Resistance Plasmids of Indigenous Pseudomonas in Egypt

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Abstract: Two hundred forty nine Pseudomonas isolates were collected from different geographical sites in Egypt. They were examined for resistance against ten heavy metals and five antibiotics. Plasmid profiles were also studied. Eight P. aeruginosa and two P. fluorescense strains were selected for further studies. They were resistant to seven to nine heavy metals and four to five antibiotics. The ten strains contain two small plasmids, but have up to five large plasmids. Plasmid curing indicated that resistant to each of cadmium, cobalt, silver, chromium, mercury, nickel or zinc is plasmid borne. It also showed that the resistance of both iron and lead are carried by the bacterial chromosome. Results also revealed that chromate resistance gene (s) were located on a large plasmid in P. aeruginosa 24 strain. The resistance of all tested antibiotic was located in plasmids.

Key words: Pseudomonas, heavy metals, antibiotic, R-plasmid, plasmid curing.

INTRODUCTION

In recent years, heavy metals, have received widespread attention because of their release into the environments. Heavy metal pollution is a growing problem in all over the world; in Egypt several reports indicated this problem in both soil and water.

Microorganisms are generally the first to be affected by discharge of heavy metals into the environment[19]. Similarly, microbial ecosystem can also drastically alter the fate of metals entering aquatic or soil environments. Bacteria, cyanobacteria and fungi can alter the form occurrence of metals through intracellular accumulation, cell wall binding, siderophore interaction, extracellular mobilization or immobilization, extracellular polymer-metal interactions, transformation and volatilization of metals that affect their bioavailability.

The resistance determinants to heavy metal are often bound on plasmids and transposons which facilitates their analysis by molecular genetic techniques[12].

Many bacterial plasmids has been found to encodes resistance to heavy metals such as Hg^{2+}, Ag^{+}, Cd^{2+}, Co^{2+}, Cr^{6+}, Cu^{2+}, Ni^{2+}, and Zn^{2+} among every tested bacterial group[11].

This study aimed to investigate heavy metal and antibiotic resistance genes in indigenous Pseudomonas in different geographical sites in Egypt. It also aimed to study the relationship between heavy metal or antibiotic resistance and the bacterial plasmids.

MATERIALS AND METHODS

Microbial Strains and Culture Conditions: Pseudomonas fluorescense 17400 and P. aeruginosa S44 (used as a reference strain), were kindly provided by Dr. A. Gaballa, Mubarak City for Scientific Research and Technology Applications, Alexandria, Egypt. A total of 249 Pseudomonas were isolated in this study from different geographical locations in Egypt. Luria -Bertani medium (LB)[4] was used for bacterial growth. Pseudomonas selective medium (cetrimide agar)[1] was used in isolation and identification of Pseudomonas isolates.

Bacterial Isolation: Soil samples were collected from different geographical areas, which have been known to be contaminated with heavy metals, i.e., Fayoum governorate, Manzalah Lagoon, Middle Delta, El-Gabal
**Table 1:** Geographical distribution of heavy metal resistance *Pseudomonas*

<table>
<thead>
<tr>
<th>Heavy metal</th>
<th>Isolate source</th>
<th>Number of resistant isolates</th>
<th>Heavy metal</th>
<th>Isolate source</th>
<th>Number of resistant isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nickel</td>
<td>M.D.</td>
<td>59</td>
<td>Cupric</td>
<td>M.D.</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>F.G.</td>
<td>90</td>
<td>chloride</td>
<td>F.G.</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>I.S.F.</td>
<td>29</td>
<td>chloride</td>
<td>I.S.F.</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>M.L.</td>
<td>12</td>
<td></td>
<td>M.L.</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>G.A.</td>
<td>12</td>
<td></td>
<td>G.A.</td>
<td>7</td>
</tr>
<tr>
<td>Zinc</td>
<td>M.D.</td>
<td>58</td>
<td>mercuric</td>
<td>M.D.</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>F.G.</td>
<td>65</td>
<td>chloride</td>
<td>F.G.</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>I.S.F.</td>
<td>28</td>
<td>chloride</td>
<td>I.S.F.</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>M.L.</td>
<td>9</td>
<td></td>
<td>M.L.</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>G.A.</td>
<td>7</td>
<td></td>
<td>G.A.</td>
<td>6</td>
</tr>
<tr>
<td>Lead Acetate</td>
<td>M.D.</td>
<td>50</td>
<td>cadmium</td>
<td>M.D.</td>
<td>49</td>
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<tr>
<td></td>
<td>F.G.</td>
<td>86</td>
<td>chloride</td>
<td>F.G.</td>
<td>6</td>
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<tr>
<td></td>
<td>I.S.F.</td>
<td>32</td>
<td>chloride</td>
<td>I.S.F.</td>
<td>25</td>
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<tr>
<td></td>
<td>M.L.</td>
<td>13</td>
<td></td>
<td>M.L.</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>G.A.</td>
<td>14</td>
<td></td>
<td>G.A.</td>
<td>9</td>
</tr>
<tr>
<td>Cobalt</td>
<td>M.D.</td>
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<td>potassium</td>
<td>M.D.</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>F.G.</td>
<td>7</td>
<td>dichromate</td>
<td>F.G.</td>
<td>5</td>
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<tr>
<td></td>
<td>I.S.F.</td>
<td>11</td>
<td></td>
<td>I.S.F.</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>M.L.</td>
<td>6</td>
<td></td>
<td>M.L.</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>G.A.</td>
<td>7</td>
<td></td>
<td>G.A.</td>
<td>8</td>
</tr>
<tr>
<td>Ferric chloride</td>
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<td>silver</td>
<td>M.D.</td>
<td>5</td>
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<tr>
<td></td>
<td>F.G.</td>
<td>123</td>
<td>nitrate</td>
<td>F.G.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>I.S.F.</td>
<td>33</td>
<td></td>
<td>I.S.F.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M.L.</td>
<td>12</td>
<td></td>
<td>M.L.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>G.A.</td>
<td>13</td>
<td></td>
<td>G.A.</td>
<td>2</td>
</tr>
</tbody>
</table>

M.D.: Middle Delta (61 isolates); F.G.: Fayoun Governorate (125 isolates); I.S.F.: Iron and Steel Factory (34 isolates); M.L.: Manzala Lagoon (14 isolates); G.A.: El-Gabal El-Asfer (15 isolates).

**Table 2:** Characterization of selected *Pseudomonas* heavy metal resistance strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid content</th>
<th>Heavy metal resistance</th>
<th>Antibiotic resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> 21</td>
<td>S, S, L, L, L</td>
<td>Cu, Cd, Pb, Ni, Hg, Ag, Zn, Fe</td>
<td>Cm, Km, Tc, Amp</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 24</td>
<td>S, S, L, L, L, L</td>
<td>Cu, Cd, Pb, Ni, Hg, Ag, Zn, Fe, Co, CrO₃</td>
<td>Cm, Km, Tc, Amp</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 25</td>
<td>S, S, L, L, L, L, L</td>
<td>Cu, Cd, Pb, Ni, Hg, Ag, Zn, Co, CrO₃, Fe</td>
<td>Cm, Km, Tc, Amp, Sm</td>
</tr>
</tbody>
</table>
Table 2: Continued.

<table>
<thead>
<tr>
<th>P. aeruginosa 26</th>
<th>Cu, Cd, Pb, Ni, Ag, Hg, Zn, Fe, Ag</th>
<th>Cm, Km, Tc, Sm</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. aeruginosa 31</td>
<td>Cu, Cd, Pb, Ni, Hg, Zn, Fe</td>
<td>Cm, Km, Tc, Amp</td>
</tr>
<tr>
<td>P. aeruginosa 36</td>
<td>Cu, Cd, Pb, Ni, Zn, Fe, Co</td>
<td>Cm, Km, Tc, Amp</td>
</tr>
<tr>
<td>P. aeruginosa 44</td>
<td>Cu, Cd, Pb, Ni, Hg, Zn, Fe, Ag</td>
<td>Cm, Km, Tc, Amp</td>
</tr>
<tr>
<td>P. aeruginosa 46</td>
<td>Cu, Cd, Pb, Ni, Zn, Fe</td>
<td>Cm, Km, Tc, Amp</td>
</tr>
<tr>
<td>P. fluorescence 13</td>
<td>Cu, Cd, Pb, Ni, Zn, Fe, Co</td>
<td>Cm, Km, Sm, Amp</td>
</tr>
<tr>
<td>P. fluorescence 16</td>
<td>Cu, Cd, Pb, Ni, Zn, Fe, Ag, CrO_3</td>
<td>Cm, Km, Tc, Amp</td>
</tr>
</tbody>
</table>

S= Small plasmid, L= Large plasmid
Cu= Copper, Cd= Cadmium, Pb= Lead, Ni= Nickel, Zn= Zinc, Hg= Mercury, Fe= Iron, Ag= Silver, Co= Cobalt, CrO_3= Chromium, Cm= chloramphenicol, Km= Kanamycin, Tc= Tetracycline, Amp= Ampicillin, Sm= Streptomycin.

El-Asfar and Iron and Steel Factory in Egypt. The soils were suspended in sterile saline solution, spreaded on LB plates and incubated at 28°C for 72 hr. Single colonies were then picked and identified according to different morphological and biochemical tests[1][2].

**Bacterial Sensitivity Tests:** Sensitivity of bacterial isolates to ten heavy metals and five antibiotics were performed by agar diffusion method[8]. The heavy metals used were cupric chloride (Cu); cadmium chloride (Cd); cobalt chloride (Co); mercuric chloride (Hg); nickel sulphate (Ni); lead acetate (Pb); potassium dichromate (CrO_3); zinc sulphate (Zn); ferric chloride (Fe) and silver nitrate (Ag). The antibiotics used were tetracycline (Tc); streptomycin (Sm); kanamycin (Km); ampicillin (Amp) and chloramphenicol (Cm).

**Plasmid DNA Isolation:** The alkaline lyses method[6] was used for plasmid DNA isolation.

**Agarose Gel Electrophoresis:** 0.8% agarose in TBE buffer was used as described[9].

**Plasmid Curing:** Several plasmid curing agents were used including acridine orange, acriflavin, norfloxacin, and ethidium bromide, each was used in combination with elevated temperature i.e., 45°C. Two methods were used, the liquid medium curing method or Disk curing method. After treatment single colonies were isolated and retested for resistance.

**RESULTS AND DISCUSSIONS**

Heavy metals have been released into the environment over long periods of time, throughout many activities of man. Once the metals have been released into the environment, they are difficult to be removed by physical or chemical means and most of them exhibit toxic effect on organisms[5]. Of all the bacterial genera that "pop" up again and again in aerobic polluted ecosystem, it is the genus *Pseudomonas* that leads the pack. Their universal distribution suggests a remarkable degree of physiological and genetic adaptability [14].

In this study *Pseudomonas* isolates were collected from five different geographical areas, which have been known to be contaminated with heavy metals, i.e.,

![Fig. 1: Plasmid profile of some Pseudomonas strains.](image_url)

Lane 1: *P. aeruginosa* 31; lane 2: *P. aeruginosa* 46; lane 3: *P. aeruginosa* 21; Lane 4: *P. fluorescence* 13; lane 6: *P. aeruginosa* 26; lane 8: *P. aeruginosa* 25.
isolates were resistant to six heavy metals, 39 isolates were resistant to five heavy metals, 44 isolates were resistant to four heavy metals, 53 isolates were resistant to three heavy metals, 46 isolates were resistant to two heavy metals, 18 isolates were resistant to single heavy metal, and 14 isolates were resistant to abiotic selection agents. The heavy metals pollution acts as stress or selection agents to develop resistant and even hyper resistant bacterial populations in these polluted areas. Thus Fayoum governorate had relatively low resistance percentages. Beside the abiotic selection to resistant microbial population in the contaminated sites, resistance transfer plays an important role. Most of these genes are found on plasmids, transposons and even on the bacterial chromosome[13] which makes it easy to be transfer intra or intergeneric leading to increase resistance levels in microbial communities[18]. Metal resistance in members of Pseudomonas species has been previously reported[7,16,17].

Ten Pseudomonas isolates were selected for further studies; their characteristics are present in Table 2. They were identified to species level[12,2]. Eight strains were P. aeruginosa and two were P. fluorescens.

**Antibiotic Resistance Patterns:** In order to study the relationship between heavy metal and antibiotic resistance, the chosen ten Pseudomonas strains were tested against five commonly used antibiotics. The antibiotic resistance patterns of the Pseudomonas strains are present in Table 2.

**Plasmid Profiles:** In order to study the relationship of multi-resistance of Pseudomonas strains and their indigenous plasmids, plasmid profiles of the selected ten strains were carried out. Several techniques have been tried for plasmid isolation. The best results were obtained using the hot alkaline lysis method[6].

Data represent in Table 2 showed that the ten Pseudomonas strains contain two small plasmids, S1 and S2 (Fig. 1) which have molecular weight larger than 2.6 kb, when compared with pUC18 plasmid, but they varied in bearing large plasmids. They contain either two large plasmids (L1 and L2) in P. aeruginosa 21, P. aeruginosa 26, P. aeruginosa 26, P. aeruginosa 31, P. aeruginosa 36, P. aeruginosa 44, P. aeruginosa 46 and P. fluorescens 13 strains, or four large plasmids (L1, L2, L3, L4) in P. aeruginosa 24 and P. fluorescens 16 strains while P. aeruginosa 25 was containing five large plasmids (L1, L2, L3, L4, L5) (Fig. 2).

**Plasmid Curing:** Plasmid DNA elimination or curing from bacterial cell is the best test to locate a genetic trait in a specific plasmid. Plasmid can be eliminated by agents that interfere with its replication or its membrane attachments sites [5]. Two methods were used in this

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**Fig. 2:** Plasmid profile of original and cured Pseudomonas strains.
Lane 1 represents the original P. aeruginosa 21, lane 2 its cured isolate, lane 3 the original P. aeruginosa 31, lane 4 its cured isolate, lane 5 the original P. aeruginosa 26, lane 6 its cured isolate; lane 7 the original P. aeruginosa 25 and lane 8 its cured isolate. S1= small plasmid 1 and S2= small plasmid 2.
Fig. 3: Plasmid contents of partially and completely cured isolates of \textit{P. aeruginosa} 24.
Lane 1: Partially cured isolate harboring only plasmid L4;
Lane 2: Completely cured \textit{P. aeruginosa} 24 isolate;
Lane 3: Partially cured isolate harboring only plasmids L3 and L4.

study for plasmid curing, i.e., liquid and disk methods. In both methods the effect of the curing agent was accompanied with elevated temperature, i.e., 45° C.

Results showed the success of liquid method in plasmid curing. The best curing agent was ethidium bromide (80 to 100 % of cells were cured), followed by acridine orange (37 to 86% of cells were cured), while the lowest curing efficiencies was obtained using acriflavin (40 to 73% of cells were cured).

The disk curing method was more efficient than the liquid method. This may be due to the accurate way to determine the sublethal dose by collecting the first bacterial growth after the zone of inhibition of each curing agent disk. The decreasing efficiencies order of the curing agents was ethidium bromide (93 – 100%), norfloxacin and acridine orange (93-100%) then acriflavin (73 – 93%).

All cured isolates were retested for heavy metals and antibiotic resistance patterns. Results showed that all cured isolated still resist to Pb and Fe while they became sensitive to all other heavy metal tested. These results indicated that Ca, Cd, Ni, Hg, Ag, Zn, Co and CrO3 resistance were plasmid borne. The obtained results were in agreement with several reports (13). On the other hand, both of Pb and Fe resistance determination were located on the bacterial chromosome.

All obtained isolates were totally plasmid cured (Figs. 2 and 3). Moreover, two partially cured isolates could be obtained from \textit{P. aeruginosa} 24 strain, the first isolate, \textit{P. aeruginosa} 24-1, had only the L4 plasmid, while the second isolate, \textit{P. aeruginosa} 24-2, still harbor L3 and L4 plasmids (Fig. 3). Both isolates were sensitive to all antibiotics tested and resist only Pb, Fe and CrO3. Science our conclusion that both Pb and Fe resistance are chromosomal genes, CrO3 resistance gene is more probable located in the plasmid L4 of \textit{P. aeruginosa} 24 strain.

Results also showed that all cured isolates had lost their antibiotic resistance to all five antibiotics tested. This indicates that the resistance determinants of tested antibiotics were located on plasmids. The two partially cured isolates of \textit{P. aeruginosa} 24 strain had also complete losing of antibiotic resistance of the original strain. This result indicated that plasmids L3 and L4 of \textit{P. aeruginosa} 24 strain do not carry antibiotic resistance genes. And these genes should be located on the other plasmids i.e., S1, S2, L1, and/or L2.

**REFERENCES**