Comparison of arbitrarily primed-polymerase chain reaction and plasmid profiles typing of *Pseudomonas aeruginosa* strains from burn patients and hospital environment

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ABSTRACT

Objectives: To identify the strengths and weakness of arbitrary primed-polymerase chain reaction (AP-PCR) and plasmid profiles for typing of *Pseudomonas aeruginosa (P. aeruginosa)* and tracking of source of infections.

Methods: Seventy-four strains of *P. aeruginosa* were isolated from burn patients and hospital environment between January to April 2003 in Ghotbadden Burn Hospital, Shiraz, Iran. The strains were classified by photo Capt Mw program, similarity and clustering of strains were assessed using NTSYS-PC version 2.02K software.

Results: Based on 50% and 64.7% and 67.5% similarity on the plotted dendrogram, 38 plasmid profiles were classified into: 2, 3 and 5 clusters, respectively. Photo Capt Mw program categorized AP-PCR products to 47 different types of 6 to 12 bands between 0.376 to 3.7 kb. Based on dendrogram pattern 3 levels (62 %, 81% and 84.6%) of similarity were selected. Using these criteria 2, 5and 11 clusters were obtained, respectively.

Conclusion: As compared with plasmid profiles, AP-PCR analysis protocol is rapid, reproducible and differentiated the isolates with higher discrimination power. These results suggest that during admission of patients in burn center a limited number of common strains cross-contaminate burn victims. However, transmissions of infection from hospital environment to patients also occur in the minority of the victims. To control cross-contamination of the patient wounds with antibiotics resistant isolates, strong disinfection of patients' bathroom after scrubbing of each patient wounds is mandatory.

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Infection is one of the most serious complications in burn patients. Serious infections caused by *Pseudomonas aeruginosa (P. aeruginosa)* remain a common complication in burn patients contributing substantially to burn morbidity and mortality. According to a survey carried out in 176 burn care centers in North America, Pseudomonas species were considered the most life threatening infections in thermally injured patients.[1] Similarly, a 25 year review of bacteremia in burn patients revealed that *P. aeruginosa* was an etiological agent for bacteremia in these patients leading to 77% mortality.[2] Burn patients colonized with Pseudomonas can be an important reservoir, and the organism persistence in the hospital environment by person-to-person transmission and by saprophytic survival and growth in a verity of hospital environments.[3] *Pseudomonas aeruginosa* strains were identified on the basis of phenotypic characteristics, but the nosocomial epidemiology was hampered during the last decade by the imprecision of the most typing methods available. High-discriminative DNA-based methods have been used to fingerprint *P. aeruginosa* isolates, particularly, analysis of DNA restriction fragment length polymorphism of rRNA gene (ribotyping)[4,5] restriction endonuclease analysis (REA),[6] and macro restriction digestion of DNA by pulsed-field gel electrophoresis (PFGE).[7,8] However, the application of these methods in clinical laboratory was restricted in the specialized centers due to technical complexity and labor-intensive nature.[9] Alternative and simpler typing techniques based on plasmid profiling and PCR with single short arbitrary primer (AP-PCR) has been used successfully for various important medical microorganisms including *P. aeruginosa*.[10,11] In this study, we reported the

results of plasmid profiles and AP-PCR fingerprinting to determined the clinical isolates from burn patients and hospital environment in our burn center, Shiraz, Iran. The aim was to determine the genetic diversity among these bacteria in order to detect DNA polymorphisms, which can indicate if there were any common types associated with *P. aeruginosa* infections in our burn center, and to evaluate the discriminatory power of both typing methods for future epidemiological studies.

Methods. Sixth-three and 11 strains of *P. aeruginosa* were isolated from patients and hospital environment, between January to April 2003 in Ghotbadden Burn Hospital, Shiraz, Iran. A sterile swab was used for sampling from burned patients or hospital environment. The samples were quickly cultured on nutrient agar media (Oxoid) and incubated over night at 37^{0}C. Any suspicious colony was then subcultured and purified. The isolates were identified as a *P. aeruginosa* based on oxidase test, Triple Sugar Iron (TSI) fermentation, color, pyocyanin pigment production and odor. Strains were preserved at -70^{0}C on nutrient broth No2 (Oxoid) containing 30% (v/v) glycerol.

Plasmid and total DNA extractions. To prepare plasmid DNA, pure single colonies form each strain was cultured into 100 ml flask containing 10 ml Leuria Burtoni (LB) medium. The flask was incubated overnight at 37^{0}C with vigorous shaking. The cells were then collected by centrifugation. Precipitated cells were then resuspended in 200 µl distilled water. Plasmid DNA was extracted based on alkaline lysis method of Birnboitm and Doly.[12] Genomic DNA was prepared by heating of 200 µl of each cell suspension at 95^{0}C for 10 min, digestion with proteinase K, extraction with phenol/chloroform and over night ethanol precipitation at -20^{0}C.

Plasmids profiles analysis. Extracted plasmid DNA was separated by horizontal electrophoresis in a 0.8% agarose slab gel in Tris-acetate-EDTA buffer at room temperature and 60 V for 4 hours. After electrophoresis the gel was stained by ethidium bromide and video image were obtained by gel documentation (Uvtec, Sigma, Germany) system. The molecular mass of unknown plasmid was assessed by comparison of their mobilities with those of supercoiled DNA ladder (Gibco-BRL, England).

Plasmid profiles typing. The Photo Capt Mw program was used to determine the molecular weights of the plasmid bands and to analyze plasmid profiles. The Discrimination Index (DI) of this typing method was calculated by application of Simpson's Index Diversity (SID).[13] The similarity among the isolates on the basis of plasmid profiles was also analyzed with NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System), version 2.02K software and its dendrogram was plotted.

Arbitrary primed-polymerase chain reaction conditions. A reaction volume of 50 µl PCR contained of 0.5mM of single primer (5>-AGG GGT CTT G-3>) (TIB MOL BIOL Syntheselabor GmbH, Berlin, Germany) 200 µM dNTPs, 2.5 mM $MgCl_2$, 2.5 unit Taq polymerase (Cinna gen, Iran) and 5 µl (50 ng of DNA). DNA amplification was carried out for 45 cycles as follows: denaturation at 95^{0}C for 1 min, annealing at 37^{0}C for 3 min and extension at 72^{0}C for 7 minutes with a final extension at 72^{0}C for 10 minutes. The PCR products were analyzed in 1.5% agarose gel. A size marker (100-bp DNA ladder, MBI Ferments, Lithuania) was used as molecular weight marker. After electrophoresis amplified DNA was stained by ethidium bromide and video image were obtained by gel documentation (Uvtec, Sigma, Germany) system.

Arbitrary primed PCR typing. The size and the number of AP-PCR bands were analyzed by Photo Capt Mw programs as compared with size marker (100-bp DNA ladder). The DI of this typing method was calculated by application of SID.[13] The similarity and clustering among the isolates on the basis of AP-PCR products was also analyzed with NTSYS-PC and its dendrogram was plotted.

Results. Thirty-eight different plasmid profiles of 6-9 bands were obtained when all isolates analyzed by photo Capt Mw program with acceptable discrimination index (DI=0.93). Plasmid profiles for some isolates are shown in **Figure 1**. Based on the numbers and size of generated bands dendrogram was plotted. To analyze data 3 points included 50% and 64.7% and 67.5% were selected on dendrogram as a low, intermediate and high similarity levels. When using these criteria patients and environmental isolates were classified to 2 (I & II), 3 (I, II & III), and 5 clusters (I, II, III, IV, & V), respectively (**Figure 2**).

Arbitrary primed-polymerase chain reaction products were analyzed by photo Capt program Mw and 47 different profiles of 3 to 12 bands between 0.376 to 3.7 kb were obtained (**Figure 3**). The DI of this typing method was high (DI=0.97). The type ability and reproducibility of the AP-PCR method were confirmed when using same annealing temperature, Deoxynucleotide Triphosphates and Magnesium Chloride ($MgCl_2$) concentrations. Dendrogram for AR-PCR was plotted. Based on dendrogram pattern to analysis similarity 3 levels (62%, 81% and 84.6%) were selected. Using these criteria 2 (I & II), 5 (I, II, III, IV & V) and 11 (I, II, III, IV, V, VI, VII, VIII, IX, X, & XI) clusters were obtained, respectively (**Figure 4**).

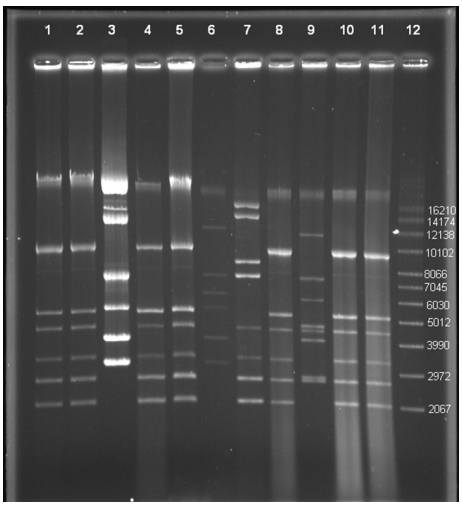

Figure 1 - Plasmid profiles of 11 isolates of *Pseudomonas aeruginosa*. Lines 1 and 2, 2 isolates with identical patterns, line 3 isolate with different patterns, lines 4 and 5, 2 isolates with identical patterns, lines 6, 7, 8, and 9; 4 different patterns, lines 10 and 11; 2 identical patterns, line 12 supercoiled DNA ladder.

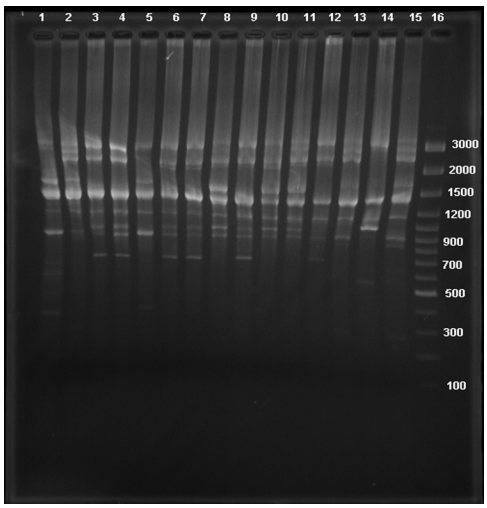

Figure 3 - Arbitrary primed-polymerase chain reaction (AP-PCR) fingerprint of 15 isolates of *Pseudomonas aeruginosa*. Lines 1 to 15 AP-PCR profiles for 15 isolates , Line 16, 100 base pair ladder.

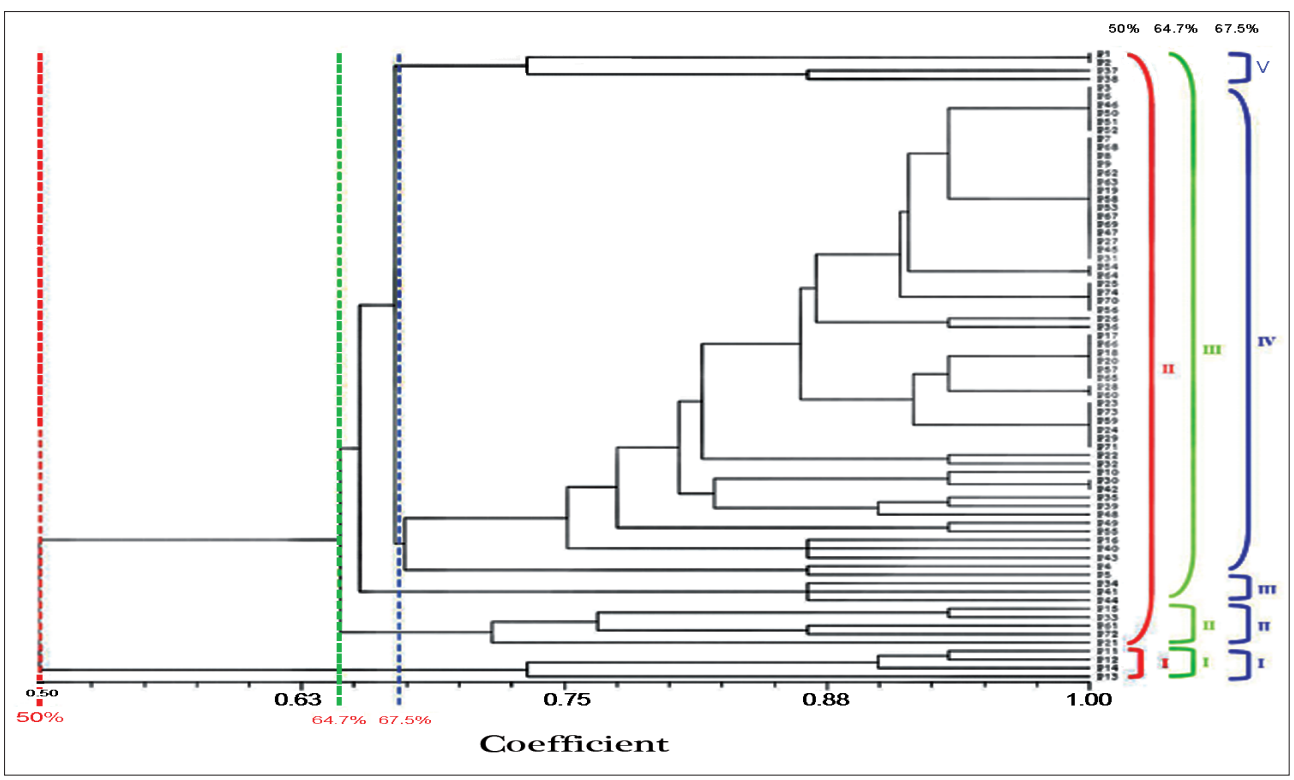

Figure 2 - Dendrogram of plasmid profiles for 74 isolates of *Pseudomonas aeruginosa*. Based on 50%, 64.7% and 67.5% similarity levels 2, 3 and 5 cluster were obtained respectively by cluster analysis. Roman numerals indicate cluster with different similarity levels.

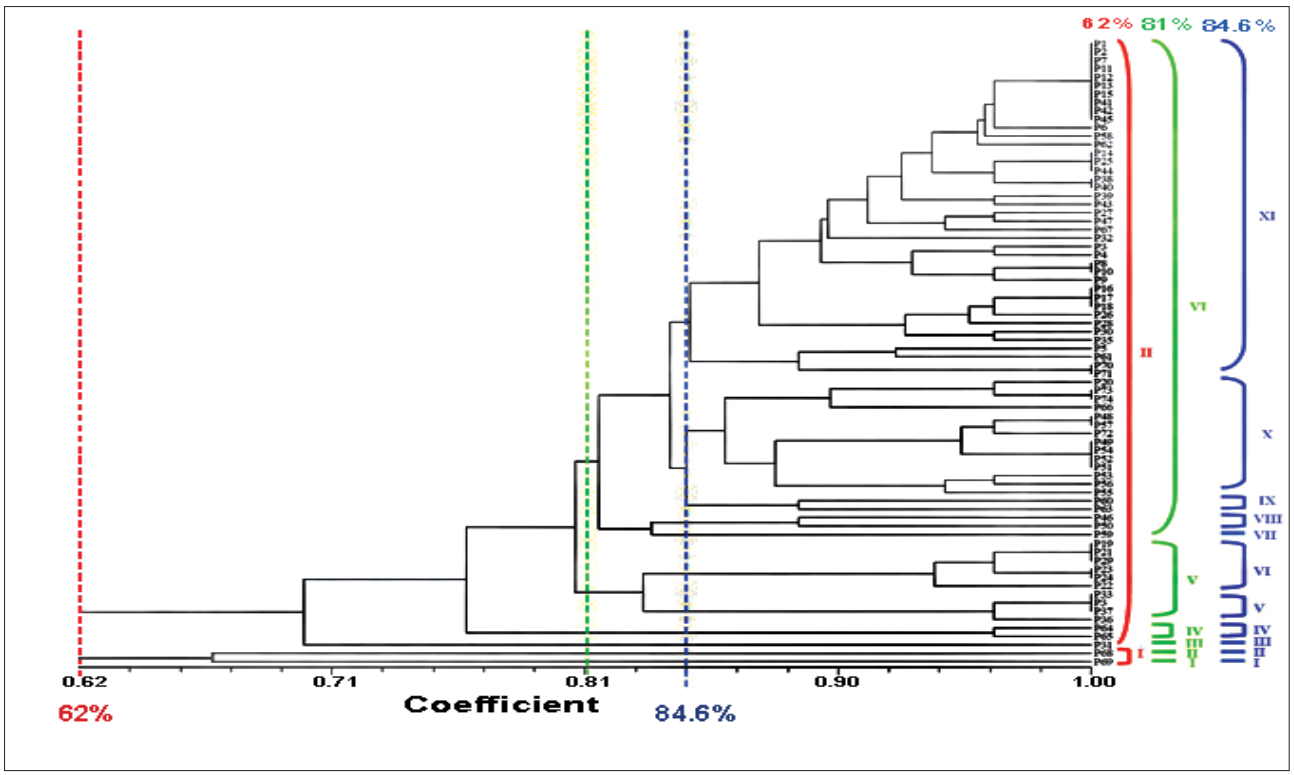

Figure 4 - Dendrogram of arbitrary primed-polymerase chain reaction for 74 isolates of *Pseudomonas aeruginosa* isolates. Based on 62%, 81 % and 84.6% similarity levels 2, 5, and 11 clusters were obtained. Roman numerals indicate cluster with different similarity level.

Discussion. To evaluate the probability of cross-transmission typing is commonly advised in epidemiological investigation. It is widely accepted that traditional phenotypic methods of *P. aeruginosa* do not permit of accurate investigation of nosocomial infections because phenotypic markers are relatively unstable.[14-16] In contrast, molecular subtyping methods offer high type ability and reproducibility, can define clonal relationships between isolates, and have contributed significantly to a better understanding of the epidemiology of *P. aeruginosa*.[17-19] *Pseudomonas aeruginosa* is an opportunistic bacteria, which frequently colonizing burn lesions.[1,2] In our study, 74 isolates of *P. aeruginosa* were typed by application of 2 molecular methods. Plasmid profiles analysis with DI of 0.93, categorized isolates into 2, 3 and 5 clusters with 50% and 64.7% and 67.5% similarity levels, respectively. Arbitrary primed-polymerase chain reaction fingerprinting typed the isolates with higher DI=0.97 and similarity levels (62%, 81% and 84%), which clustering them into 2, 5 and 11 clusters, respectively. It has been suggested that typing method with DI of >0.90 was acceptable.[13] Additionally, it has been proposed that isolates with >80% similarity level can be considered as identical isolates.[20] Taken together these criteria indicate that AP-PCR is preferable as compared with plasmid profiling to type *P. aeruginosa*.[11] Furthermore, AP-PCR protocol is a rapid and reproducible when compared with plasmid profiles analysis. However, variation at an annealing temperature, dNTPs and MgCl2 concentrations, and even PCR machine can affect in the reproducibility of AP-PCR typing.[21,22] Nevertheless, comparison of the genetic clustering of the 74 isolates showed a high degree of resemblance by application of both molecular methods. These results indicate a limited number of common types associated with *P. aeruginosa* infections in our burn center. Although some isolates were located in different clustering groups but they showed more than 80% similarity indicating these isolates originated from a limited number of primary clones. These isolates might tolerate genetic divergence arising from point mutations, insertion or deletion of chromosomal DNA. Recently, we showed *P. aeruginosa*, which colonized burn wound, were resistant to the most of administrated antibiotics.[23] Application of high quantity of antibiotics to treat burn victims could accelerate genetic diversion of a few resistant isolates to overcome antibiotics pressure. In general, these results indicate that transmission of *P. aeruginosa* to burn patients predominantly originated

from a limited number of patients sources. When burn patients admitted to the hospital, their lesions have already been infected with *P. aeruginosa*. However, during admission of patients in the Burn Center, various patients' strains cross-contaminate burn victims mostly when their lesions scrubbed in the bathroom.[1] Consequently, when cross-contaminating isolates colonized burn wounds only isolates be able to survive that could tolerate antibiotics pressure. These strains mostly probably driven from antibiotic resistant clones because they were more adapted to hostile conditions. However, transmissions of infection from hospital environment to patients also occur in the minority of the victims. According to results of this study and our recent one;[23] to eliminate antibiotics resistant isolates of *P. aeruginosa* in our burn center, strong disinfection of patients' bathroom after scrubbing of patient wounds are recommended. Control of antibiotics resistant strains could accelerate treatment of fewer antibiotics resistant isolates whenever colonizing burn wounds.

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