Imipenem Resistance Mechanisms in Multidrug Resistant Pseudomonas aeruginosa clinical isolates from south India

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Abstract
This study reports the resistance profiles of 45 P. aeruginosa isolates from 85 burn patients (52.94%). Among them, 27(60%) of the isolates showed multidrug resistance to more than eight antibiotics and 14 (30%) isolates were found to be imipenem resistant (IMRPA). All the 45 P. aeruginosa strains screened showed resistance to ciprofloxacin, ceftazidine, cefepime and piperacillin. 96% gentamycin, 44% to tobramycin, 25% amikacin and 40% aztreonam.

However, low range of resistance was observed to imipenem 30%, ticarcillin (20%) colistin (12%) and polymyxin B 14%. Genotype examination revealed that the multidrug isolates produced the integron located integrase gene-specific (intI3) and acc(6')-Ib gene-specific primers found to be well conserved among blaIMP-positive strains. The P. aeruginosa isolates were separated into two genotypes: genotype A had high prevalence (12 strains) than genotype B (3 strains). Routine detection of metallo beta lactamase in vitro testing along with antibiotic use may help in prevention and treatment of burn patients infected with metallo beta lactamase producing P. aeruginosa.

Keywords: P. aeruginosa, imipenem, metallo beta lactamase, TEM.

Introduction
Burn patients are at high risk for infection because of the immunocompromising effects, prolonged hospital stays and intensive diagnostic and therapeutic procedures. Infection is the most common cause of death following burn injury. P. aeruginosa is a rapidly emerging nosocomial pathogen and causes severe infections that include bacteremia, pneumonia, meningitis, urinary tract and wound infections.

P. aeruginosa colonization of severe burn wounds and its rapid proliferation within the damaged tissues often leads to disseminated infections resulting in bacteremia and septic shock3 and high rates of mortality and morbidity. The innate and acquired resistance of P. aeruginosa to many antimicrobials complicates treatment of such infections16. It has been estimated that at least 50% of all deaths caused by burns are the result of infection and untreatable infections have become a tragically frequent occurrence in patients infected with P. aeruginosa.

Unfortunately, this pathogen has been developing increased resistance to antimicrobial agents5. Due to their broad spectrum of activity and stability to hydrolysis by most beta lactamases, the carbapenems have been the drug of choice for treatment of infections caused by penicillin or cephalosporin-resistant gram-negative bacilli especially extended spectrum β-lactamase (ESBL) producing gram-negative infections17. Since the first isolation of plasmid mediated metallo beta lactamase from P. aeruginosa in 1991,27 increasing rates of metallo beta lactamase (MBL) P. aeruginosa producing strains have become a serious problem. Such strains are resistant to multiple antibiotics as they hydrolyze all beta-lactams and are insensitive to clinically available inhibitors like clavulanic acid4.

Carbapenem, mainly imipenem had been considered the useful agent for the treatment of infections. However, numerous outbreaks caused by multidrug resistant (MDR) P. aeruginosa from different parts of India are appearing very rapidly. Recently, resistant strains producing metallo beta lactamase have been recovered from ICU patients in India9. Despite increased susceptibility of patients to bacterial infections is well corroborated, the long-lasting illness associated with these infections remains poorly understood.

Our phenotypic analysis clearly demonstrated that P. aeruginosa isolates obtained were resistant to all clinically significant antibiotics including carbapenems (imipenem). Truly little finite information is available on the molecular epidemiology of P. aeruginosa clinical isolates in Gulbarga. In the present study, the aim is to investigate the antimicrobial resistance profiles, virulence determinants profiles and molecular characteristics of P. aeruginosa isolates.

Material and Methods
Bacterial strains: Forty-five P. aeruginosa isolates were obtained from burnt wound patients between April – December 2011 from Dist. Government hospital and diagnostic center. They were identified to species level as per Gilardi Scheme. For each study, an overnight culture was
diluted in fresh BHI broth and further incubated to ensure exponential growth conditions.

**Antimicrobial susceptibility testing:** Testing was performed in accordance with the guidelines established by the Clinical and Laboratory Standards Institute with the Kirby-Bauer method using nine antibiotic discs including: gentamicin, amikacin, tobramycin, ceftazidime, cefepime, imipenem, ticarcillin, pipercillin, aztreonam, colistine, polymixin-B and ciprofloxacin (Hi-media, Mumbai) on Mueller Hinton agar medium. The plates were incubated at 37°C for 24 h to check the zone of inhibition. Bacterial strains that demonstrated resistance to three or more categories of antibiotics were defined as MDRP. The P. aeruginosa ATCC 27853 strain were adopted as the standards for control.

**MIC:** MICs were determined on plates of Mueller-Hinton broth containing serial two-fold dilutions of each antibiotic. Bacterial suspensions of 10^4 colony-forming units (CFU)/mL were inoculated onto the surface of the plates and results were recorded after overnight incubation at 35°C in an aerobic atmosphere. The MIC was defined as the lowest antibiotic concentration with no visible growth.14

**Detection of MBL’s:** MBL producing P. aeruginosa was suspected when the isolate was resistant to ceftazidime and imipenem. Various methods have been recommended for screening MBL. These include the modified Hodge test, double disc synergy test using imipenem and EDTA discs or ceftazidime and EDTA discs, EDTA impregnated imipenem discs and EDTA impregnated meropenem discs.

We used zone enhancement with EDTA impregnated imipenem discs for phenotypic determination of MBL’s. Test organisms were inoculated on to plates with Mueller Hinton agar as recommended by the NCCLS. A 0.5 M EDTA solution was prepared by dissolving 186.1 g of disodium EDTA, 2H_2O in 1000 ml of distilled water and adjusting it to pH 8.0 using NaOH. The mixture was sterilized by autoclaving. EDTA solution was added to ceftazidime discs to obtain a desired concentration of 750 µg.

The EDTA impregnated antibiotic discs were dried immediately in an incubator and stored at -20°C in airtight vials. Then, 10 µg imipenem discs (with and without EDTA) were placed on the surface of an inoculated agar plate. The inhibition zones of imipenem and imipenem EDTA discs were compared after 18-18 h of incubation in air at 35°C. Strains with enhancement zone in imipenem EDTA discs were recognized as MBL producing P. aeruginosa.15

**Preparation of chromosomal DNA:** Cells from an overnight culture in BHI broth collected by centrifugation were suspended in lysis buffer (Phosphate-buffered [PBS] containing 1% sodium dodecyl sulfate [SDS] and 100 µg/ml Proteinase K. The cell suspension was incubated at 37°C for 1 hr and equal volume of phenol: chloroform (1:1) mixture was added to cell suspension and vortexed. The samples were centrifuged and aqueous phase was transferred to fresh tube. The DNA was precipitated by adding 100µl of 3M sodium acetate and 3 vol of cold absolute alcohol, air-dried and suspended in 50 µl of TE buffer [10mM Tris -HCl [pH 8.0], 0.1mM EDTA].

**PCR for detection of AME, intI3 and IPM gene:** The presence of the bladMP gene and intI3 gene amplification was done using the primers (Table 1). Amplification was also done to check the presence of aminoglycoside modifying gene aac(6’)-Ib gene with oligonucleotide primers selected from published sequences. The total reaction volume (50.0 µl) consisted of template DNA 1.0 µl, PF (100ng/ml), PR (100ng/ml), 2.0 µl 1 of dNTP mix (2.5mM each), 10X ChromTaq Assay buffer 5.0 µl, 0.5 µl of ChromTaq enzyme (3U/ml), distilled water 37.5 µl.

The PCR conditions were performed as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1 min and DNA extension at 72°C for 1.5 min. After agarose gel electrophoresis, the ethidium bromide-stained PCR products were visualized under UV light. In the amplification of the intI3 gene, the annealing step was carried out at 57°C.

**Electron Microscope:** Ten ml of Imipenem resistant *Pseudomonas aeruginosa* liquid culture was sedimented by centrifugation at 8000 rpm for 10min at 4°C, the supernatant fluids were discarded and the cell pellets were washed twice with 50mM potassium phosphate buffer (pH 7). Bacteria were then fixed in 3% glutaraldehyde (in 0.1M sodium phosphate buffer, pH 7.2) for overnight at 4°C followed by 1% osmium tetra oxide for 1hr at 4°C. For contrast amplification, the cells were treated with 2% uranyl acetate in 95% alcohol for 1hr at 20°C in the dark. Cells were subjected to infiltration with araldite propylene oxide (1:1) solution for four times. Cells were dehydrated with 70% ethanol for 18hrs and embedded in acrylic resin (TAAB Laboratory, UK).

Ultra-thin sections were prepared with an ultra-microtome of 400-500 A° (Lieca EM UE 6), stained with uranyl acetate and lead citrate and examined with a Transmission electron microscope (Techn G2 Spirit TEM, at 80 kV). Cell wall thickness was calculated using photographs taken at a magnification of x80, 000. To measure the cell wall thickness, 30 individual bacterium per population were considered and results were considered and results were expressed as the mean ±SD. Statistical calculations were made using Parametric Statistic program, version 1.01 (Lundon Software, Inc., Chagrin Falls, OH, USA).

**Genotyping:** Clonal distribution between the strains was studied by enterobacterial repetitive intergenic consensus (ERIC)-PCR genotyping; ERIC2-PCR amplification with the primer was performed using previously described primer by Versalovic et al. in a total volume of 20µl amplification
buffer (10 mM Tris–HCl, pH 9.0, 1.5 mM MgCl₂, 50 mM KCl and 0.01% Gelatin), containing 0.25mM of each dNTP, 20 picomoles of primer, 25 ng of template DNA and 1U / reaction of Taq DNA polymerase. The thermal cycler was programmed for 35 cycles of 1 min at 94 °C, 1 min at 53 °C and 2 min at 72 °C with 8 min final extension period. Minor differences in band intensity as well as the weak bands were not considered to define the types.

Results

Antibiotic susceptibility profile among P. aeruginosa isolates: From the 85 burn wound samples, 45 P. aeruginosa were isolated. Based on the resistance to antibiotics, isolates were classified into MDR, ARPa and IMPa. Out of 45 strains, 27 were found to be MDR and were found to be MDR (60%) and 55% were found to be ARPa and 30% were found resistant when tested for IMP. It was also notable that all the isolates were susceptible to ticarcillin (80%, n=36), colistin (88%; n=40) and polymyxin B (86%; n=38).

MIC’s: The MIC’s of imipenem varied slightly and were in the range of 2-64 μg/ml; for 4 isolates it was 16 μg/ml; for 3 isolates it was 32 μg/ml whereas for 3 strains it was 64 μg/ml. All the strains had MIC’s of 32 μg/ml-2048 μg/ml range for gentamycin. MIC’s of ceftazidime resistant isolates ranged from 32-256 μg/ml

Detection of MBL’s: Of the 45 isolates of P. aeruginosa, 10 isolates (20%) were found resistant to carbapenems (imipenem) and 20 (44%) were found to be MBL producers confirmed by disc potentiation method. The P. aeruginosa ATCC 27853 did not exhibit any zone size enhancement with EDTA impregnated imipenem discs.

Distribution of Genes: All the high-level aminoglycoside resistant isolates were positive for PCR amplification to aac(6')-Ib gene genes in their genome. The intI3 gene was detected in 8 of the 10 blalMP positive P. aeruginosa strains. The sizes of the amplified products for blalMP, aac(6')-Ib and intI3 were approximately 500bp, 300bp and 700bp respectively for the strains tested (Table 2).

Transmission electron microscopy: The transmission electron microscopic observation revealed the effect of imipenem on the cell size of P. aeruginosa. The cell diameter of the control strain and ATCC was 0.39 and 0.41μm respectively (Fig. 1A and 1B) whereas the imipenem resistant had a cell diameter of 0.43 μm (Fig. 1C) which was comparatively higher to the Imipenem susceptible strain which had a diameter of 0.40 μm (Fig. 1D). It was also noted that imipenem induced peeling of the outer membrane leading to an increased irregularity of the cell surface (Table 3).

![Fig. 1: Transmission electron micrographs of strains, A-Control strain, B- ATCC control strain, C- IMP resistant strain and D- IMP susceptible strain, Bar=100nm](image-url)
Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward sequence</th>
<th>Backward sequence</th>
<th>Base pair (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>blaIMP</td>
<td>CTACCGCAGACAGATCTTTG</td>
<td>AACCAGTTTTGCTTACCAT</td>
<td>587</td>
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<tr>
<td>intI3</td>
<td>GCAGGGTGTTGAGAACATACG</td>
<td>ACAGACGAGAAGCTTTATG</td>
<td>760</td>
</tr>
<tr>
<td>aac(6')-Ib</td>
<td>TATGAGTGCGTAAATCGAT</td>
<td>CCGCTTTTCGCTAGCA</td>
<td>395</td>
</tr>
</tbody>
</table>

*bp, size in pairs of expected amplicons.

Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Antibiotic pattern</th>
<th>blaIMP</th>
<th>intI3</th>
<th>aac(6')-Ib</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA1</td>
<td>Pc, Cp, Ca, Cf, G, Tb</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PA2</td>
<td>Pc, Cp, Ca, Cf, G, Ak, Tb, I, Pb, Ao, Ti</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PA3</td>
<td>Pc, Cp, Ca, Cf, G, Ak, Tb, Pb, I</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PA4</td>
<td>Pc, Cp, Ca, Cf, G, Ak, Pb, Ti</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PA5</td>
<td>Pc, Cp, Ca, Cf, G, Ak, Tb, Ao, Ti, Cl1</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PA6</td>
<td>Pc, Cp, Ca, Cf, G, Ak, Tb, Pb, Ao, Ti, Cl, I</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PA7</td>
<td>Pc, Cp, Ca, Cf, G, Tb, Pb</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PA8</td>
<td>Pc, Cp, Ca, Cf, G, Ak, Pb, Ao, Ti, Cl, I</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PA9</td>
<td>Pc, Cp, Ca, Cf, G, Ak, Pb, Ao, Ti, Cl, I</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PA10</td>
<td>Pc, Cp, Ca, Cf, G, Ak, Pb, Ao, Ti, Cl, I</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PA11</td>
<td>Pc, Cp, Ca, Cf, G, Ak, Ao, Ti, Cl, I</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PA12</td>
<td>Pc, Cp, Ca, Cf, G, Ak, Ao, I</td>
<td>+</td>
<td>+</td>
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<tr>
<td>PA13</td>
<td>Pc, Cp, Ca, Cf, G, Ak, Ao, I</td>
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<td>+</td>
<td>+</td>
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<td>PA14</td>
<td>Pc, Cp, Ca, Cf, G, Ak, Ao, I</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PA15</td>
<td>Pc, Cp, Ca, Cf, G, Ao</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Tobramycin (Tb), Gentamycin (G), Amikacin (Ak), Ticarcillin (Ti), Piperacillin (Pc), Cefepime (Cp), Ceftazidime (Ca), Imipenem (I), Aztreonam (Ao), Colistin (Cl), Polymyxin - B (Pb), Ciprofloxacin (Cf)

Table 3

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cell growth (%)</th>
<th>Cell diameter in µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>A- Strain Control</td>
<td>100</td>
<td>0.39±0.003*</td>
</tr>
<tr>
<td>C- IMP resistant</td>
<td>87</td>
<td>0.43±0.040</td>
</tr>
<tr>
<td>B- ATCC 27853</td>
<td>100</td>
<td>0.41±0.03</td>
</tr>
<tr>
<td>D- Imp Susceptible</td>
<td>2</td>
<td>0.40±0.02</td>
</tr>
</tbody>
</table>

*Each mean value was the average of the size of 30 cells based on the transmission electron microscopy photos

Genotyping: From the multidrug resistant P. aeruginosa isolates, two visually different genotypic patterns were generated. The amplicon size was in the range of 200-2650bp. Amongst the 15 multidrug resistant P. aeruginosa isolates, 12 isolates exhibited one genotype and were monomorphic while 3 isolates exhibited polymorphism and was classified into second genotype.

Discussion

It is well known that P. aeruginosa is the predominant opportunistic pathogen particularly in the burn patients. Several immune deficits have been described among burns patients including impaired cytotoxic T lymphocyte response, myeloid maturation arrest causing neutropenia, impaired neutrophil function and decreased macrophage production. In India, it has been found that 31.84% of infection in burn patients is predominant by P. aeruginosa along with Klebsiella and Staphylococcus Sp*[13]. It is also reported that 96% of the P. aeruginosa isolates from burn wounds were resistant to four or more antibiotics*21.

The study presented here describes the hetero resistant P. aeruginosa isolates producing the carbapenem hydrolyzing metallo beta lactamase, which has been identified as sources of nosocomial outbreaks worldwide including India. The isolation frequency of imipenem-resistant strains of P. aeruginosa is speculated to have been increasing. In the present study, 53% of the burn wounds are infected with P.
aeruginosa infection. The percentage of multi drug resistant strains is significantly high (86%) in comparison to 40-70% reported earlier.13,23

The subinhibitory antibiotic concentration in wounds due to the administration of an inappropriate dosage of antibiotic or the regular administration of aminoglycoside in combination with beta lactam provides optimal conditions for the selection and persistence of multidrug resistant P. aeruginosa strains and their subsequent local invasion and hematogenous dissemination in infected wound patients.22

In this study imipenem resistance by Kirby-Bauer method showed that 30% of the P. aeruginosa were IMPRA with the MIC’s in the range of 2-64 μg/ml; the difference in the level of imipenem resistance was among the isolates which harboured the metallo beta lactamases gene. Four strains showed moderate resistance (MIC’s 16 μg/ml) and 3 strains showed high level imipenem resistance (MIC’s 64 μg/ml). This finding is explained in part by the permeation efficiency of imipenem through each bacterial outer membrane.16

The expression of high-level aminoglycoside resistance (MIC’s 32-2048 μg/ml) against gentamycin and tobramycin evaluates the extensive use of these antibiotics in the treatment of bacterial infection. The resistance is majorly exhibited by genome with the secretion of enzyme which inactivates the broad rage use of aminoglycoside action especially gentamycin and tobramycin by catalyzing the acetyltransferase reactions.

The presence of blalMP resistance can also be detected by the expression of metallo beta lactamase. In our study, the production of metallo beta lactamase was identified and showed that long term exposure to carbapenem may lead to the emergence of strains with elevated expression of blalMP gene because of mutations. Since the antimicrobial susceptibility studies are not sufficient for the early identification of blalMP carrying strain, it is necessary that every clinical isolate showing the high-level resistance should be subjected to PCR method for the detection of blalMP gene.

The results confirmed that 10 of 15 strains of P. aeruginosa tested have the metallo-b-lactamase gene like the blalMP gene. Therefore, strains producing IMP-I are difficult to control with β-lactams and related drugs in combination. It is reported that the metallo-β-lactamase gene (blalMP) cassette of Serratia marcescens AK9373 is located in the space between the integrase gene (intI3) and the aminoglycoside acetyltransferase gene [aac(6′)-Ib]10,12. Further, this is transposed into other plasmids or the chromosomes of gram-negative bacteria by this integron element which is mediated by large plasmids with wide host ranges. This finding warned of the further dissemination of the blalMP gene cassette into various gram-negative rods. Hence, we have directed special attention to the early recognition of metallo-b-lactamase-producing clinical isolates.12

RAPD analysis shows that the isolates showing a genotype of monomorphic form encoding the beta-lactamase gene families are equally prevalent in all the types. Besides this, isolates showed single different RAPD type that was specific for carrying metallo beta lactamase gene. The genotype pattern differed in their antibiogram profile which justifies the conjugational transfers of drug resistant determinants.

In-vitro changes in the morphology of many organisms by antibiotics are well documented. IPM treatment of P. aeruginosa showed marked differences in the resulting bacterial morphologies. IPM treatment induced rod-shaped bacteria to become rounded. This result is consistent with the findings reported by Jackson and Kropp.11 The TEM studies of IMP resistant strains reveal the spheroplast formation of the cells with altered cell shape. The increase in cell size was observed, large cells can tolerate antibiotic stress alternate way than the susceptible and normal size cells has been reported earlier.

Further, our findings also suggest that minor outer membrane proteins might be involved in the permeation of imipenem through the bacterial outer membrane. Another possible explanation for the differences in resistance to imipenem among these strains would be the varying expression levels of the metallo-β-lactamase gene in resistant strain compared with those of the non-susceptible and control strains.20

Conclusion
Our results support the important role that clinical microbiology laboratories must play to distinguish metallo beta lactamase producing P. aeruginosa strains with other mechanisms responsible for carbapenem resistance. The early detection of these would help in approach to antimicrobial therapy and prevent the dissemination of these multi drug resistant strains.

References


18. Neumann G., Veeranagouda Y. and Karegoudar T.B., Cells of Pseudomonas putida and Enterobacter sp. adapt to toxic organic compounds by increasing their size, Extremophiles, 9, 163–168 (2009)


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