Phenotypic characters of urinary isolates of *Pseudomonas aeruginosa* & their association with mouse renal colonization

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**Background & objectives:** Very few studies regarding production of virulence factors in different predominant serotypes of uropathogenic *Pseudomonas aeruginosa* are available and they have not been correlated to *in vivo* pathogenicity in the urinary tract. This study was carried out with the objective to analyze the phenotypic characters of uroisolates of *P. aeruginosa* *in vitro* and to study the association of these virulence traits with their ability to cause nephropathogenicity in mouse model of ascending urinary tract infection (UTI).

**Methods:** Protease, elastase, alginate, haemolysin, pyochelin, pyoverdin and phospholipase C were measured using standard protocols in 18 uroisolates of *P. aeruginosa* isolated from patients suffering from complicated UTIs. An ascending model of pyelonephritis was established in Swiss Webster (LACA) female mice with these isolates. Quantitative bacterial count and histopathological evaluation of mouse renal tissue was done which were then assessed for a possible association with elaboration of virulence factors.

**Results:** All isolates of *P. aeruginosa* were able to colonize renal tissue of mice. However, renal counts varied amongst different isolates producing different virulence factors. Isolates producing high levels of haemolysin along with other virulence factors were able to colonize and multiply more in mouse renal tissue as compared to those producing low levels of haemolysin.

**Interpretation & conclusion:** The findings of this study indicated an association between haemolysin production and renal colonization. High level of haemolysin production *in vitro* could be used as surrogate information for assessing pyelonephritic potential of *P. aeruginosa*.

**Key words** Mouse model - *Pseudomonas aeruginosa* - urinary tract infections - virulence factors

*Pseudomonas aeruginosa* is an opportunistic pathogen, which causes complicated infections mainly of the lower respiratory tract, wounds and urinary tract in immunocompromised, immunodeficient or surgically manipulated hosts. This organism has been reported in 35 per cent cases of catheterized patients of complicated urinary tract infections (UTIs) and is difficult to manage.
Predisposing factors particularly in urinary tract infections are instrumentation and catheterization during surgery or other urological diseases that enhance the susceptibility for \textit{P. aeruginosa} colonization leading to infection\(^3\). Virulence of \textit{P. aeruginosa} is related to the elaboration of a number of products including exotoxin A, exoenzyme S, alginate, haemolysin, phospholipase C, elastase, alkaline protease and siderophores\(^4,5\). Although many workers have tried to establish correlation between virulence factors of \textit{P. aeruginosa} and pathogenesis by employing different animal models such as burn mouse model, mouse corneal infection model and rat acute and chronic lung infection model\(^6,7\), but paucity of literature exists in relation to UTIs caused by \textit{P. aeruginosa}.

Relative contribution of each of the many exoproducts implicated in virulence of \textit{P. aeruginosa} may vary depending on the site and type of infection\(^3\). Variation in all the exoproducts produced by \textit{P. aeruginosa} isolates in samples obtained from different infections has been reported by Woods \textit{et al}\(^4\). Visca \textit{et al}\(^8\) compared the \textit{in vitro} production of virulence related factors amongst different epidemiological types of uropathogenic \textit{P. aeruginosa} isolates and observed high degree of heterogeneity among predominant O serotypes in terms of protease, elastase and phospholipase C production. No association of any virulence determinant with the pathogenesis of UTIs caused by \textit{P. aeruginosa} was observed. The present study was undertaken to investigate relative contribution of the bacterial virulence traits to \textit{in vivo} nephropathogenicity of \textit{P. aeruginosa} in mouse model of ascending urinary tract infection.

**Material & Methods**

\textit{Bacterial isolates:} A total of 18 isolates of \textit{P. aeruginosa} were obtained from 50 urine samples of patients with complicated UTIs attending Government Medical College and Hospital, Chandigarh, during February 2000 to September 2000. These isolates were identified biochemically\(^9\). Standard strain of \textit{P. aeruginosa}, (PAO), producing most of the recognized virulence factors, was procured from Barbara H. Iglewski, University of Rochester, New York (USA). All the strains were stored in lyophilized forms. At the time of experiment, suspension was made in sterile normal saline, absorbance (A\(_{540}\)) adjusted to 0.4 and inoculated into nutrient broth. Cultures were incubated overnight at 37\(^\circ\)C under static conditions.

\textit{Serotyping:} O-serotyping was performed according to the scheme of the international antigenic typing system (IATS)\(^10\) by Laboratory of Healthcare Associated Infection (LHCAI), London.

\textit{Cell surface hydrophobicity:} Cell surface hydrophobicity was measured by the method of phase separation with p-xylene\(^11\). Initial optical density and optical density of the aqueous phase was taken and surface hydrophobicity was expressed as percentage.

\textit{Alginate determination:} Alginate in the culture supernatant was precipitated using an equal volume of 2 per cent (w/v) cetylpyridinium chloride. Amount of alginate was determined using a borate/carbazole method\(^12\).

\textit{Assay of extracellular products:}

\textit{Siderophore production –} Cell free supernatants (CFS) were extracted with ethyl acetate by the method of Cox and Adams\(^13\). For pyochelin, 1 ml each of 0.5 N HCl, nitrite molybdate reagent and 1 N NaOH were added. Final volume was made to 5 ml with sterile distilled water and absorbance was read at 510 nm according to the method of Sokol\(^14\). For pyoverdin estimation, fluorescence of 3 ml of culture supernatant in 50 mM Tris HCl (pH-7.4) was measured at 460 nm while the samples were excited at 400 nm in a Gibson spectrofluorometer\(^15\).

\textit{Phospholipase C activity –} The method of Haberman and Hardt\(^16\) was employed for estimation of phospholipase C activity. Diameter of hydrolysis of egg yolk agar was measured in centimeters.

\textit{Elastase production –} Elastolytic activity was assessed on 0.1 per cent elastin (Sigma, USA) agar plates and zone of clearance was noted\(^17\).
Protease production – Proteolytic activity was measured according to the method of Woods et al. Three ml of supernatant in 10 mM Tris HCl buffer (pH 7.5) was incubated with 15 mg of Hide powder at 37°C for 1 h while being shaken vigorously. The undissolved substrate was removed by centrifugation at 3000 g for 10 min and the protease activity was determined in the supernatants at 595 nm.

Haemolysin production – Quantitative determination of both cell free and cell bound haemolysin using 2 per cent suspension of washed human erythrocytes was done following the method of Linkish and Vogt. For cell bound haemolysin, cells in stationary phase were taken. The amount of haemolysin was determined using lyophilized haemoglobin to calibrate a standard curve.

All the experiments were carried out in triplicate in two sets.

Induction of acute ascending pyelonephritis – The method of Hagberg et al. was followed for inducing acute pyelonephritis in 6-8 wk old female LACA (Swiss Webster) mice, free of bacteriuria. Bacterial inoculum (50 µl of 10⁸ cfu/ml) was introduced by the ascending route using a soft intramedic non radio-opaque polyethylene tubing with an outer diameter of 0.61 mm (Clay Adams, USA). A set of 8 mice in triplicate was used for each isolate of P. aeruginosa. Urine of mice was collected by gently pressing the abdomen of mice. It was cultured on nutrient agar and MacConkey agar plates. Plates were incubated at 37°C and quantitated after overnight incubation. On post-infection day 7, mice were sacrificed, kidneys were removed aseptically and were subjected to bacteriological and histopathological examination.

The study protocol was approved by the institutional ethics committee for animal experimentation.

Statistical analysis: Chi square ($\chi^2$) test was applied to find association between virulence traits and mouse renal colonization.

Results & Discussion

In the present study, range for production of each virulence factor was arbitrarily selected and isolates were placed in low or high producer category. It was observed that 7 (39%) isolates of P. aeruginosa showed strong hydrophobicity (>20%) (Table I). Strongly hydrophobic isolates showed an average log count of 5.47 per g of renal tissue (Table II). No significant association ($P>0.1$) was found between hydrophobicity of P. aeruginosa and its colonization in mouse renal tissue. However, in case of Escherichia coli, Topley et al. observed that isolates bearing mannose sensitive fimbriae were significantly more hydrophobic and more destructive than those bearing MR/P fimbriae or non-fimbriate organism indicating a direct correlation of extent of renal scarring with the relative surface hydrophobicity. Although Lerrer and Garber identified PA-IL and PA-IIL as the lectins of P. aeruginosa, which resembled P-fimbriae and type I fimbriae of E. coli respectively, but their role in attachment of P. aeruginosa to the uroepithelium remains to be seen.

In the present study, high alginate production (>100 µg/ml), pyochelin production (OD > 0.016) and pyoverdin production (RF > 7.5) (Table I) was observed in 8 (44%), 11 (61%) and 9 (50%) isolates of P. aeruginosa respectively (Table II). These isolates showed a log count of 3.15, 4.63 and 4.83/g of mouse renal tissue (Table II). However, no association of renal colonization with these properties was observed in mice. Similarly isolates elaborating PLC 12 (67%), elastase 11 (61%) and high protease activity of > 1.0 units/ml 9 (50%) although were able to colonize kidney tissue but the renal bacterial count was found to be low (Table II). No association of these properties with renal colonization was observed. Woods et al. also showed that relative contribution of a given factor may vary with the type of infection. Although role of extracellular products such as elastase, protease, exotoxin A and exoenzyme S have been shown in vivo studies in models of
Table I. Phenotypic expression of virulence factors of urinary isolates of *Pseudomonas aeruginosa*

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Serotype</th>
<th>Hydrophobicity (%)</th>
<th>Alginate conc. (µg/ml)</th>
<th>Pyochelin (OD at 510 nm)</th>
<th>Pyoverdin (RF at 460nm)</th>
<th>Phospholipase C (hydrolysis zone in cm)</th>
<th>Elastase (hydrolysis zone in cm)</th>
<th>Protease (units/ml)</th>
<th>Haemolysin (hemoglobin released mg/ml)</th>
<th>Log bacterial count/g of renal tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>PU-1</td>
<td>O11</td>
<td>7.7±0.2</td>
<td>107.5±1.3</td>
<td>0.030±0.005</td>
<td>23.6±1.5</td>
<td>2.9±0.1</td>
<td>1.3±0.06</td>
<td>1.19±0.09</td>
<td>0.90±0.05</td>
<td>0.67±0.03</td>
</tr>
<tr>
<td>PU-2</td>
<td>O6</td>
<td>11.7±0.4</td>
<td>108.6±1.1</td>
<td>0.014±0.001</td>
<td>3.4±1.2</td>
<td>1.0±0.09</td>
<td>0.7±0.05</td>
<td>2.51±0.1</td>
<td>0.87±0.07</td>
<td>0.60±0.04</td>
</tr>
<tr>
<td>PU-3</td>
<td>O11</td>
<td>14.3±0.5</td>
<td>107.3±1.7</td>
<td>0.017±0.002</td>
<td>12.5±1.1</td>
<td>0.7±0.08</td>
<td>1.0±0.07</td>
<td>0.47±0.08</td>
<td>0.62±0.05</td>
<td>0.40±0.03</td>
</tr>
<tr>
<td>PU-4</td>
<td>O3</td>
<td>17.3±0.3</td>
<td>39.4±0.8</td>
<td>0.009±0.001</td>
<td>3.4±1.2</td>
<td>2.2±0.09</td>
<td>1.5±0.08</td>
<td>2.44±0.2</td>
<td>0.70±0.06</td>
<td>0.50±0.05</td>
</tr>
<tr>
<td>PU-5</td>
<td>O11</td>
<td>23.7±0.2</td>
<td>106.9±1.5</td>
<td>0.021±0.004</td>
<td>22.5±1.5</td>
<td>1.1±0.08</td>
<td>-</td>
<td>0.90±0.08</td>
<td>2.55±0.09</td>
<td>2.00±0.07</td>
</tr>
<tr>
<td>PU-6</td>
<td>O4</td>
<td>11.7±0.3</td>
<td>109.9±2.1</td>
<td>0.015±0.003</td>
<td>4.5±1.1</td>
<td>-</td>
<td>-</td>
<td>0.56±0.08</td>
<td>0.50±0.07</td>
<td>0.50±0.03</td>
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<tr>
<td>PU-7</td>
<td>PT</td>
<td>15.7±0.6</td>
<td>161.0±2.5</td>
<td>0.026±0.005</td>
<td>3.0±1.2</td>
<td>0.6±0.07</td>
<td>1.9±0.05</td>
<td>11.7±0.3</td>
<td>0.52±0.06</td>
<td>0.47±0.03</td>
</tr>
<tr>
<td>PU-8</td>
<td>O4</td>
<td>22.8±0.2</td>
<td>102.7±1.3</td>
<td>0.019±0.002</td>
<td>1.2±1.3</td>
<td>-</td>
<td>-</td>
<td>0.41±0.09</td>
<td>0.50±0.05</td>
<td>0.45±0.04</td>
</tr>
<tr>
<td>PU-9</td>
<td>PT</td>
<td>19.1±0.2</td>
<td>51.0±0.9</td>
<td>0.024±0.005</td>
<td>10.4±1.7</td>
<td>-</td>
<td>-</td>
<td>0.43±0.09</td>
<td>0.47±0.05</td>
<td>0.21±0.03</td>
</tr>
<tr>
<td>PU-10</td>
<td>O4</td>
<td>5.7±0.4</td>
<td>48.6±1.1</td>
<td>0.014±0.002</td>
<td>2.3±1.9</td>
<td>0.8±0.05</td>
<td>0.3±0.04</td>
<td>4.86±0.2</td>
<td>0.70±0.07</td>
<td>0.80±0.04</td>
</tr>
<tr>
<td>PU-11</td>
<td>O4</td>
<td>21.1±0.3</td>
<td>46.4±0.9</td>
<td>0.025±0.003</td>
<td>10.2±1.5</td>
<td>1.2±0.06</td>
<td>0.7±0.05</td>
<td>5.64±0.4</td>
<td>2.60±0.09</td>
<td>0.95±0.05</td>
</tr>
<tr>
<td>PU-12</td>
<td>O1</td>
<td>31.7±0.5</td>
<td>145.4±1.8</td>
<td>0.029±0.006</td>
<td>18.4±1.3</td>
<td>2.7±0.2</td>
<td>2.9±0.08</td>
<td>2.88±0.1</td>
<td>0.58±0.05</td>
<td>0.27±0.03</td>
</tr>
<tr>
<td>PU-13</td>
<td>O6</td>
<td>27.6±0.4</td>
<td>76.4±1.2</td>
<td>0.018±0.004</td>
<td>7.4±1.1</td>
<td>3.3±0.1</td>
<td>2.3±0.09</td>
<td>2.78±0.2</td>
<td>2.96±0.08</td>
<td>0.97±0.03</td>
</tr>
<tr>
<td>PU-14</td>
<td>O11</td>
<td>20.6±0.6</td>
<td>58.1±1.1</td>
<td>0.016±0.003</td>
<td>13.6±1.3</td>
<td>-</td>
<td>1.8±0.06</td>
<td>5.04±0.5</td>
<td>0.96±0.06</td>
<td>0.70±0.04</td>
</tr>
<tr>
<td>PU-15</td>
<td>O11</td>
<td>13.2±0.4</td>
<td>34.0±0.9</td>
<td>0.021±0.002</td>
<td>2.3±1.2</td>
<td>-</td>
<td>-</td>
<td>0.37±0.06</td>
<td>3.20±0.09</td>
<td>1.75±0.06</td>
</tr>
<tr>
<td>PU-16</td>
<td>O11</td>
<td>6.7±0.3</td>
<td>49.4±1.3</td>
<td>0.004±0.001</td>
<td>3.1±1.1</td>
<td>3.0±0.1</td>
<td>1.9±0.07</td>
<td>0.72±0.07</td>
<td>3.22±0.08</td>
<td>1.80±0.07</td>
</tr>
<tr>
<td>PU-17</td>
<td>O11</td>
<td>24.7±0.4</td>
<td>87.0±1.7</td>
<td>0.025±0.005</td>
<td>30.5±1.3</td>
<td>0.7±0.05</td>
<td>-</td>
<td>0.43±0.09</td>
<td>3.59±0.08</td>
<td>0.95±0.05</td>
</tr>
<tr>
<td>PU-18</td>
<td>O11</td>
<td>17.6±0.5</td>
<td>69.4±1.4</td>
<td>0.009±0.003</td>
<td>7.7±1.1</td>
<td>-</td>
<td>-</td>
<td>0.85±0.1</td>
<td>2.98±0.09</td>
<td>0.98±0.05</td>
</tr>
<tr>
<td>PAO</td>
<td>PT</td>
<td>33.4±0.6</td>
<td>158.3±2.4</td>
<td>0.035±0.005</td>
<td>100.0</td>
<td>1.9±0.07</td>
<td>2.2±0.09</td>
<td>9.75±0.2</td>
<td>3.00±0.09</td>
<td>2.00±0.08</td>
</tr>
</tbody>
</table>

**PAO, Pseudomonas aeruginosa strain O; PT, polytypable; RT, relative florescence**

Table II. Association of phenotypic expression of virulence factors of urinary isolates of *Pseudomonas aeruginosa* with mouse renal colonization

<table>
<thead>
<tr>
<th>Virulence traits</th>
<th>Hydrophobicity</th>
<th>Alginate</th>
<th>Pyochelin</th>
<th>Pyoverdin</th>
<th>Phospholipase C</th>
<th>Elastase</th>
<th>Protease</th>
<th>Cell free hemolysin</th>
<th>Cell bound hemolysin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Number of isolates (%)</td>
<td>7 (39)</td>
<td>11 (61)</td>
<td>8 (44)</td>
<td>10 (56)</td>
<td>11 (61)</td>
<td>7 (39)</td>
<td>9 (50)</td>
<td>9 (50)</td>
<td>12 (67)</td>
</tr>
<tr>
<td>Average log bacterial count per g of renal tissue</td>
<td>5.47</td>
<td>3.85</td>
<td>3.15</td>
<td>5.54</td>
<td>4.63</td>
<td>4.23</td>
<td>4.83</td>
<td>4.12</td>
<td>4.73</td>
</tr>
</tbody>
</table>

*P<0.01 between cell free and bound haemolysin and log bacterial count/g*
corneal infection, respiratory infection and burn wounds, no reports are available on *P. aeruginosa* induced UTIs.

In the present study, 10 (56%) isolates were high producers of cell free haemolysin (>0.75 mg/ml of haemoglobin released) and 11 (61%) were high producers of cell bound haemolysin (>0.50 mg/ml of haemoglobin released) (Table I) and showed an average log count of 6.30 and 6.01 per g of renal tissue respectively (Table II). Isolates possessing high haemolytic property along with other virulence factors showed significantly high bacterial counts and marked inflammatory changes in cortex and medulla along with destruction of tubules in the mouse renal tissue (*P*<0.01) when checked after post-infection day 7 (Fig. 1). On the contrary, in case of isolates, which were low producers of haemolysin but elaborating other virulence attributes, mild inflammatory changes were observed in calyceal, cortical and medullary regions (Fig. 2). This indicated a direct association of haemolysin production with renal colonization. It was observed that majority of isolates showing high haemolytic activity and high bacterial counts in renal tissue belonged to serotype O11. Relative contribution of haemolysin production in case of urinary tract infections caused by *P. aeruginosa* cannot be ruled out wherein provirulence activity of haemolysin is also reported to be multifactorial which include release of iron from erythrocytes, disruption of phagocyte function and direct toxicity to host tissues. Similar association of haemolysin production and increased bladder colonization as well as nephropathogenicity was found in case of invasive uropathogenic *E. coli*. In a normal clinical situation all the virulence factors in conjunction may be deciding the outcome of an infection and hence all should be considered. Our results indicated that as in other infections, uropathogenicity of *P. aeruginosa* was also multifactorial. Besides considering levels of all extracellular enzymes, high levels of haemolysin production *in vitro* could be used as surrogate information for pyelonephritic potential of *P. aeruginosa*.

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**Fig. 1.** Photomicrograph revealing marked inflammation in cortical region (H & E x 100) (indicated by arrows).

**Fig. 2.** Photomicrograph showing mild interstitial inflammation in cortical region (H & E x 100) (indicated by arrows).
References


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