Genetic Diversity of *Pseudomonas aeruginosa* Strains Isolated from Hospitalized Patients

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ABSTRACT

Background: Pseudomonas aeruginosa is one of the most common nosocomial pathogens often causing major problems in Intensive Care Units. This study aimed to investigate the genotypic diversity of Pseudomomas aeruginosa strains isolated from hospitalized patients in National Research Institute of Tuberculosis and Lung Disease (NRITLD) with random amplified polymorphic DNA (RAPD) method and also to determine the antibiotic resistance pattern.

Materials and Methods: Seventy three P. aeruginosa isolates from different specimens were analyzed. These strains were isolated from patient admitted in Intensive Care Unit (ICU) (31), non-ICU inpatient (40), and two environmental specimens one from ventilator and one from soap specimen in ICU. All strains were identified with biochemical testing and antimicrobial susceptibility testing which carried out according to National Committee for Clinical Laboratory Standards (NCCLS). Random Amplified Polymorphic DNA typing (RAPD) was used to study the genetic diversity of Pseudomonas aeruginosa using 2 sets of primers and electophoretic banding patterns were analyzed visually and by GelCompar II software.

Results: Phylogenic analysis of the RAPD pattern showed rates of genetic similarity ranging from 40-100%. Four epidemiologically and genetically related isolates (clones) each containing 2-3 isolates were identified. Most of them were from ICU. We detected high antimicrobial resistance rate to Chloramphenicol, Ceftriaxon, Cefepime, Ceftazidime (75-97%) and relatively low resistance rate to Imipenem, Amikacin, Ciprofloxacin and Gentamicin (42-53%).

Conclusion: Although a few epidemiologically related clones are found with RAPD method, most of the isolates are probably emanate from the host itself. There is also a high rate of antibiotic resistance especially in ICU. **(Tanaffos 2008; 7(1): 32-39)**

Key words: Pseudomonas aeruginosa, Typing, Random amplified polymorphic DNA (RAPD), Susceptibility pattern

INTRODUCTION

Pseudomonas aeruginosa is one of the most common nosocomial pathogens often causing major problems in Intensive Care Units (ICU) (1). The

worldwide emergences of multi-resistant bacterial infections, by this pathogen are associated with highest mortality rate and are difficult to demolish (2).

From the epidemiological point of view, it is often necessary to determine the relatedness (clonality) of the bacterial isolates. This is particularly important in

endemic and epidemic nosocomial outbreaks of bacterial infections in order to improve their management. Genomic fingerprinting methods are now regarded as the most accurate methods for the typing of microorganisms for epidemiologic purposes. These methods include pulse field gel electrophoresis (PFGE), ribotyping and PCR-based fingerprinting (3). However, their use in clinical microbiology laboratories has been limited because they are time consuming and labor intensive. Random amplified polymorphic DNA known as RAPD is a new method and is based on the use of simple arbitrary primers in PCR of low stringency. This technology is proved to be useful in typing strains of bacteria and is highly reproducible (4, 5).

Many studies have been directed at *Pseudomonas aeruginosa*. However, most studies are confined to the epidemiology and infections in patients with cystic fibrosis and also concern outbreaks (6-9). Only a few articles present in endemic situation (3). Using RAPD method, we analyzed the genotypes of *P.aeruginosa* isolates, in a setting of endemicity from clinical specimens of Masih Daneshvari Hospital in a 1 year period and also determined antibiotic susceptibility pattern.

MATERIALS AND METHODS

Pseudomonas aeruginosa isolates were obtained from routine clinical specimens sent for bacterial culture from 71 patients admitted to different units of Masih Daneshvari Hospital from 2003 to 2004. Thirty-one cases were from ICU, 40 cases from other locations including surgical unit, infectious diseases and internal medicine wards, and 2 environmental specimens from ventilator and soap in ICU. All isolates were identified as *P.aeruginosa* on the basis

of their typical colonial appearance, characteristic pigments, positive oxidase test and growth at 42°C. The antimicrobial susceptibility test was carried out through disk diffusion method according to national committee for clinical laboratory standards, NCCLS 2002 recommendation. Quality control was performed using standard strains of *P. aeruginosa* (ATCC27853).

Isolation of *P. aeruginosa* genomic DNA:

A single colony was inoculated into 2 ml of brain heart infusion broth and incubated overnight at 37°C. After harvest by centrifugation (3000g for 10 minute) the bacterial pellet was resuspended in 567 micro-liter TE buffers, 30µl SDS 10%, and then 3µl proteinase K was added to the mixture. After overnight incubation, 100µl NaCl 5M was added and tubes were incubated at 37°C for 10 minutes. Approximately equal volume of chloroform/isoamyl alcohol (24:1) was added, mixed thoroughly and spined 15 minutes in micro-centrifuge. Aqueous-viscous supernatant was removed to a fresh tube and 0.6 volume of isopropanol was added to precipitate the nucleic acid. The DNA stored at -20°c.

RAPD analysis:

To select primers that generated reproducible polymorphic random amplified polymorphic DNA patterns, two 10 mers primers set; (primer 208, ACGGCCGACC and primer 272, AGCGGGCCAA, Sinagen Company), which had been previously used by others (4, 10, 11) were used. RAPD PCR mix was set up. Reaction mixture (25 µl) consist of 10x reaction buffer, 3Mm $MgCl_2$, 200µm dNTP, 20pm primer and 2 unit Taq DNA polymerase using template DNA and was amplified with a DNA

thermal cycler as follows: (I) 4 cycles with 1 cycle consisting of 5 minutes at 94ºC, 5 minute at 36ºc and 72ºc. (II) 30 cycles with 1cycle consisting of 1 minutes at 94ºC, 1 minute at 36ºC and 2 minutes at 72ºC and followed by a final extension step at 72ºC for 10 minutes. RAPD products were then separated by electrophoresis in 1.5% agarose gel. (17 well 21x15 cm) with 5x TBE running buffer at 9 V/cm for 3 hours.

Molecular size standards were stained with Ethidium bromide. The RAPD fingerprints were analyzed both by naked eye and by computer with GelCompar II software with the molecular size standards used to correct for gel to gel migration variation.

The similarities between fingerprints were determined by construction of a similarity matrix using the Dice's coefficient with 1.5% position tolerance and optimization of 1% and a dendrogram generated using unweighted pair group method with an average linkage (UPGMA) algorithm. Definitions of clonal structures of *P. aeruginosa* strains were made according to Tenover et al. (12).

RESULTS

Seventy three cases of *Pseudomonas aeruginosa* were isolated in the microbiology laboratory in Masih Daneshvari Hospital during a one-year period. Thirty one cases were from patients admitted to ICU, 40 cases from departments of surgery, medicine, and infectious disease, two from environmental source; one from ventilator and one from liquid soap in ICU. The specimens were collected from sputum (28 cases), bronchus (5 cases), trachea (15 cases), pleura (12 cases), lung (4 cases), urine (4 cases), and other sites (3 cases). The details of the isolates are summarized in Table 1.

Using primer 272, 3 to 12 bands and primer 208, 2 to 8 bands from 100 to 3500 bp were detected. The resultant dendrogram produced by GelCompar II soft ware (Figure 1) showed 40-100% genetic homology between the isolates. With the 73 isolates studied, the PCR with both primers generated 67 different patterns, including 4 clones detected mostly from ICU. Three clones were related genetically and epidemiologically (clones I, II, IV).

Clone I included 2 isolates from respiratory system in 2 patients admitted to ICU one month apart. Clone II was isolated from the trachea of 3 patients in ICU, 2 patients were admitted at the same day, 10 days apart from the first patient. Two isolates in clone III were isolated one month apart from different patients in different locations (ICU and surgery).

Using criteria of Tenover et al (12), with maximum one band difference on visual inspection, we identified another clone, isolated from ventilator and 2 patients in ICU one month apart. This clone had more than 90% homology using software, which was rechecked with both sets of primers and showed similar bands or up to at most one non-homologus band.

Results of antibiogram:

There were 24 distinct antibiotypes (against 67 genotypes), as shown in Table 1. Multi-drug resistant rate was 30% (22 from 73 cases), in which most of them were from ICU (81%). Imipenem was the most invitro active antibacterial agent (42.4%) followed by Amikacin (49.2%), Ciprofloxacin (53%) and Gentamicin (59%).

There was a high resistance rate to Chloramphenicol (97.2%), Ceftriaxone (89.8%), Cefepime (75%), and Ceftazidime (77%).

Table 1. Origin and characteristics of the *P. aeruginosa* isolates

Antibiogram type	Patient's code	Date	Specimen	Unit	Amikacin	Ceftazidim	Ceftriaxone	Chloramphenicol	Ciprofloxacin	Gentamicin	Imipenem
1	OR-61	6/11	Trachea	Surgery	S	R	R	R	S	S	S
1	009-13	29/11	Sputum	Medicine	S	R	R	R	S	S	S
1	I-40	24/2	Pleura	ICU	S	R	R	R	S	S	S
1	004-44	31/3	Sputum	Medicine	S	R	R	R	S	S	S
1	004-60	28/5	Sputum	Medicine	S	R	R	R	S	S	S
1	005-73	12/6	Sputum	Infectious	S	R	R	R	S	S	S
1	OR-74	14/8	Sinus secretion	Surgery	S	R	R	R	S	S	S
1	OP-39	23/1	Sputum	Medicine	S	R	R	R	S	S	S
2	I-62	16/9	Oral lesion	ICU	R	R	R	R	R	R	R
2	I-VENT	2/5		ICU	R	R	R	R	R	R	R
2	I-33	9/7	Trachea	ICU	R	R	R	R	R	R	R
2	I-4	5/8	Sputum	ICU	R	R	R	R	R	R	R
2	I-72	13/12	Pleura	ICU	R	R	R	R	R	R	R
2	I-60	26/12	Trachea	ICU	R	R	R	R	R	R	R
2	I-9	27/12	Urine	ICU	R	R	R	R	R	R	R
2	I-28	13/1	Bronchus	ICU	R	R	R	R	R	R	R
2	I-17	16/7	Trachea	ICU	R	R	R	R	R	R	R
2	I-23	24/10	Trachea	ICU	R	R	R	R	R	R	R
2	I-27	6/11	Trachea	ICU	R	R	R	R	R	R	R
2	I-29	6/11	Trachea	ICU	R	R	R	R	R	R	R
2	008-37	17/1	Urine	Medicine	R	R	R	R	R	R	R
2	OR-38	20/2	Lung	OR	R	R	R	R	R	R	R
2	I-41	17/3	Trachea	ICU	R	R	R	R	R	R	R
2	I-46	1/4	Urine	ICU	R	R	R	R	R	R	R
2	I-48	4/4	Trachea	ICU	R	R	R	R	R	R	R
2	I-49	10/4	Trachea	ICU	R	R	R	R	R	R	R
2	004-50	16/4	Sputum	Medicine	R	R	R	R	R	R	R
2	I-63	19/6	Trachea	ICU	R	R	R	R	R	R	R
2	I-68	15/4	Bronchus	ICU	R	R	R	R	R	R	R
2	OP-69	30/4	Sputum	Medicine	R	R	R	R	R	R	R
3	I-Soap	2/5		ICU	R	S	I	R	R	S	R
4	OR-57	17/1	Pleura	Surgery	S	R	R	R	S	R	R
4	OR-15	7/7	Pleura	Surgery	S	R	R	R	S	R	R
4	I-16	26/6	Pleura	ICU	S	R	R	R	S	R	R
5	004-12	19/12	Bronchus	Medicine	S	R	R	R	S	S	R
6	009-14	21/6	Blood	Medicine	S	R	R	R	S	R	S
6	OR-41	14/2	Pleura	Surgery	S	R	R	R	S	R	S
6	005-71	24/6	Sputum	Infectious	S	R	R	R	S	R	S
7	008-18	18/9	Pleura	Medicine	S	S	R	R	S	S	S
7	004-19	5/11	Sputum	Medicine	S	S	R	R	S	S	S
7	OP-20	8/10	Sputum	Medicine	S	S	R	R	S	S	S
7	005-57	17/1	Pleura	surgery	S	S	R	R	S	S	S
7	009-58	12/5	Sputum	Medicine	S	S	R	R	S	S	S
7	006-62	14/6	Bronchus	Infectious	S	S	R	R	S	S	S
8	005-21	17/10	Sputum	Infectious	S	R	R	R	S	R	S
9	OR-22	15/3	Lung	OR	I	S	R	R	S	R	S
10	006-25	28/10	Sputum	Infectious	R	R	R	R	S	I	S
11	I-30	18/1	Trachea	ICU	R	R	R	R	R	R	S
11	I-34	22/1	Trachea	ICU	R	R	R	R	R	R	S
11	005-35	6/2	Urine	Infectious	R	R	R	R	R	R	S
11	I-36	4/3	Trachea	ICU	R	R	R	R	R	R	S
11	I-43	28/3	Bronchus	ICU	R	R	R	R	R	R	S
11	007-53	29/4	Sputum	Medicine	R	R	R	R	R	R	S
11	I-51	5/5	Sputum	ICU	R	R	R	R	R	R	S
11	I-65	24/8	Trachea	ICU	R	R	R	R	R	R	S
11	I-66	1/3	Sputum	ICU	R	R	R	R	R	R	S
12	I-31	18/1	Sputum	ICU	S	S	I	R	R	S	S
13	006-32	19/1	Sputum	Infectious	I	R	R	R	S	I	S
14	OR-42	17/3	Pleura	Surgery	S	R	R	R	S	R	R
14	I-54	30/4	Lung	ICU	S	R	R	R	S	R	R
15	004-43	23/3	Sputum	Medicine	S	R	R	R	R	S	S
15	009-55	25/4	Sputum	Medicine	S	R	R	R	R	S	S
15	008-56	7/5	Sputum	Medicine	S	R	R	R	R	S	S
15	008-75	25/5	Sputum	Medicine	S	R	R	R	R	S	S
16	I-44	24/3	Sputum	ICU	I	R	R	R	R	S	R
17	004-45	12/5	Sputum	Medicine	I	I	I	R	I	S	S
18	OR-47	3/4	Pleura	Surgery	I	R	R	R	S	R	S
19	I-52	31/1	Sputum	Medicine	R	R	R	R	S	S	R
20	009-40	31/4	Sputum	Medicine	S	S	R	R	S	R	S
21	OR-64	22/6	Lung	Surgery	I	I	R	R	S	R	S
22	005-67	25/1	Sputum	Infectious	I	S	R	R	S	I	S
23	OR-70	20/9	Pleura	Surgery	I	S	S	R	S	S	S
24	008-72	13/12	Pleura	ICU	I	S	R	R	S	I	R

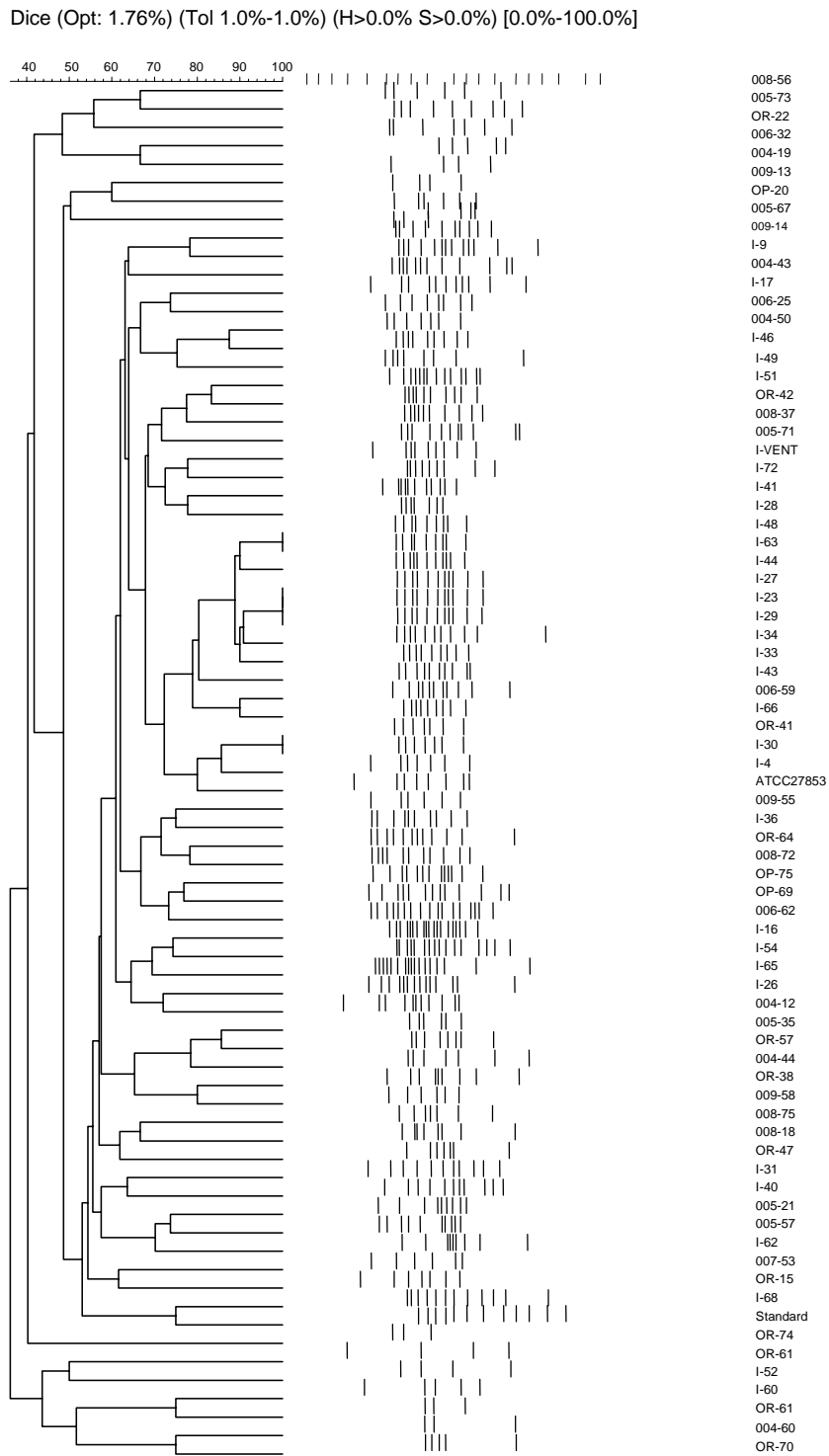Dice (Opt: 1.76%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]

Figure 1. Dendrogram of Pseudomonas aeruginosa isolates showing percent similarities of patterns.

DISCUSSION

The goal of strain typing studies is to provide laboratory evidence that the epidemiologically related isolates collected during an out break of disease are also related genetically and thus represent the same strain. This information is helpful for understanding and controlling the spread of disease in hospital and communities (12).

In this study, we have determined the genetic diversity of *Pseudomonas aeruginosa* strains isolated from different departments of Masih Daneshvari Hospital in endemic situation, using RAPD analysis. The results were compared with those of antibiogram base on NCCLS. In agreement with other studies, there was substantial diversity among the strains. The large numbers of genotypes suggests that most *P. aeruginosa* strains were derived from the patients themselves, as shown previously (3, 5).

In this study, a few genetically related isolates (4 clones) detected were mostly from ICU. However, the epidemiological data should always be taken in account when deciding whether genetically related strains are also related epidemiologically.

Epidemiologically related species are defined as species cultured from patients' specimens collected in a limited period of time or from a defined area as a part of epidemiologic study and these might have a common source (12).

However, cross-acquisition was established for only 7 patients (3 pairs isolates in three clones: 1, 2, 4), these results are suggestive of a common exogenous source. This was only found for clone 4, in which one environmental sample from ventilator was obtained.

Speijer et al (3), in their study on Pseudomonas isolated from ICU patients in endemic conditions showed that most patients were infected during their stay in ICU, which indicated that a common exogenous source or cross-acquisition was an important route of *Pseudomonas aeruginosa*

transmission. In our study, isolation of clone IV from ICU ventilator was in accord with their findings. However, in their study, most patients were infected with different species of Pseudomonas; this could be indicative of an endogenous source which was not detected on admission. We also had multiple different genotypes of Pseudomonas.

Computer analysis of banding pattern revealed different groups of genetically related species. Discrepancies were present in results of computer analysis and visual observation, which have been present in other studies as well (1). For example, cluster IV were similar at visual exam, but showed 90% homology by computer analysis. To identify whether the genetically related species are also epidemiologically related, in addition to comparison of the eye observation (visual exam) and GelCompar, epidemiologic information should also be included (5).

RAPD method is recommended as an excellent screening method for many bacterial species; this has had comparable results with the PFGE reference method which is very expensive and time consuming (2, 5, 13).

The choice of primers for use in RAPD analysis is one of the most critical factors. It appears that some arbitrary primers may work better than others and may provide results that are more reproducible (14). We used the two sets of primers which were used in previous studies and had comparable results with PFGE method (4).

No association was observed between genotype and antibiotype as isolates of the same genotype displayed different antibiotype and vice versa, as already shown by others (15).

The most effective antibiotic agent in our study was Imipenem, followed by Ciprofloxacin.

The incidence of resistance is dependent on the patterns of antibiotic usage and is different in other

countries.

Sader et al. (16), in a multi-centric study, performed in Brazil, showed that Imipenem is the most active agent against *P. aeruginosa* followed by Ciprofloxacin.

There was a high antibiotic resistance rate mostly in our ICU, in which a multi-drug resistance rate was 71%.

Loureiro et al. (2) showed 75-100% resistance in specimens obtained in NICU. The least antibiotic resistance was to Tazosin (0-35%).

CONCLUSION

Control of infections is based on the identification of the organisms and their mode of spread; the molecular technique used in this study makes this possible in the shortest possible time with a reasonable cost.

Antibiotic resistance of *Pseudomonas aeruginosa* is increasing rapidly in ICU which makes it difficult to eradicate.

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