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

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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', C', K' and Gm' along with Pb' to a sensitive strain of P. aeruginosa PTCC1074.1 (Rif') but not to E. coli K12 HB101.1 (Rif'). 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 (50μM) within 60 min. and reached a plateau afterwards.


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. P. aeruginosa can be found in most moist environments and occasionally in the normal intestinal or skin flora. In the hospital, skin, respiratory equipment and humidifiers can be important sources of infections. Due to its ubiquitous presence, the organism
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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. Involvement of the respiratory tract especially from contaminated respirators results in necrotizing pneumonia. *P. aeruginosa* also causes severe invasive otitis externa in diabetic patients. 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. Wadman et al. reported *P. aeruginosa* resistant to aminoglycosides in cystic fibrosis patients. More than 90% of resistant strains were impermeable to these antibiotics. Carmeli et al. 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. However, no such report existed in Iran.

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.

**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 (ceftoxime), CAZ (ceftizidime), 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, mercuric chloride, chromieum 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. For MIC experiment 50, isolated Pseudomonas strains were grown for 8 hours in 20 mL sterile Muller-Hinton broth separately and 0.1 mL log-phase (10⁵-10⁶ 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(NO₃)₂ and other metal ions initially a stock solution of 2500 μM 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 μM. The MIC was defined as the lowest concentration of the antibiotic/metal that inhibited the growth. Concentrations above 10 μM were designated as resistant and below 10 μM as sensitive. The sensitivity and resistance of the isolates were calculated according to published papers.

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⁺ strains was extracted using the Qiagen- large plasmid prep kit and sized related to phage-λ DNA digested with enzyme HindIII. Electrophoresis was carried out in horizontal bed apparatus using 1% 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⁺) [donor] and *P. aeruginosa* PTCC1074.1 (sponaneous rifampin resistant mutant) and *Escherichia coli* K12 HB101.1 (Rif⁺) [recipients] were added to 20 mL Luria bertani 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 μm 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 10⁻⁹). Each dilution (0.1 mL) was spread onto a MHA agar plate selective for transconjugants (50 μMPb + 100 μg/mL Rif) and for recipient (100 μg/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 DH5α was transformed with 10 μL of purified plasmid preparation of strain 16 in medium containing 50 μM Pb + 100 μg/mL NaI. For competence generation, the cells were incubated in 50 mM ice cold CaCl₂ for 2-hours.¹⁷

**Pb accumulation**
The accumulation of Pb at different time intervals was studied by inoculating freshly prepared culture of *P. aeruginosa* strain 16 (Pb) [10⁵ cells/mL] into 20 mL LB broth in 100 mL Erlenmeyer flask and incubated for 24-hours on a shaker (200 rpm). Pb(NO₃)₂ was added to a final concentration of 50 μM. 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] (PIPEC) buffer pH 6.7 and centrifuged at 10,000 rpm. Similarly, samples were withdrawn at 5, 10, 20, 30, 60 and 80 minutes and treated as above. The cell pellets were thoroughly resuspended in 1 mL 6N ultrapure HNO₃ 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.¹⁸

**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>
<thead>
<tr>
<th>Percentage of burn</th>
<th>1-10 No. (%)</th>
<th>11-20 No. (%)</th>
<th>21-30 No. (%)</th>
<th>31-40 No. (%)</th>
<th>&lt;50 No. (%)</th>
<th>Total No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-30</td>
<td>2 (15.4)</td>
<td>4 (28.6)</td>
<td>1 (8.3)</td>
<td>-</td>
<td>-</td>
<td>7 (14.0)</td>
</tr>
<tr>
<td>31-40</td>
<td>6 (46.1)</td>
<td>4 (28.6)</td>
<td>4 (33.3)</td>
<td>4 (57.1)</td>
<td>3 (75.0)</td>
<td>21 (42.0)</td>
</tr>
<tr>
<td>41-50</td>
<td>5 (38.5)</td>
<td>3 (21.4)</td>
<td>5 (41.7)</td>
<td>-</td>
<td>1 (25.0)</td>
<td>14 (28.0)</td>
</tr>
<tr>
<td>&lt;50</td>
<td>-</td>
<td>3 (21.4)</td>
<td>2 (16.7)</td>
<td>3 (42.0)</td>
<td>-</td>
<td>8 (16.0)</td>
</tr>
<tr>
<td>Total</td>
<td>13 (100.0)</td>
<td>14 (100.0)</td>
<td>12 (100.0)</td>
<td>7 (100.0)</td>
<td>4 (100.0)</td>
<td>50 (100.0)</td>
</tr>
</tbody>
</table>

*Age: (21±14.3)

Figures in parenthesis indicates percentage of infected patients.

<table>
<thead>
<tr>
<th>Metal ions</th>
<th>Antibiotics</th>
<th>MIC Pseudomonas strain 16</th>
<th>MIC PTCC1074.1</th>
<th>MIC PTCC1074.1 (T)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Metal (μM)</td>
<td>Antibiotic (μg/mL)</td>
<td>Metal (μM)</td>
</tr>
<tr>
<td>Zn</td>
<td>tetracycline</td>
<td>200</td>
<td>258</td>
<td>10</td>
</tr>
<tr>
<td>Cd</td>
<td>chloramphenicol</td>
<td>5</td>
<td>512</td>
<td>5</td>
</tr>
<tr>
<td>Cu</td>
<td>gentamycin</td>
<td>100</td>
<td>126</td>
<td>50</td>
</tr>
<tr>
<td>Pb</td>
<td>kanamycin</td>
<td>50</td>
<td>126</td>
<td>5</td>
</tr>
<tr>
<td>Hg</td>
<td>ceftoloxacin</td>
<td>10</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>As</td>
<td>cefotaxime</td>
<td>20</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>Cr</td>
<td>cepfazidime</td>
<td>200</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>Ag</td>
<td>piperacillin</td>
<td>10</td>
<td>0.5</td>
<td>10</td>
</tr>
</tbody>
</table>

*T: transconjugant
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Conjugation experiments with *P. aeruginosa* strain 16 as donor and *P. aeruginosa* PTCC1074.1 (Rif') and *E. coli* K12 HB101.1 (Rif') as recipients are shown in Table III. 50 Pb' 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' plasmid was further transformed to *E. coli* K12 DH5α 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' strain of *S. aureus* displayed plasmid linked resistance to penicillin, erythromycin, and several toxic heavy metals.1-3 Jordan and Barton4 reported lead immobilization by Gram-negative encapsulated bacteria. Levinson et al.10 reported the presence of a large plasmid in *Citrobacter freundii* ATCC8090 (CF) encoded Cd and ampicillin resistance genes. Levinson et al.10 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.11 studied production of TEM-24 plasmid mediated extended spectrum β-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.15 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*.

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

1: Plasmids in strain 16.
2: DNA standard markers.
3: Plasmids in PTCC1074.1 transconjugant.
4: Plasmid in strain 35.

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

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Selection based on</th>
<th>Conjugation frequency</th>
<th>Marker co-transferred</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain 16</td>
<td>PTCC125.1</td>
<td>MHA+Pb'+ Rif'</td>
<td>$1 \times 10^4$</td>
<td>Te.C.Gm.K</td>
</tr>
<tr>
<td>Strain 16</td>
<td>HB101.1</td>
<td>MHA+Pb+ Rif</td>
<td>$1 \times 10^9$</td>
<td>-</td>
</tr>
</tbody>
</table>

a= Muler-Hinton agar, b= 50μM, c= 100μg/mL.

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

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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

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REFERENCES