RIBOTYPING AND INCREASING TREND OF ANTIBIOTIC RESISTANCE OF *PSEUDOMONAS AERUGINOSA* ISOLATED IN IRAN

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ABSTRACT

The trend of antibiotic resistance, ribotyping and serotyping patterns of *Pseudomonas aeruginosa* collected since 1987 in Iran were investigated. The results showed that among the aminoglycosides, amikacin was the most effective antibiotic against *P. aeruginosa* with 98.4% susceptibility rate followed by tobramycin (73%) and gentamicin (71%). Of the cephalosporins, susceptibility to ceftazidime was 93%. Among the antibiotics tested *in vitro*, ciproflaxacin was found to be the most effective against the strains, with 98.4% susceptibility rate. The most predominant monovalent serotype was O:11 (34%). Other dominant serotypes were O:5 (20%), O:1 (16%) and O:6 (15%). Thirteen percent of the isolates showed no agglutination with the tested sera. A high percent of the O:11 serotype isolates (68%) were resistant to ≥ 3 antibiotics. The collected *P. aeruginosa* isolates were classified into 3 ribotypes using *Puv*II restriction enzyme. The results suggest that the antibiotic resistance among *P. aeruginosa* increased significantly rate in Iran in the last decades, with no changes in the ribotype and serotype patterns.

Key words: *Pseudomonas aeruginosa*, Iran, Genotyping

INTRODUCTION

Pseudomonas aeruginosa is a major opportunistic pathogen in cystic fibrosis, burn patients and immunocompromised patients (11). Its ubiquitous nature such as the ability to survive in the moist environment and resistance to many antibiotics make *P. aeruginosa* a common pathogen in the sporadic or clustered cases and particularly in intensive care units of the hospitals (5,9). It has been documented that the antibiotic resistance among *P. aeruginosa* is increasing at an alarming rates worldwide (6,9). The high mortality and the antibiotic resistance rate of *P. aeruginosa* emphasize the need for analyzing the development of the phenotypic and genotypic diversity of the *P. aeruginosa* worldwide. The data regarding *P. aeruginosa* infections are scarce in Iran and there are only a few recent publications about the phenotypic characteristics of the isolates. Lack of the data from the strains isolated in the precedent years prohibits us to illustrate the development of diversity of *P. aeruginosa* in Iran. This is the first report

analyzing in detail the phenotypic and genetic characterizations of *P. aeruginosa*.

MATERIAL AND METHODS

Bacterial strains

The nosocomial *P. aeruginosa* strains were from our collection obtained during 1987-1988 from 86 patients hospitalized in the adult ICU in Tehran, Iran. The bacteria isolates were identified with API 20E system (bioMerieux SA, Montalieu Vercieu, France) and serotyped by slide agglutination test according to the international serogrouping schema for *P. aeruginosa* by using a panel of O monovalent specific antisera numbered O1 to O16 (Difco Laboratories, Detroit, MI, USA).

Antimicrobial susceptibility testing

Antibiotic susceptibility patterns were tested by standard disk technique according to NCCLS guidelines (2). The following antibiotics were used; amikacin (30 µg), azlocillin (75 µg),

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ciprofloxacin (5 µg), ceftazidime (30 µg), cefoperazone (75 µg), ticarcillin (75 µg), tobramycin (10 µg) and gentamicin (10 µg). All antibiotics were purchased from Difco laboratories.

PCR of exotoxin A gene

For DNA extraction, a single colony was grown in Trypto casein soy broth for 24 h at 37ºC after which the cultures were centrifuged and the pellets were lysed with the lysing buffer solution containing 0.1 M NaCl, 50 mM EDTA, 0.1 M Tris-Hcl (pH 8.0) supplemented with SDS (0.5%) and 0.5 mg/ml proteinase K. PCR was performed in a 50 µl reaction mixture containing sterile water 30 µl, 5 µl of 10x *Taq* polymerase buffer, 4 µl of each nucleotide phosphate (2.5 mM), 50 pmol of each primer ETA1 and ETA2, 0.25 µl *Taq* DNA polymerase (0.5 U/µl) (Gibco-BRL Life Technologies, Rockville, MD, USA) and 1 µl DNA template. The primers used were: ETA1: 5'-GACGCCCTCAGCATCACCAGC-3' and ETA2: 5'-CGCTGGCCCATTCGCTCCAGCGCT-3' (18).

The cycling conditions were as follows: pre-incubation at 95ºC for 2 min, and 35 cycles of 1 min at 94ºC, 1 min at 68ºC, 72ºC for 1 min and the final incubation for 7 min at 72ºC.

Ribotyping

Ribotyping was performed as described previously (13). Briefly, the extracted DNA from *P. aeruginosa* isolates was cleaved by restriction endonucleases *Puv*II (Amersham Biosciences, Piscataway, NJ, USA). The fragments were separated by electrophoresis and then transferred onto nylon membrane by alkali blotting procedure with a vacuum blotter. Hybridization was performed with the probes labeled with digoxigenin-11-dUTP (DIG). The membranes were then visualized by addition of alkaline phosphate-conjugated anti-digoxigenin antibody (Roche Diagnostic GmbH, Mannheim, Germany) and 5-bromo-4-chloro-3-indolyl phosphate substrate and nitroblue tetrazolium.

RESULTS

The results showed high level of resistance to the β-lactams and a low level of resistance to ciprofloxacin. The most active antimicrobial agents were ciprofloxacin, amikacin and ceftazidime with 1.6, 2.3 and 7% resistance rates, respectively (Table 1). Kanamycin (66%) showed the lowest in vitro antibacterial activity followed by carbenicillin (63%), ticarcillin (58%) and mezlocillin (57%). The number of strains showed resistance to cefoperazone, tobramycin, azlocillin and gentamicin were 17, 27, 28 and 29%, respectively.

Table 1 also shows *P. aeruginosa* cross-resistances between antibiotics. The highest level of resistance was observed with

Table 1. Cross-resistances of *P. aeruginosa* isolates.

	AN	AZ	CIP	CAZ	CFP	CB	K	GM	MZ	TIC	TM
					Percent of isolates resistant to:						
AN	2.3	2	0	0	2	2	2	2	2	2	2
AZ	0	28	0	3	13	20	16	15	21	19	13
CIP	0	0	1.6	0	1	1	0	1	1	1	1
CAZ	2	3	0	7	2	3	3	2	2	3	2
CFP	2	13	1	2	17	15	13	15	15	15	12
CB	2	20	1	3	15	63	43	23	35	36	21
K	2	16	0	3	13	43	66	19	31	31	16
GM	2	15	1	2	14	23	19	29	25	24	22
MZ	2	21	1	2	15	35	31	25	57	39	23
TIC	2	19	1	3	15	36	31	24	39	58	22
TM	2	13	1	2	12	21	16	22	23	22	27

AN= amikacin, AZ= azlocillin, CIP= ciprofloxacin, CAZ, ceftazidime, CFP, cefoperazone, CB= carbenicillin, K= kanaymcin, GM= gentamicin, MZ= mezlocillin, TIC= ticarcillin and TM= tobramycin. Underlines indicate the percent of isolates resistant to a single drug.

kanamycin and carbenicillin (43%), followed by ticarcillin and mezlocillin (39%), carbenicillin and ticarcillin (36%) and carbenicillin and mezlocillin (35%). The highest level of concomitant resistance to 3 antibiotics (34%) was observed with carbenicillin, mezlocilli, ticarcillin or carbenicillin, kanamycin, ticarcillin (data not shown). Among the isolates 55%, 35% and 30% showed resistance to 3, 4, and 5 different antibiotics, respectively.

All of the isolated *P. aeruginosa* strains were examined with monovalent anti-O serum. The results showed that the strains could be serotyped with 8 of 16 monovalent sera (Table 2). Taking polyagglutinable strains into consideration, however, the strains could react with all of the monovalent sera with an exception of anti-O:15 serum. Out of the 86 strains, 13% and 23% were nontypeable or polyagglutinable, respectively. Table 2 showed that the majority of the isolates belonged to serotype O:11 (34%) followed by O:5 (20%) and O:1 (16%) among the monovalent serotype reacting strains. These three serotypes altogether constitute 70% of the total isolates and O:11 accounted for over a third of the collection.

To confirm the identity of strains, the *exotoxin A* (ETA) gene was amplified by a pair of oligonucleotide primers ETA1 and ETA2. Eighty one strains (94%) gave a DNA band of ETA gene with 367 bp amplified product.

Figure 1 shows the ribotypes of *P. aeruginosa* isolates digested with *Puv*II restriction endonucleases which resulted in 3 different patterns. Lane 1 was recognized as the predominant ribotype with 59% of the isolates. This pattern contained five distinct bands, ranging from 9.6 to 4.3 kb. Lane 2 showed bands similar to the lane 1 with the presence of two additional distinct

Table 2. Percent distribution of *P. aeruginosa* O serotypes amongst the isolates reacting with one[a] or more[b] anti-sera.
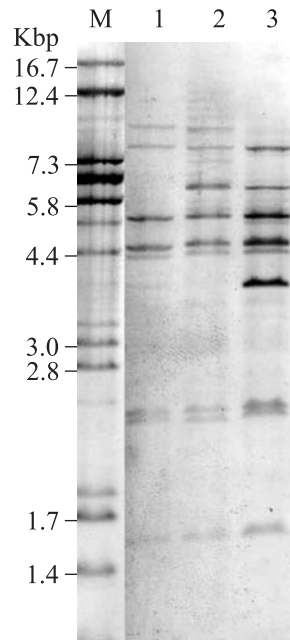
O. serotype	% isolates reacting with a single anti serum[a]	% isolates reacting with more than one anti-sera[b]
1	16	13
2	0	11
3	4	5
4	0	3
5	20	19
6	15	10
7	0	3
8	0	4
9	5	3
10	4	5
11	34	17
12	0	1
13	2	6
14	0	1
15	0	0
16	0	2

Figure 1. Ribotype patterns of *P. aeruginosa* DNA digested with *Puv*II restriction endonucleases. M= molecular size marker of *Citrobacter koseri* DNA cleaved with *Mlu*I.

bands with the molecular sizes of 6.5 and 6.4 kb. This pattern constituted 8% of the total *P. aeruginosa* isolates. Lane 3 was considered as the pattern 3 with 33% of the isolates which was similar to lane 2 with an absence of 9.6 kb band. A unique band at the lower part of the gel with molecular size 3.9 kb was evident that could not be observed in either lane 1 or 2.

DISCUSSION

Resistance of gram-negative aerobic bacteria to aminoglycoside antibiotics differs by the region and country (7). In Europe the resistance rates of *P. aeruginosa* isolates have changed little over the last several years to commonly prescribed antibiotics (12). Our results showed that the antipseudomonal effects of amikacin (97.7%) were greater than gentamicin (71%) for the isolates obtained in 1988. Since then, the trend of resistance has been altered progressively. In 1997, *P. aeruginosa* resistant to amikacin was 49% (14) and in 2003 reached over 95% (16). Nowadays almost all of the *P. aeruginosa* isolates are resistant to both amikacin and gentamicin.

As 1997, 95% of the *P. aeruginosa* isolates were resistant to gentamicin, carbenicillin, co-trimoxazole, ceftizoxime and tetracycline (14,16). By comparing the data presented in our study and the recent isolates, we have found that *P. aeruginosa* stains have become significantly resistant to the antibiotics in a decade. For example, an increase of about 50 and 4 folds in the antibiotic resistance to amikacin and tobramycin was evident.

In comparison with neighboring countries such as Turkey, we find comparable antibiotic susceptibility trend. They have observed an increase in the resistance to amikacin (25 folds) and tobramycin (2 folds) in the isolates obtained within a 10 year period (1,7). Of course, the comparison of resistance data between studies is affected as the strain collections, patient populations and the difference in the methods of studying.

The results presented here demonstrated that the quinolone had a considerable in vitro activity against our isolates. Resistance rate of *P. aeruginosa* to ciprofloxacin was 1.6% whereas the isolates obtained nowadays is about 40%, more than what have been reported by other investigators elsewhere. The level of ciprofloxacin resistant has been registered to be about 23% in Spain (4), 32% in Italy (3) and 27% in Latin America (9). Ceftazidime is one of the most common 3rd generation antibiotics in ICU protocols (15).

At the present time, the resistant rate is about 70% while only 7% of our isolates were resistant to ceftazidime which in part may be due to the restricted use of this drug in the 80's. According to other reports, *P. aeruginosa* resistance to ceftazidime is 13 to 50% worldwide (3,4,7). In our collection, the multidrug-resistant *P. aeruginosa* to ciprofloxacin, ceftazidime and gentamicin were apparently a minor challenge back in the late 80's, with only 1 strain isolated in this study. The trend has now been altered with the occurrence of a significant multidrug-resistant *P. aeruginosa* in the recent years (16).

Cross-resistance data are useful to indicate alternative drugs for treatment. In our study, the results showed that the combination of any two drugs would have resulted in a significant reduction in the resistance rates of the isolates. For example, 66% of *P. aeruginosa* showed resistant to kanamycin alone, but a significant reduction in the resistance rate was observed when kanamycin was combined with ciprofloxacin (0%, lowest resistance) or carbenicillin (43%, highest resistance). This affect was evident when a combination of any of the two antibiotics was used.

The serotype O:11 is a common serotype worldwide and frequently associated with the outbreaks in hospitals and multi-drug resistance (8,18). The pattern of *P. aeruginosa* serotypes in Iran has remained the same with O:11 strains currently to be the predominant serotype. Furthermore, the serotype O:11 is one of the most frequently reported serotype in Europe and elsewhere (4). In our study, the three dominant serogroups, O:11, O:5 and O:6, showed a high degree of multidrug resistance with 15%, 12.7%, 12%, respectively. The first two O serotypes are still reported as the predominant and the latter serotype has, however, been changed to O:12 (16). This may be due partly to the expansion of the multidrug resistant O:12 clone in Iran which has also been reported to have reached all over Europe (10). In our study, this serotype accounted for a low percentage in the old isolates.

With ribotyping, the typeability reached 100% for all strains, whereas serotypeability did not exceed more than 87% of the total strains. No association between the ribotype and the serotyping or antibiotic resistance pattern was observed among the 86 isolates. On the whole, the presence of high frequency of nontypeable and polyagglutinable strains in our study has indicated the limited usefulness of serotyping in *P. aeruginosa*. In conclusion, it seems that the predominant ribotype pattern labeled as 1 and O:11 serotype populations have remained the major strains since 1987. In comparison with the recent surveys that have examined the susceptibility to various antibiotics in *P. aeruginosa*, a significant and clear tendency toward decreased susceptibility for all groups of antibiotics is evident in Iran.

ACKNOWLEDGEMENT

RESUMO

Ribotipagem e perfil de resistência a antibióticos de *Pseudomonas aeruginosa* isolada no Irã

Investigou-se o perfil de resistência a antibióticos, os ribotipos e os sorotipos de *Pseudomonas aeruginosa* isolada no Irã a partir de 1987. Os resultados indicaram que, entre os antibióticos testados, a amicacina foi o antibiótico mais eficiente, com 98.4% de inibição, seguida por tobramicina (73%) e gentamicina (71%). Entre as cefalosporinas, a sensibilidade a ceftazidima foi 93%. Entre os antibióticos testados in vitro, ciprofloxacina foi a mais eficiente. O sorotipo de maior prevalência foi O:11 (34%). Outros sorotipos encontrados foram O:5 (20%) e O:6 (15%). Treze por cento dos isolados não aglutinaram com os soros utilizados. Uma elevada porcentagem dos isolados pertencentes ao sorotipo O:11 foi resistente a pelo menos três antibióticos. Utilizando-se a enzima de restrição *Puv*II, as cepas foram classificadas em três ribotipos. Os resultados sugerem que a resistência a antibióticos em *P. auruginosa* aumentou significantemente nas últimas décadas, sem modificação dos padrões de ribotipos e sorotipos.

Palavras-chave: *Pseudomonas aeruginosa,* Irã, genotipagem

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