in the Alzzahra Medical Center for evidence of *P. aeruginosa* colonization of the respiratory tract.

Sample collection

Sputum sample from hospitalized patients and those referred to clinic for persistence of therapy was taken. Sputum sample after examination by a experienced physician was collected directly in a sterile vial after coughing. Throat swabs were collected from children under two years or who were not able to expectorate sputum.[8,15] Throat swabs carried to the microbiology laboratory by using Trypticase Soy Broth medium.

Isolation and characterization of *P. aeruginosa* strains

Sputum samples were vortexed for complete homogenization. Throat swabs samples and the sputum sample cultured on MacConkey agar, Blood agar, selective agars such as cetrimide agar and Pseudomonas agar. Then the plates were incubated at 37°C for 24 h. *P. aeruginosa* colonies was identified by standard bacteriological methods[4,16,17] and confirmed on 16S rDNA- based PCR assay.[18]

Antibiotic susceptibility

Antibiotic sensitivity test for the mucoid morphotype and non-mucoid morphotype were performed by using the Kirby Bauer method, the antibiotics were; gentamicin, ciprofloxacin, piperacillin, amikacin and ceftazidime (Hi Media, padtan teb). *P. aeruginosa* ATCC 27853 served as the control strains.

Molecular typing method

DNA extraction: A 24 h single colony picked up from cultured on Mueller-Hinton agar and suspended in the microtube contained 100 ul of distilled water (Cinnagen Co.). The Tube was boiled for 15 min in boiling water. Equal volume of 24:1 chloroform: Isoamyl alcohol (Qualigens) was added and centrifuged at 12,000 rpm for 15 min. The supernatant used as crude DNA for 16S rDNA- based PCR assay and PCR-ribotyping.[5]

16S rDNA-based PCR assay

16S rDNA sequence has been used as a taxonomic "gold standard" in determining the phylogenies of bacterial species.[18] In order to confirm the isolates as *Pseudomonas aeruginosa* we used a PCR assay that based on 16S rDNA sequence with specific primers as described by Theodore *et al*.[18]

PCR-ribotyping and digestion with Hinf1 restriction enzyme

PCR- ribotyping of *P. aeruginosa* isolates and digestion with Hinf1 (fermentas) restriction enzyme was carried out as described by Agarwal.[5] PCR-ribotyping amplification reaction was performed in a total volume of 50 µl containing 200 µM dNTPs mix, 2.5 mM MgCl2, 2.5U Taq DNA polymerase, 20 pm of each primer and 6 µl of the DNA template. Amplification programmed for 25 cycles with each consisting of 94°C for 1 min, 41°C for 1 min and

72°C for 1 min. A final extention at 72°C for 7 min applied. Amplification product was loaded on agarose gel. 10 µl of PCR-ribotyping amplification product was restricted with 5U Hinf1 restriction enzyme in 37°C for 24 h and restricted fragments loaded on 2 per cent agarose gel or on 10% polyacrylamide gel for better resolution.[5]

RESULTS

P. aeruginosa was isolated from 21 of the 59 patients (35.5 %) (13 men/8 female) (Minimum age 3 month, Maximum 24 years), Out of 21 patients, 9 patients (42.8%) were culture positive for mucoid morphotype (Type 5) of *P. aeruginosa* [Tables 1 and 2]. 38 patients out of the total patients (64.5 %) were culture positive for organism other than *P. aeruginosa* such as *Staphylococcus aureus*, *Escherchia coli*, *Enterobacter*, *Klebsiella sp* and *Citrobacter sp* by cultured samples on MacConkey agar, Blood agar, selective agars such as cetrimide agar and Pseudomonas agar.

Table 1: Colony morphotypes and PCR-ribotyping patterns of *P. aeruginosa* isolated from cystic fibrosis patients

Patient. No	Age (yr/mth)	Gender	Morphotype		Sample	PCR-ribotyping
			NM	M		
1	2/7	M	*	–	TS	P1
2	2/6	M	*	–	TS	P1
3	2/2	M	*	–	TS	P1
4	1/8	F	–	*	TS	P1
5	3/7	M	*	–	TS	P1
6	1/9	M	*	–	TS	P1
7	13	M	–	*	SP	P1
8	9	F	*	–	SP	P1
9	13	M	–	*	SP	P1
10	1	M	–	*	TS	P1
11	24	M	–	*	SP	P5
12	5/5	F	*	–	TS	P1
13	5/5	F	–	*	TS	P1
14	8	M	*	–	TS	P1
15	0/3	F	–	*	TS	P1
16	12/5	F	–	*	SP	P1
17	14	M	–	*	SP	P1
18	0/3	F	*	–	TS	P1
19	6/5	M	*	–	TS	P1
20	5/2	M	*	–	TS	P1
21	4/5	F	*	–	TS	P1

M: Male, F: Female, SP: Sputum, TS: Throat swab, P: Pattern, NM: Non-mucoid, M: Mucoid, *Positive sign, – : Negative sign

Table 2: Mucoid/Non-Mucoid ratio in *Pseudomonas aeruginosa* isolated from cystic fibrosis patients

Morphotype	Number of *P. aeruginosa* isolated from cystic fibrosis patients (percent)	Resistance isolates to at least one of four antibiotics (GM, AN, PIP and CP) (percent)	Mean age (year)
Mucoid	9 (42.9)	9 (81.8)	9/4
Non-Mucoid	12 (57.1)	2 (18.2)	4/3
Total	21 (100)	11 (100)	—

Antibiotic susceptibility

Antibiotic susceptibility of the 21 *P. aeruginosa* CF isolates were investigated by 5 antibiotic and the resistance rates according to CLSI guidelines were as follows: Amikacin (9.5%), Gentamicin (9.5%), Ciprofloxacin (14.2%), Pipracilin (19%) and Ceftazidim (86%) [Figure 1]. The result showed that the majoritiy of *P. aeruginosa* isolates were the most resistance to ceftazidime (86%). Out of 11 resistance cases to at least one of four antibiotics (GM, AN, PIP and CP), 9 (81.8%) isolates were mucoid that is higher than in compared with two nonmucoid isolates (18.2%). Nineteen isolates (90.5%) were found sensitive to gentamicin and amikacin. The 85.7% of patients (6/7) harbored the isolates that was displayed resistance to at least 2 antibiotics had repetitive hospitalization (at least two time).

16S rDNA- based PCR assay

All *P. aeruginosa* isolated from 21 patients that was identified by biochemical and microscopic tests, confirmed by16S rDNA- based PCR assay. All DNA isolated from samples positive for *P. aeruginosa*

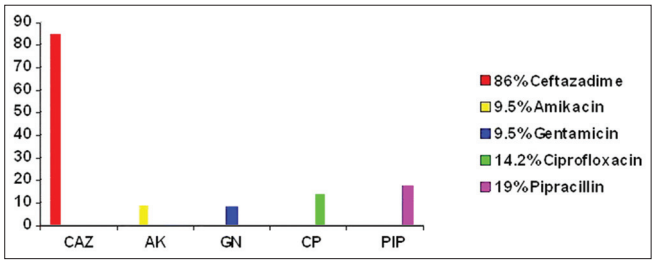

Figure 1: Resistance rates of P.aeruginosa isolated from cystic fibrosis Patients value < 0.001, Ceftazadime (CAZ), Amikacin (AK), Gentamicin (GN) Ciprofloxacin (CP), Pipracillin (PIP)

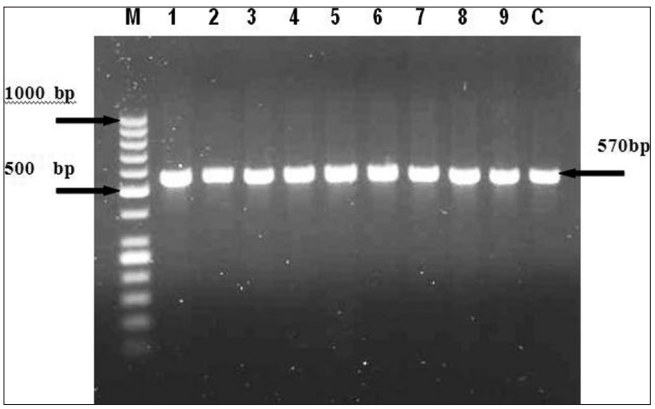

Figure 2: PCR-ribotyping pattern 1(570 bp): Lane M: 50 bp ladder, lane C: control strain (27853) lanes 1-9: CF Patients No.1-9

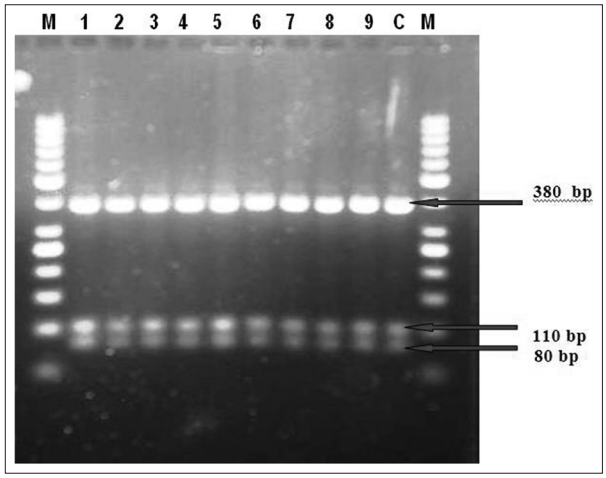

Figure 3: Hinf1-Restriction of pattern1 (380, 110, 80 bp): Lane M: 50 bp ladder, lane C: control strain (27853) lanes 1-9: CF Patients No.1-9

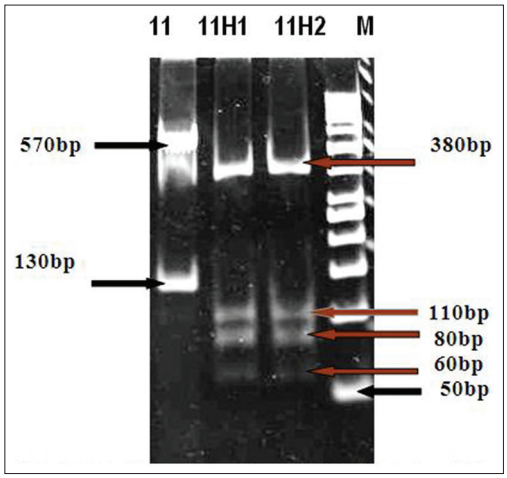

Figure 4: PCR-ribotyping pattern 5(570, 130 bp) and Hinf1-Restriction of pattern 5(380, 110, 80, ~60 bp) on 10% Polyacrylamide gel electrophoresis: Lane M: 50 bp ladder, lanes 11: CF Patient No.11, lanes 11H1 and 11H2 Hinf1-Restriction of CF Patient No.11

(21 patients) showed bands where corresponding with special band of *P. aeruginosa* (956 bp).

PCR-Ribotyping and digestion with hinf1 restriction enzyme

Two PCR- ribotyping patterns were identified as P1: 570 bp, P5: 570 bp, 130 bp as showed in [Figures 2 and 4]. Twenty of 21 patients harbored *P. aeruginosa* with a single PCR- ribotyping (Pattern P1). *P. aeruginosa* isolated from one Patient (No. 11) shown PCR- ribotyping pattern P5 [Figure 4]. Digestion profile with Hinf1 enzyme for about the isolates that are include P1 of PCR-ribotyping, yielded bands as molecular weight (bp): 380 bp, 110 bp, 80 bp [Figure 3] and for patient No. 11 was; 380 bp, 110 bp, 80 bp,~60 bp on 10% polyacrylamide gel electrophoresis [Figure 4]. The isolate from patient No. 11 was mucoidity. PCR- ribotyping along with the restriction of the amplified product was repeated at least 3 times.

DISCUSSION

In present study, *P. aeruginosa* infection rate, was 35.5% and the rest percent (64/5%) totally related to other organisms that correspondence to Anthony Hart, Trust and Pedersen reports.[19-21] They reported that *P. aeruginosa* infection rate vary from 20-85% in most CF patients, but with a higher prevalence in adults units. Comparison our results with Anthony Hart, about *P. aeruginosa* infection rate (35.5%/up to 85%) may be due to the fact that the median age of patients that we were investigated was less than 10 years (6.2 years). During lung infections in CF patients, *P. aeruginosa* usually undergoes a transition from a non-mucoid to a mucoid phenotype. Usually, CF patients acquired non-mucoid *P. aeruginosa* until one year and mucoid *P. aeruginosa* until 13 years after acquisition of disease, and our data showed non-mucoid *P. aeruginosa* occurs during infancy and early childhood, while mucoid *P. aeruginosa* generally begins later. In present study out of 21 patients, 9 isolates (42.8%) were culture positive for mucoid *P. aeruginosa*. and mean age was (9.4 years), while the median age of patients harbored non-mucoid *P. aeruginosa* was (4.3 years). Approximately, Our finding in agreement with Zhanhai Li report.[22] Transition of initial non-mucoid *P. aeruginosa* to mucoid *P. aeruginosa* prevented/postponed by aggressive anti-*P. aeruginosa* treatment, eradication of mucoid *P. aeruginosa* seems impossible, and is a life-threatening situation.[23-26] Studies showed that prolonged therapy with antibiotics possibly lead to antibiotic resistance and may not eradicate the organisms. In our study, Ceftazadime showed the most resistance (86%) to *P. aeruginosa* isolated from CF patients (median age 6.2 years). Both Amikacin and Gentamicin were the most sensitive antibiotics (9.5% antibiotic resistance for each other).

About the influence of these antibiotics on adult CF patients, different results have been reported by L. Leone and M. R. O Carroll *et al*.,[27,28] and contradicted with our result. Therefore, It seems that adult CF patients and those (urinary Patients, Hospital personnel exposed to antibiotics and the Hospital Environment (sets, wards, showers, sinks, drug shelfs, blankets,…) were colonized with *P. aeruginusa* for a long time are in antibiotic resistance risk. About high resistance to Ceftazidime in CF patients in Isfahan/ Iran, it may be due to frequent administration of this antibiotic, or it may be due to the transmission of high resistance strains among CF patients and environmental source.

Based on molecular typing of these isolates, the CF patients can be colonized by different genotype as has been reported.[4,5,7-10] In our study, majority of isolates showed the single PCR-ribotyping (Pattern P1), This was in agreement with the earlier reports[4,5] On these reports and our study, it seems that the majority of PCR-ribotyping Patterns (isolated from newly infected CF patients (who are in lower age)) is similar. and also *P. aeruginosa* to adapt and survive in the face of host immune mechanisms easily exposed to mutation[29] and genetic diversity in patients with long-time infections (predominantly adults). Thus, it is possible that the *P. aeruginosa* with genetic diversity reported from patients with long-time infections, the other results (data not showed) of our study showed that the PCR-ribotyping pattern of prevalence isolates (isolated from newly infected CF patients (who are in lower age)) similar to the most prevalence pattern of *P.aeruginusa* isolated from urinary out Patients, personnel and the Hospital Environment. The predominance of pattern P1, in strains isolated from newly infected CF patients. and strains isolated from other patients or common source (has been cited, before) suggests a possible cross-colonization between CF patients and other source.

The limitation of this study that must be mentioned is identification of single colony from each cultured sample and attributed to the colonized pathogen of the relevant patient, while patients may be infected by different type of microorganism, as well as different strains of pseudomonas.

In conclusion, *Pseudomonas aeruginosa* in comparison with other organisms is the most common pathogen in CF patients at Alzahra Hospital in Isfahan/Iran. Mucoid to non-mucoid isolates ratio of *Pseudomonas aeruginosa* is lower among 21 CF patients in our samples. Most mucoid cases of *Pseudomonas aeruginosa* detected in upper age of CF patients. The isolates of *P. aeruginosa* showed meaningful difference between drug resistance to ceftazidime in compared to other antibiotics (PIP, CP, AK, GM). Pattern1 of PCR – Ribotyping is the most prevalence pattern in CF patients in Isfahan/Iran.

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