ABSTRACT

Background & objectives: *Pseudomonas aeruginosa* is an important nosocomial pathogen since it can survive in minimal medium. There is a global emergence of multidrug resistant strains of *Pseudomonas*. These strains are the main causes of nosocomial infections causing morbidity and mortality as these infections are difficult to eradicate. The objective of the present study was to determine the prevalence of strains of *P. aeruginosa* in hospital environment, its incidence, clinical infections caused and to detect source of nosocomial infection by characterization of the *Pseudomonas* isolates. Methods: A total of 613 strains of *Pseudomonas* were isolated from different clinical specimens. 68 strains were isolated from environmental sites like Intensive Care Unit, Operation Theatre and Wards. Strains were identified by standard methods. Antibiogram and Pyocin typing was carried out for further characterization. Genotyping was performed by Pulsed Field Gel Electrophoresis. Results: Highest percentage of *P. aeruginosa* was from urine samples, followed by pus, tracheal secretions and sputa. Prevalence of the organism was highest in Intensive Care Unit followed by Intensive Care Medical Unit. The antibiogram showed maximum sensitivity to Piperacillin/Tazobactum, followed by Carbenicillin. About 47% of the strains belonged to pyocin type 10, 2.04% belonged to type 33 and 49% could not be typed. Two PFGE patterns were found among the isolates designated as SP and SP1. Out of 47, 44 isolates showing SP pattern were of pyocin type 10 and resistant to Sepran, Lomefloxacin, Colistin, PolymyxinB, Ampicillin and Augmentin. The rest 3 isolates showing SP1 pattern were resistant to Gentamycin, Amikacin, Netilmycin, Carbenicillin, Ciproflloxacin and Chloramphenicol in addition to those mentioned earlier. Interpretation & conclusions: Majority of clinical and environmental strains showed a single PFGE pattern and a similar antimicrobial susceptibility pattern. Thus it is presumed that the strains of *P. aeruginosa* have a common origin. Out of 545 strains, 221 were isolated from post-operative cases. It can be concluded that patients acquired these infections from the hospital.

KEY WORDS: Antibiogram, Pyocin typing, Pulsed Field Gel Electrophoresis.

1. INTRODUCTION

Nosocomial or hospital acquired infections are caused by a wide variety of pathogens; including *Pseudomonas aeruginosa*, *Klebsiella* spp., *Escherichia coli*, *Enterococci*, *Staphylococci* etc. *Pseudomonas aeruginosa* is an important nosocomial pathogen. This organism is a gram-negative motile bacillus, which is invasive, toxigenic & often multi-drug resistant, leading to complications during treatment. It causes infections of wounds, burns, iatrogenic meningitis, urinary tract infections, pneumonia etc. Strains of *Pseudomonas aeruginosa* produce pyocin, which has characteristic property and can be used for typing of strains. The worldwide emergence of multidrug resistant bacterial strains is of growing concern, especially in nosocomial infections caused by *P. aeruginosa*. These infections are difficult to eradicate due to resistance to many antimicrobials, thus major cause of morbidity and mortality, leading directly and indirectly to an enormous increase in cost of hospital stay for the patients and also emergence of new health hazards for the community.

2. MATERIALS AND METHODS

2.2. Sample collection for the study: Multiple environmental swabs were collected from various sites of Intensive Care Units.
(ICU), Operation theatres (OT) & Wards. Sites included patient’s table, trolley, sink, wall, floor, oxygen & suction tubings, A/C filters, etc. Swabs were also collected from different instruments like endoscopes, bronchoscopes, stethoscopes and ventilators. Cleaning solutions, disinfectants, detergents, mops, etc were also screened for *P. aeruginosa*. Hand swabs, throat swabs and nasal swabs from attending staff were collected and processed. Strains of *P. aeruginosa* isolated from different clinical specimens were collected from the Dept. of Pathology.

2.3. Morphological identification of various isolates of *Pseudomonas*:
Strains were identified by standard methods like morphology, colony characteristics, O/F test, gelatine liquefaction and oxidase test.

2.4. Antibiotic Sensitivity Testing:
Antibiotic susceptibility tests were carried out by disk diffusion technique according to CLSI guidelines [5]. Mueller Hinton Agar was used for growing the lawn of culture of the strains by swabbing the culture onto the agar plate. Different antibiotic discs are then placed equidistant and the plate is incubated at 37°C for 24 hrs. (Bauer et al, 1966) [6].

Following antibiotic discs were used:
- Ampicillin (10μg), Augmentin (30μg), Cefoperazone (75μg), Cefotaxime (30μg), Cefazidime (30μg), Ceftizoxime (30μg), Ceftriaxone (30μg), Gentamycin (10μg), Amikacin (30μg), Septran (25μg), Netilmix (30μg), Ciprofloxacin (5μg), Piperacillin (100μg), Piperacillin / Tazobactum (100/10μg), Ticarcillin / Clavulanic acid (75/10μg), Carbenicillin(100μg), Tobramycin (10μg), Levofloxacin (5μg), Lomefloxacina (10μg), Colistin (5μg), Imepenem (10μg), Chloramphenicol(30μg), Polymixin-B(10μg), Cefoperazone / Sulbactum (30/10μg).

2.5. Pyocin Typing for characterization of strains:
A total of 245 strains were typed employing Gillies and Govan Pyocin Typing Technique [1]. The strain to be typed was streaked diamentrically across the surface of Tryptone Soya Agar to give an inoculum width of approximately 1 cm. After incubation for 14-18hrs at 32°C, the growth was exposed to chloroform for 15 mins. The plate was then opened and the traces of chloroform vapour were eliminated by exposing the medium to air for few minutes & then the culture was scrapped out with the help of a sterile slide. Cultures of the eight standard indicator strains were grown under static conditions in Nutrient broth (Oxoid No. 2) for 4hrs at 37°C & were then streaked on to the medium by means of a loop inoculator at right angles to the line of the original inoculum, starting from the original test strain growth area. Indicator strains no: 1-5 were applied on the left side of the plate and strains no: 6-8 were on the right side. A control strain was also streaked along with the test strains. The plate was then incubated at 37°C for 18hrs. Plates were observed for growth inhibition of indicator strains. Inhibition of the test cultures greater than the control was defined as positive. Pyocin type was determined as per the protocol.

2.6. Pulse-Field Gel Electrophoresis (PFGE):
Cells from an overnight culture of *P. aeruginosa* in Luria Bertanii (LB) broth were harvested by centrifugation and resuspended in a cell suspension buffer containing Tris, EDTA and NaCl (pH 7.2). A 0.5 ml aliquot of this suspension was added in 0.5 ml of molten 2% low-melting Agarose (Sea Plaque, Rockland) made in Milli-Q™ water and equilibrated to about 50°C. This agarose embedded culture was then pipetted into plug molds (Bio-Rad Laboratories) and allowed to solidify. Each plug was then
subjected to lysis with a buffer containing lysozyme (1mg/ml) and incubated at 37°C for 1 hr. Following incubation, the lysis buffer was replaced with Proteinase K buffer and further incubated for 48 hr at 50°C. After incubation, the plugs were washed with 0.1 X TE and subjected to overnight digestion with Spe I restriction endonuclease at 37°C. After digestion, plugs were washed in dilute TE and sliced using a sterile blade before loading onto the gel.

The digested DNA was electrophoresed in a 1.2% Agarose- 0.5X TBE- gel with a CHEF-DR® III apparatus for 24.4 h at 6V with switching times ramped from 2 – 60s at 14°C. After electrophoresis, the gels were stained with ethidium bromide (EtBr) solution (1mg/ml) for 1 hour and destained with distilled water for 4 hours and the banding patterns were viewed using the UVI Band/Map V.99 software.

The criteria of Tenover et al [2, 3] were used to interpret the PFGE banding patterns. Isolates were marked genetically indistinguishable or identical, if their restriction patterns demonstrated the same number of bands with the same apparent size, these were designated as genomic pattern SP. Isolates were considered to be closely related if their PFGE patterns showed two to three band differences, consistent with a single genetic event, and were classified as type SP1. Isolates were possibly related if their PFGE patterns showed four to six band differences, consistent with two independent genetic events and these were to be classified as type SP2.

3. RESULTS:
3.1. Prevalence and incidence of strains of P. aeruginosa leading to nosocomial infections:
A total of 613 strains of P. aeruginosa were collected, out of which 545 strains were isolated from clinical samples e.g. urine, tracheal secretion, pus, blood, sputum, catheter tip and other body fluids. 68 strains were isolated from environmental sites like from various sites of ICU, operation theatres and wards. Among the isolates from clinical specimens, 211 strains were isolated from post-operative cases.

Among the clinical isolates, highest percentage was from urine, followed by pus, tracheal secretions and sputum (Table I); Fig I illustrates percentage of infection at different sites. Incidence was found to be more in male than in females except for gastrointestinal tract. Prevalence of Pseudomonas aeruginosa at different sites was evaluated and was found to be highest in ICU followed by IMCU (Fig. II). Strains of P. aeruginosa were detected from suction apparatus as well as sinks (Fig.III).

3.2. Interpretation of Antimicrobial Susceptibility test:
A total of 613 isolates were tested for sensitivity to various antibiotics. The Antibiogram (Fig.IV) shows maximum sensitivity to Piperacillin/Tazobactum (Pt) [55.8%], followed by Carbenicillin (Cb). The isolates were assigned to 3 antibiotic profiles based on their susceptibility to the mentioned 23 antibiotics. Overall, out of 613 isolates, 382 (62.3%) strains showed resistance to Septran, Lomefloxacin, Colistin, Polymixin B, Ampicillin and Augmentin (Profile A), 222 (36.2%) strains showed resistance to Ampicillin, Augmentin, Gentamycin, Amikacin, Carbenicillin, Netilmicyn, Ciprofloxacins, Septran, Lomefloxacins and Chloramphenicol (Profile B), and 9 (1.47%) Strains showed resistance to Ampicillin, Augmentin, Gentamycin, Carbenicillin, Netilmicyn, Septran, Lomefloxacins, Chloramphenicol, Ciprofloxacins, Levofloxacins and Tobramycin (Profile C) [Table-II].
Table -1
Percentage of occurrence of *P. aeruginosa* in clinical specimens

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Specimen</th>
<th>% Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Urine</td>
<td>31</td>
</tr>
<tr>
<td>2</td>
<td>Pus</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>Tracheal secretion</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>Sputum</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>Wound</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>Fluids</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>Swabs</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>Tips</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>Blood</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>Secretions</td>
<td>3</td>
</tr>
<tr>
<td>11</td>
<td>Others</td>
<td>1</td>
</tr>
</tbody>
</table>

Table -2
Antibiotic resistance profile of *Pseudomonas aeruginosa* strains isolated from clinical and environmental specimens

<table>
<thead>
<tr>
<th>S.No</th>
<th>Profile</th>
<th>Resistance to antibiotics</th>
<th>No. of strains</th>
<th>Percentage frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>Septran, Lomefloxacin, Colistin, PolymixinB, Ampicillin &amp; Augmentin</td>
<td>382</td>
<td>62.3%</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>Ampicillin, Augmentin, Gentamycin, Amikacin, Carbenicillin, Netilmicin, Ciprofloxacin, Septran, Lomefloxacin &amp; chloramphenicol</td>
<td>222</td>
<td>36.2%</td>
</tr>
<tr>
<td>3</td>
<td>C</td>
<td>Ampicillin, Augmentin, Gentamycin, Carbenicillin, Netilmicin, Ciprofloxacin, Septran, Lomefloxacin, Chloramphenicol, Levofloxacin &amp; Tobramycin</td>
<td>9</td>
<td>1.47%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>TOTAL</strong></td>
<td><strong>613</strong></td>
<td></td>
</tr>
</tbody>
</table>

Table III.
Pyocin types of *Pseudomonas aeruginosa* from clinical and environmental specimens

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Pyocin Type</th>
<th>No. of Strains</th>
<th>Percentage frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P10</td>
<td>115</td>
<td>47</td>
</tr>
<tr>
<td>2</td>
<td>P33</td>
<td>5</td>
<td>2.04</td>
</tr>
<tr>
<td>3</td>
<td>P97</td>
<td>2</td>
<td>0.8</td>
</tr>
<tr>
<td>4</td>
<td>Others (6, 55, 1, 92, 18, 95, 99, 3, 26, 72, 102, 20)</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>5</td>
<td>Non-Pyocinotypable</td>
<td>122</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td></td>
<td><strong>245</strong></td>
</tr>
</tbody>
</table>
Table IV.
Comparison of Pyocin type and PFGE pattern showed by *Pseudomonas aeruginosa* strains from clinical and environmental specimens

<table>
<thead>
<tr>
<th>Type of specimen</th>
<th>Clinical</th>
<th>Environmental</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyocin type</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>PFGE pattern</td>
<td>SP, SP1</td>
<td>SP SP1 SP SP1 SP SP1 SP SP1 SP SP1</td>
</tr>
<tr>
<td>Number of isolates</td>
<td>27a</td>
<td>3a 2b 1a 13a 1a</td>
</tr>
</tbody>
</table>

a : isolates resistant to septran (co-trimazole), lomefloxacin, colistin, polymyxin B, ampicillin and augmentin

b : isolates resistant to gentamycin, amikacin, netilmicin, carbenicillin, ciprofloxacin and chloramphenicol in addition to those mentioned in a

Fig I.

Percentage rate of infection in different clinical sites

Fig. II

Prevalence of *P.aeruginosa* in different Intensive Care Units (ICUs).
Fig. III

Prevalence of *P. aeruginosa* at different sites and instruments.

![Graph showing prevalence of *P. aeruginosa* at different sites and instruments.](image)

HOSPITAL SITES OF INSTRUMENTS

Fig IV.

Antibiogram of *Pseudomonas aeruginosa* isolated from both clinical and environmental specimen

![Antibiogram graph showing % sensitivity of various antibiotics.](image)

Ampicillin (A), Augmentin (Ag), Cefoperazone (Cs), Cefotaxime (Ce), Ceftazidime (Ca), Ceftizoxime (Ck), Ceftriaxone (Ci), Gentamycin (G), Amikacin (Ak), Septran (Co), Netilmicyn (Nt), Ciprofloxacin (Cf), Piperacillin (P), Piperacillin/Tazobactum (Pt), Ticarcillin/Clavulanic acid (Tc), Carbencillin (Cb), Tobramycin (Tb), Levofoxacin (Le), Lomefloxacin (Lo), Colistin (Cl), Imepenem (I), Chloramphenicol (C), Polymixin-B (Pb),
Fig.V.
PFGE patterns of SpeI digests of genomic DNA from Pseudomonas aeruginosa isolates from samples from patients and of hospital origin.

3.3. Identification of pyocin types:
A total of 245 strains were typed and assigned to Type 10, 33, 97 and others. 115 out of 245, (47%) belonged to Type 10, 2.04% type 33 and 122 (49%) could not be typed (non-pyocinotypable) [Table III].

3.4. Analysis of Pulse-Field Gel Profiles:
A total of 47 isolates were subjected to DNA macro-restriction analysis by Pulse-field Gel Electrophoresis. Analysis of PFGE profiles was performed by visual inspection of photographs of EtBr stained-gels. The patterns of the bands were compared. Once isolates were recognized having identical patterns, a representative of the group was used to compare its pattern with similar isolates in respect to their antibiotic profile or pyocin type. Procedure was repeated and reproducibility was established. All isolates were digested with SpeI and the PFGE patterns were visually compared (Fig.VA & VB). A and B show representative PFGE patterns of DNA digested with SpeI from isolates taken from clinical specimen and hospital ICUs respectively.

Totally two patterns were found among the isolates and designated as SP and SP1. SpeI digest yielded patterns of 8 fragments of 4.36kb to 97.0kb (Pattern SP) and another subtype showing difference in one band size (kb) (Pattern SP1). Pattern SP consisted of majority isolates and was found to be the dominant type in the hospital, which was found in both clinical (30 out of 47) and
environmental (14 out of 47) samples. Isolates showing PFGE pattern SP1 were interpreted to be closely related to those of pattern SP and were prevalent only in clinical samples (3 out of 47). All the isolates (44 out of 47) showing SP pattern for PFGE were of the pyocin-type 10 and resistant to Septran (co-trimoxazole), Lomefloxacin, colistin, polymyxin B, ampicillin and Augmentin, whereas the rest 3 isolates, showing SP1 pattern were resistant to Gentamicin, Amikacin, Netilmicin, Carbenicillin, Ciprofloxacin and Chloramphenicol in addition to those mentioned earlier (Table IV).

4. DISCUSSION

Typing of strains is essential for determining the epidemiology of nosocomial infections and aiding in the design of rational pathogen control methods. Earlier biotyping, antibiotyping, pyocino typing and other phenotyping methods were used for this purpose. In the present era genotyping methods like PFGE, ribotyping, PCR and Nucleotide Sequence based analysis like SLST and MLST gained considerable importance due to high specificity & sensitivity.

The present study was carried out over the period of 2002-2007 for a survey of incidence of nosocomial infections caused by Pseudomonas aeruginosa at Sir H.N. Hospital & Research Center Mumbai, India. A total of 613 strains of P. aeruginosa were isolated from clinical specimens from infected patients and hospital environment, out of which 545 strains were from clinical specimens and 68 strains were from hospital environment. Among the clinical specimens, P. aeruginosa infections were most common in urinary tract infections followed by respiratory tract and wound infections. Intensive care patients are more prone to infection because of the debilitating effect of a prolonged hospitalization and instrumentation [12]. In this study, the highest incidence of P. aeruginosa (28.35%) was found in ICU, which was followed by IMCU (26.86%), NICU (16.41%), IPCU (14.92%), and ICCU (13.43%). ICUs are generally considered epicenters of multidrug-resistant (MDR) organisms. The most important risk factors are excessive use of antibiotics exerting selective pressure on bacteria, the frequent use of invasive devices and relative density of immuno-suppressed patient population with severe underlying diseases [13].

Nowadays, prevalence of MDR strains of P. aeruginosa are seen mainly in hospital acquired infections due to the selective pressure exerted on the bacteria by over-usage of broad-spectrum antibiotics. In a study carried out in Turkey, Inan et al. [14] isolated 60-83% multidrug-resistant Pseudomonas aeruginosa strains from ICU patients. These strains were resistant to Ceftazidime (34%), Imipenem (26%), Gentamicin (67%), and Amikacin (26%). In another survey in Italy Bonfiglio et al, (1998) [15] reported that Meropenem followed by Amikacin was most effective drug against Pseudomonas infections. In Spain, Bouza et al (1999) [16] found that isolates from their intensive care units were more resistant to Aztreonam, Cefepime, Ceftazidime, Imipenem, Ticarcillin, piperacillin, and piperacillin/tazobactam than those from other clinical settings; isolates from their indoor patients were significantly often resistant to Ceftazidime, Imipenem, and Meropenem; and isolates from their outdoor patients were more often resistant to ciprofloxacin than were nosocomial isolates. Resistance to aminoglycosides is higher in Southern Europe than in Central and Northern Europe [17]. Reports of the susceptibility of
*Pseudomonas aeruginosa* to Gentamicin and Tobramycin have ranged from 49.8% to 77.7% in Greece, to as high as 96.6% to 99.2% in the United Kingdom [17]. Previous studies reported that antipseudomonas effects of Amikacin were greater than those of Gentamycin [18, 19].

In the present study, however, the rate of susceptibility to aminoglycosides was found to be variable. (susceptibility to Amikacin; 27.6%, Netilmycin; 25.4%, and Gentamycin; 23.2%) and to Tobramycin (51.4%). Lowest rate of susceptibility was observed for Ampicillin (4%), Co-trimazole (9.4%) and Augmentin (9.6%), whereas the combination of Piperacillin/Tazobactum was found to be the most effective (55.8%), followed by Carbenicillin (53.8%). Among broad-spectrum Cephalosporins, Cefotaxime (52.8%), Cefoperazone (45.4%) and Cefoperazone/sulbactum (46.6%) were found to be moderately effective. In general, this pattern was exhibited by most of the isolates. In this context the incidence of resistance to an antibiotic was dependent on the origin of the strains, probably reflecting the patterns of antibiotic usage in the hospital.

Pyocin typing of 245 strains yielded 3 major types. Type10 was most prevalent followed by type 33 and 97 with isolation frequencies of 47%, 2.4% and 0.8% respectively. Out of 245 strains, 122 could not be typed by Govan’s [1] method. Other authors [20, 21] also reported pyocino-type 10 as the frequently occurring strains of *P. aeruginosa* worldwide.

Analysis of PFGE profiles of the 47 isolates from the phenotypically typed strains revealed two patterns (SP & SP1). SP1 as the subtype of the other SP. Out of 44 isolates showing pattern SP, 27 clinical strains and 13 environmental strains were of the same pyocino-type (i.e. type 10), 3 clinical strains were of type 33 and 1 environmental strain was of type 97 (Table.3). All of these 44 isolates showed similar antibiotic susceptibility pattern. Pattern SP1 was shown by only 3 isolates (2 of pyocino-type 33 and 1 of pyocino-type 97) suggesting that they are probably the mutants of the isolates showing pattern SP with an enhanced capacity of resistance to a broader range of antibiotics.

Since majority of clinical and environmental strains showed a single PFGE pattern and a similar antimicrobial susceptibility pattern, it can be concluded that the strains of *Pseudomonas aeruginosa* isolated from both clinical and environmental specimens of the hospital have a common origin. Moreover, 221 clinical strains out of 545 cases were isolated from post-operative cases. Thus, it can be concluded that pseudomonas infection among patients was of nosocomial origin. The results obtained in this study show that it is necessary to focus on tracing the source of infections to control & prevent nosocomial infections.

5. REFERENCES


