

## *Basic Science in Medicine*

# PLASMID MEDIATED METAL AND ANTIBIOTIC RESISTANCE IN *PSEUDOMONAS AERUGINOSA* STRAINS ISOLATED FROM BURN PATIENTS

MOHAMMAD REZA SHAKIBAIE, Ph.D.

*From the Department of Genetics, Research Center, Kerman  
University of Medical Sciences, Kerman, I.R. Iran.*

### ABSTRACT

*Pseudomonas aeruginosa* is a leading cause of burn infections, and antibiotic-resistant strains of this bacterium are emerging due to extensive application of antibiotics in the burn unit of hospitals. In this study 50 strains of *P. aeruginosa* were isolated from burn patients infected with this micro-organism in the burn unit of a general hospital in Kerman, Iran over one year [May 1999-April 2000]. Sensitivity/ resistance of the isolates for antibiotics and metals was determined by MIC test. 46% of the isolates were resistant to ciprofloxacin, kanamycin (K), gentamicin (Gm), tetracycline (Te) and chloramphenicol (c). 35% were resistant to amikacin (AN), ceftriaxone (Ceft), and cefotaxime (CTX), and 10% were resistant to imipenem (Imp) and piperacillin (PIP). The isolates exhibited varied MIC's to metal ions. 87% were sensitive to cadmium (Cd), 62% to lead (Pb), 91% to mercury (Hg), 54% to zinc (Zn), 85% to chromium (Cr), and 83% to arsenate (Ars). Among them, strain 16 was found to be resistant to Pb, Cr, and Zn as well as Te, C, Gm, and K. Conjugation and transformation experiments revealed the transfer of  $Te^r$ ,  $C^r$ ,  $K^r$  and  $Gm^r$  along with  $Pb^r$  to a sensitive strain of *P. aeruginosa* PTCC1074.1 ( $Rif^s$ ) but not to *E. coli* K12 HB101.1 ( $Rif^s$ ). Subsequent plasmid isolation and agarose gel electrophoresis (0.7%) confirmed the presence of three-plasmid bands in strain 16 and the transconjugant. Furthermore, strain 16 accumulated a maximum amount of Pb (50 $\mu$ M) within 60 min. and reached a plateau afterwards.

*MJIRI, Vol. 16, No. 3, 159-163, 2002.*

**Keywords:** *Pseudomonas*, antibiotic resistance, lead resistance, plasmid

### INTRODUCTION

*P. aeruginosa* is one of the leading causes of burn

infections, ranking second among Gram-negative pathogens reported to the national nosocomial infection surveillance system.<sup>1,2</sup> *P. aeruginosa* can be found in most moist environments and occasionally in the normal intestinal or skin flora.<sup>2</sup> In the hospital, skin, respiratory equipment and humidifiers can be important sources of infections.<sup>6</sup> Due to its ubiquitous presence, the organism

**Correspondence address:** Mohammad Reza Shakibaie, Ph.D., Department of Genetics, Research Center, Kerman University of Medical Sciences, Kerman, I.R. Iran. Tel-0098 34157789, Fax- 341 263249, E-mail: Shakibaie@arg3.uk.Ac.Ir

## Metal and Antibiotic Resistance of *P. aeruginosa*

can be found in clinical samples as a contaminant without any relation to diseases, however, it can be responsible for serious and lethal infections in immunocompromised patients such as infection of wounds and burns with bluish green pus, meningitis, fulminant septicemia and urinary tract infections.<sup>5</sup> Involvement of the respiratory tract especially from contaminated respirators results in necrotizing pneumonia.<sup>14</sup> *P. aeruginosa* also causes severe invasive otitis externa in diabetic patients.<sup>7</sup> In spite of antibiotic toxicity, antibiotic-resistant *P. aeruginosa* are emerging from various clinical samples in the hospitals and creating problems in the treatment of infections caused by this organism.<sup>21,7</sup> Wadman et al.<sup>20</sup> reported *P. aeruginosa* resistant to aminoglycosides in cystic fibrosis patients. More than 90% of resistant strains were impermeable to these antibiotics. Carmeli et al.<sup>3</sup> compared the risks of emergence of resistance associated with four antipseudomonal agents, it was observed that ceftazidime was associated with the lowest risk and imipenem had the highest risk. Plasmid mediated antibiotic resistance is the most frequently reported in this micro-organism and is often transmissible by conjugation process not only within the genus but to other Gram negative pathogens as well.<sup>4,11</sup> However, no such report existed in Iran.<sup>19</sup>

The present investigation deals with isolation of plasmid mediated Pb and multiple antibiotic resistance in *P. aeruginosa* isolated from burn patients capable of accumulating Pb.

### MATERIAL AND METHODS

#### Patients and bacterial source

Out of 250 patients studied in the burn unit of Kerman Hospital over a period of one year, 50 were infected with *P. aeruginosa* strains. 22 Isolates were collected from females and 28 from males. The mean±standard deviation (SD) for their age was 21.3±14.3. The patients who had dysautonomic features including severe burns in hands, legs, face or body were included in this study. The genus and species of the isolates were identified by various standard microbiological tests as described previously.<sup>4,6</sup>

#### Antibiotics and metals

All antibiotic discs used in this study were provided by Darupaksh Company Ltd. Iran including Te, K, Gm, C, Cp (ciprofloxacin), CXT (cefotaxime), CAZ (ceftazidime), Rif (rifampin) and Nal (nalidixic acid). Powders of the above antibiotics were received from Razak Company Ltd. Iran with 99.5% purity. The salts of the following heavy metals were purchased from Sigma (USA): silver nitrate, lead nitrate, sodium arsenate, copper sulfate, cadmium chloride, mercury chloride,

chromium sulfate and zinc chloride. The chemicals for plasmid isolation were obtained from E.Merck (Dermstadt, Germany).

#### Antibiotic and metal sensitivity

The primary antibiotic sensitivity test was carried out by disc diffusion break point assay and minimum inhibitory concentration (MIC) of the above antibiotics and metals was determined by agar dilution method.<sup>16</sup> For MIC experiment 50, isolated *Pseudomonas* strains were grown for 8 hours in 20mL sterile Muller-Hinton broth separately and 0.1mL log-phase ( $10^5$ - $10^6$  cells/mL) cultures were inoculated into 19.9 mL sterile Muller-Hinton agar (without NaCl) containing appropriate concentrations of the antibiotics and metals. For MIC of  $Pb(NO_3)_2$  and other metal ions initially a stock solution of 2500  $\mu$ M for each metal ion in sterile double distilled water was prepared and then diluted to the following concentrations: 0.05, 0.1, 0.5, 1.0, 2.0, 5.0, 10.0, 50.0  $\mu$ M. The MIC was defined as the lowest concentration of the antibiotic/metal that inhibited the growth. Concentrations above 10 $\mu$ M were designated as resistant and below 10  $\mu$ M as sensitive. The sensitivity and resistance of the isolates were calculated according to published papers.<sup>18,15,4</sup>

A sensitive standard culture of *P. aeruginosa* PTCC1074.1 (Persian type culture collection) was included in the MIC test.

#### Plasmid DNA extraction

Plasmid DNA from  $Pb^r$  strains was extracted using the Qiagen- large plasmid prep kit<sup>17</sup> and sized related to phage  $\lambda$  DNA digested with enzyme HindIII. Electrophoresis was carried out in horizontal bed apparatus using 1mM Tris-EDTA-Borate (TEB) buffer (pH 7.2) either at 60V for 4-hours or 90V for 2-hours.

#### Conjugation by membrane filter

Freshly prepared cultures of *P. aeruginosa* strain 16 ( $Pb^r$ ) [donor] and *P. aeruginosa* PTCC1074.1 (spontaneous rifampin resistant mutant) and *Escherichia coli* K12 HB101.1 (Rif<sup>r</sup>) [recipients] were added to 20 mL Luria bertanii broth (L/B) in 100 mL Erlenmeyer flasks separately and incubated on a shaker (200 rpm) for 18-hours at 37°C. Donor (3 mL) and recipient (2 mL) were mixed in a sterile filter assembly containing 0.25  $\mu$ m pore size membrane filter (Sartorius, Germany). Membrane was then placed onto the surface of Muller-Hinton (MHA) agar and incubated at 37°C for 24-48 hours. Mating was disrupted by vigorous shaking in 5 mL sterile normal saline (0.8%). The suspension was then serially diluted ( $10^{-2}$  to  $10^{-8}$ ). Each dilution (0.1 mL) was spread onto a MHA agar plate selective for transconjugants (50  $\mu$ MPb + 100  $\mu$ g/mL Rif) and for recipient (100  $\mu$ g/mL Rif). The frequency of conjugation

was calculated as number of transconjugants divided by number of recipients multiplied by dilution factor. Simultaneously, controls for donor and recipient were carried out to check spontaneous mutants.

### Transformation

Recombinant deficient mutant of *E. coli* K12 DH5 $\alpha$  was transformed with 10  $\mu$ L of purified plasmid preparation of strain 16 in medium containing 50  $\mu$ M Pb + 100  $\mu$ g/mL NaI. For competence generation, the cells were incubated in 50mM ice cold CaCl<sub>2</sub> for 2-hours.<sup>17</sup>

### Pb accumulation

The accumulation of Pb at different time intervals was studied by inoculating freshly prepared culture of *P. aeruginosa* strain 16<sup>(Pb<sup>r</sup>)</sup> [10<sup>8</sup> cells/mL] into 20 mL L/B broth in 100 mL Erlenmeyer flask and incubated for 24-hours on a shaker (200 rpm). Pb(NO<sub>3</sub>)<sub>2</sub> was added to a final concentration of 50  $\mu$ M. One set was kept as control without added Pb. 1 mL of culture was withdrawn

immediately and centrifuged in sterile microfuge tube at 10,000 rpm at 4°C for 10 min. Pellet was washed once with 10 mM phosphate buffer pH 8.0 and then with 2 mM piperazine N,N bis[2-ethane sulfonic acid] (PIPES) buffer pH 6.7 and centrifuged at 10,000 rpm. Similarly, samples were withdrawn at 5, 10, 20, 40, 60 and 80 minutes and treated as above. The cell pellets were thoroughly suspended in 1 mL 6N ultrapure HNO<sub>3</sub> and incubated overnight at room temperature (25°C). The digests were diluted with 10 mL sterile DD/W and heated at 80°C for 30 minutes using a reflux condenser. The Pb content of each sample was measured with a Perkin-Elmer atomic absorption spectrophotometer equipped with a graphite analyzer.<sup>18</sup>

## RESULTS AND DISCUSSION

The distribution of number and percentage of the samples obtained from burn patients in Kerman hospital are shown in Table I. As shown, the number of burned

**Table I.** Distribution of number and percentage of samples obtained from patients in the burn unit of the hospital.

Percentage of burn	Age group*					Total No. (%)
	1-10 No. (%)	11-20 No. (%)	21-30 No. (%)	31-40 No. (%)	<50 No. (%)	
1-30	2 (15.4)	4 (28.6)	1 (8.3)	-	-	7 (14.0)
31-40	6 (46.1)	4 (28.6)	4 (33.3)	4 (57.1)	3 (75.0)	21 (42.0)
41-50	5 (38.5)	3 (21.4)	5 (41.7)	-	1 (25.0)	14 (28.0)
<50	-	3 (21.4)	2 (16.7)	3 (42.0)	-	8 (16.0)
Total	13 (100.0)	14 (100.0)	12 (100.0)	7 (100.0)	4 (100.0)	50 (100.0)

\*Age: (21 $\pm$ 14.3)

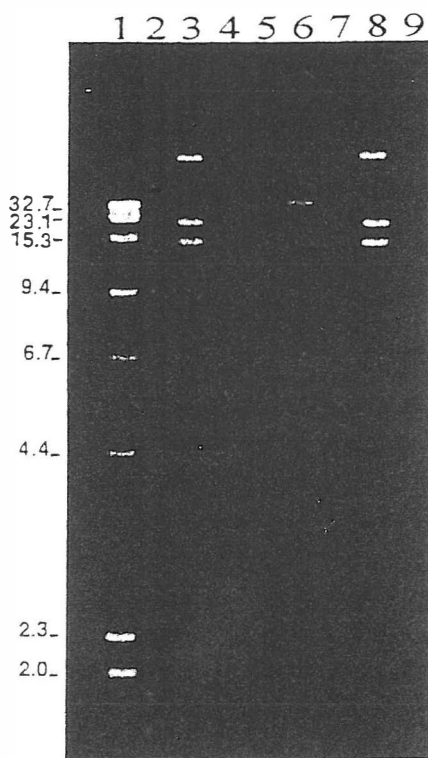
Figures in parenthesis indicates percentage of infected patients.

**Table II.** *In vitro* susceptibility of *Pseudomonas aeruginosa* strain 16, *P. aeruginosa* standard culture PTCC1074.1 and PTCC1074.1 transconjugants to different antibiotics and metals.

Metal ions	Antibiotics	MIC					
		Pseudomonas strain 16		PTCC1074.1		PTCC1074.1 (T)*	
		Metal ( $\mu$ M)	Antibiotic ( $\mu$ g/mL)	Metal ( $\mu$ M)	Antibiotic ( $\mu$ g/mL)	Metal ( $\mu$ M)	Antibiotic ( $\mu$ g/mL)
Zn	tetracycline	200	258	10	16	10	126
Cd	chloramphenicol	5	512	5	25	5	256
Cu	gentamycin	100	126	50	16	50	126
Pb	kanamycin	50	126	5	25	50	64
Hg	ciprofloxacin	10	8	5	0.5	5	0.5
As	cefotaxime	20	25	20	0.5	20	0.5
Cr	ceftazidime	200	16	20	1	20	1
Ag	piperacillin	10	0.5	10	0.5	10	0.5

\*T: transconjugant

## Metal and Antibiotic Resistance of *P. aeruginosa*



**Fig. 1.** Agarose gel electrophoresis of the plasmids from *P. aeruginosa* strain 16 and 35, *P. aeruginosa* PTCC1074.1 transconjugant.

Lane 1: Plasmids in strain 16.

Lane 2: DNA standard markers.

Lane 3: Plasmids in PTCC1074.1 transconjugant.

Lane 4: Plasmid in strain 35.

patients increases with age, however, the peak incidence is between 1 to 10 years old. The severity of burns was maximum at age 21 to 30, being 70%. The isolated strains exhibited varied MICs to antibiotics and  $Pb(NO_3)_2$ . Twenty-one isolates showed MIC 2  $\mu M$ , twenty-four demonstrated MIC 10  $\mu M$  and the remaining 5 isolates had MIC 50  $\mu M$  respectively. Among the isolates, strain 16 exhibited the highest MIC to Pb, Cr and Zn as well as Te, C, Gm and K as shown in Table II.

Conjugation experiments with *P. aeruginosa* strain 16 as donor and *P. aeruginosa* PTCC1074.1 ( $Rif^r$ ) and *E. coli* K12 HB101.1 ( $Rif^r$ ) as recipients are shown in Table III. 50  $Pb^r$  transconjugant colonies were examined for marker simultaneously co-transferred. It was observed that all exhibited co-transfer of Te, C, Gm and K along with Pb. However, any other metal or antibiotic marker was co-transferred.

Plasmid DNA was extracted from  $Pb$  and antibiotic resistant colonies of strain 16 as well as PTCC1074.1 transconjugants. Three bands were observed in strain 16 and in the transconjugants (Fig. 1) and one plasmid band was found in strain 35. The  $Pb^r$  plasmid was further transformed to *E. coli* K12 DH5 $\alpha$  with a frequency of  $4.0 \times 10^{-6}$ .

The results of accumulation of  $Pb$  in *P. aeruginosa* strain 16 are shown in Fig. 2.  $Pb$  was taken almost immediately and reached a maximum within 60 minutes; further, there was no change in accumulation process.

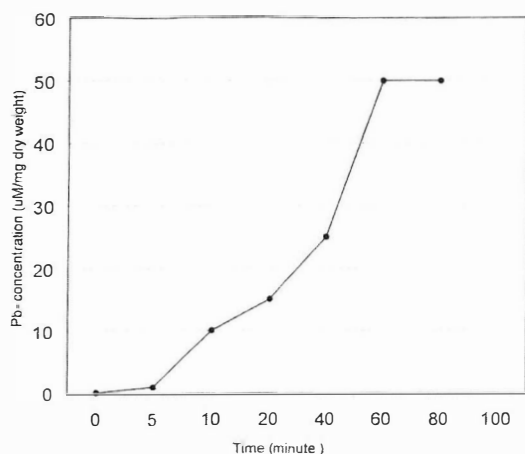
Previous reports on  $Pb^r$  strain of *S. aureus* displayed plasmid linked resistance to penicillin, erythromycin, and several toxic heavy metals.<sup>1,13</sup> Jordan and Barton<sup>8</sup> reported lead immobilization by Gram-negative encapsulated bacteria. Levinson et al.<sup>10</sup> reported the presence of a large plasmid in *Citrobacter freundii* ATCC8090 ( $Cf^r$ ) encoded Cd and ampicillin resistance genes. Lotard et al.<sup>9</sup> isolated ceftazidime susceptible and resistant *P. aeruginosa* from pulmonary specimens. The ceftazidime resistant strains harbored a self transmissible plasmid which expressed oxacillinase activity. Similarly, Marchandin et al.<sup>11</sup> studied production of TEM -24 plasmid mediated extended spectrum  $\beta$ -lactamase by clinical isolates of *P. aeruginosa*. TEM and resistance markers for aminoglycosides, chloramphenicol and sulfonamide were encoded by a transferable plasmid. Plasmid mediated metal and antibiotic resistance in marine *Pseudomonas* were studied by Rajini and Mahadevan.<sup>15</sup> They transferred 146 kb plasmid conferred inducible resistance to Hg, Ars, and cadmium into *E. coli*. However, there is no report on plasmid mediated lead and antibiotic resistance in *P. aeruginosa*.

**Table III.** Intra-genetic and inter-genetic conjugation between *P. aeruginosa* strain 16 [donor] and *P. aeruginosa* PTCC1074.1 and *E. coli* K12 HB101.1 [recipients] by membrane filter technique.

Donor	Recipient	Selection based on	Conjugation frequency	Marker co-transferred
Strain 16	PTCC125.1	MHA <sup>a</sup> +Pb <sup>b</sup> + Rif <sup>c</sup>	$1 \times 10^{-4}$	Te.C.Gm.K
Strain 16	HB 101.1	MHA+Pb + Rif	$1 \times 10^{-9}$	-

a= Muler-Hinton agar, b= 50 $\mu M$ , c= 100 $\mu g/mL$ .

A control of conjugation was carried out to check the presence of spontaneous mutants. It was  $<10^{-9}$ .



**Fig. 2.** Pb-accumulation in *Pseudomonas aeruginosa* strain 16.

### CONCLUSION

The data presented here suggest that Pb and antibiotic resistance gene(s) in *P. aeruginosa* strain 16 reside on a mobile element and transferring these traits from resistant to sensitive strains is possible using conjugation and the presence of the plasmid was confirmed by plasmid isolation and subsequent agarose gel electrophoresis.

### ACKNOWLEDGEMENTS

Our sincere thanks are due to Dr. Taban from Kerman hospital and the staff of the microbiology department, Kerman school of medicine for their help during this research. Kerman University of Medical Sciences supported this investigation.

### REFERENCES

1. Aikin RM, Dean ACR: Lead accumulation by *Pseudomonas fluorescens* and by *Citrobacter sp.* Microb Lett 9: 55-66, 1979.
2. Bdey GP, Bolivar R, Fainstein V, Jadeja L: Infections caused by *Pseudomonas aeruginosa*. Rev Infect Dis 5: 279-289, 1983.
3. Carmeli Y, Troillet N, Eliopoulos G, Samore M: Emergence of antibiotic resistant *Pseudomonas aeruginosa*, comparison of risk associated with different antipseudomonal agents. Antimicrob Agent Chemother 43: 1379-1382, 1999.
4. Devicente A, Ariles M, Codina JC, Borrego JJ, Romero P: Resistance to antibiotics and heavy metals of *Pseudomonas aeruginosa* isolated from natural water. Appl Bacteriol 68: 625-632, 1990.
5. Doggett G (ed): *Pseudomonas aeruginosa: Clinical Manifestations of Infection and Current Therapy*. New York: Academic Press, 1979.
6. Farmer JJ, Weinstein RA, Zierdt CH, Brokop CD: Hospital outbreak caused by *Pseudomonas aeruginosa*, importance of serogroup. J Clin Microbiol 16: 266-270, 1982.
7. Hanberger H, Hofmann M, Linderen S, Nilsson LE: High incidence of antibiotic resistance among bacteria in intensive care unit at university in Sweden. Scan J Infect Dis 6: 607-614, 1997.
8. Jordan FL, Barton LL: Lead immobilization by Gram-negative encapsulated bacteria. Abstr Am Soc Microbiol 95: 447, 1995.
9. Leotard S, Poirel L, Leblanc PE, Nordmann P: *In vivo* selection of oxacillinase mediated ceftazidime resistance in *Pseudomonas aeruginosa*. Microb Drug Resist 7(3): 273-275, 2001.
10. Levinson HS, Mahler I: Phosphatase activity and lead resistance in *Citrobacter freundii* and *Staphylococcus aureus*. FEMS Lett 161: 135-138, 1998.
11. Marchandin H, Sirot D, Darbas H, Carriere C: Production of a TEM-24 plasmid mediated extended spectrum beta-lactamase by clinical isolate of *Pseudomonas aeruginosa*. Antimicrob Agent Chemother 1: 213-216, 2000.
12. National Committee for Clinical Standards: Methods for dilution in antimicrobial susceptibility tests for bacterial growth aerobically. 4th ed. Approved standard M70-A4, NCCLS, 1996.
13. Novick RP, Roth C: Plasmid linked resistance to inorganic salts in *Staphylococcus aureus*. J Bacteriol 95: 1335-1342, 1968.
14. Pier GB: Pulmonary diseases associated with *Pseudomonas aeruginosa* in cystic fibrosis, current status of the host bacterium. J Infect Dis 151: 575-581, 1985.
15. Rajini DB, Mahadevan A: Plasmid mediated metal and antibiotic resistance in marine pseudomonas. Biometals 5: 73-80, 1992.
16. Sabry SA, Ghzlam HA, Abou-zeid DM: Metal tolerance and antibiotic resistance patterns of bacterial population isolated from seawater. Appl Microbiol 82: 245-252, 1997.
17. Sambrook J, Fritsch EF, Maniatis T: Molecular cloning - a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor N.Y., 1989.
18. Shakibaie MR, Kapadnis BP, Dhakephalkar P, Chopade BA: Removal of silver from photographic waste water effluent using *Acinetobacter baumannii* BL54. Can J Microbiol 45: 995-1000, 1999.
19. Shakibaie MR, Adeli S, Nikian Y: Emergence of ciprofloxacin resistance among *Pseudomonas aeruginosa* isolated from burn patients. Irn J Med Sci 26 (3&4): 155-159, 2000.
20. Wadman S, Sherman DR, Hickey MJ, Warrenner P, Shawar RM, Floger KR, Stover CK: Characterization of a *Pseudomonas aeruginosa* efflux pump contributing to aminoglycoside impermeability. Antimicrob Agent Chemother. 43 (12): 2975-2983, 1999.
21. Williams JR, Heyman LD: Containment of antibiotic resistance. Science 279: 1153-1155, 1998.