Spread of Multi Drug Resistant *Pseudomonas aeruginosa* clones in a University Hospital

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**Running title:** Study of a *Pseudomonas aeruginosa* outbreak

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Abstract

An outbreak of multidrug-resistant *Pseudomonas aeruginosa* (MDRPA) infections in a University Hospital is described. Phenotypic and genotypic analysis of 240 isolates revealed that 152 patients, mainly in the ICU, were colonized or infected with MDRPA, the majority O11. All MBL positive isolates carried *bla*\textsubscript{VIM-2} or *bla*\textsubscript{VIM-1} genes. One or more, type III secretion system toxin genes, was detected in most isolates. Five dominant PFGE types were characterized associated with ST235, ST111, ST253, ST309 and ST639.

Keywords: *P. aeruginosa*, outbreak, clones, MLST, toxins, ICU.
*Pseudomonas aeruginosa* is an opportunistic pathogen causing severe invasive disease in critically ill and immunocompromised patients. Because of its ubiquitous nature, ability to survive in moist environments, and innate resistance to many antibiotics and antiseptics, constitutes a common pathogen in hospitals, particularly in intensive care units (ICUs). Its treatment is a therapeutic challenge because of the intrinsic resistance and the ability to easily acquire resistance determinants (1). Multidrug-resistant *P. aeruginosa* (MDRPA) infections mainly occur in ICU patients (2). The prevalence and epidemiology of MDRPA have become the focus of numerous single and multi-center surveillance studies (3). The large number of secreted and cell-associated virulence factors is implicated in the pathogenesis of severe infections. The type III secretion system (TTSS), a complex of three proteins, is associated with lung injury, sepsis, and a 6-fold greater risk of mortality, constituting an important virulence determinant (4, 5). Results on emergence and spread of MDRPA isolates in the University Hospital of Patras (UHP) and their phenotypic and genotypic characteristics are presented in this study.

During a two-year period, a total of 952 *P. aeruginosa* isolates were recovered from 430 patients hospitalized in our tertiary-care hospital, located in southwestern Greece, with 700 acute care beds and about 100,000 admissions annually. Two hundred and forty, the first ten from every month no replicate isolates (one isolate per patient), from different wards and a variety of clinical specimens including true infections and carriage, were selected for further study. Colonizing isolates were recovered from stool and respiratory tract specimens from patients without signs of infection.
P. aeruginosa was identified by standard methods. Colonizing (83) and infection-related (157) isolates were compared for their phenotypes and genotypes. For further analyses, isolates were divided into two groups: those recovered from ICU (92) and those from non-ICU patients (148). Antibiotic susceptibility testing was performed by the agar disk diffusion method against antipseudomonal agents according to CLSI guidelines (6). All isolates resistant to at least three classes of antibiotics were defined as MDRPA (7). The MIC of colistin was determined by the Etest (AB Biodisk, Solna, Sweden). All imipenem non-susceptible isolates, (IMP-NS), (MICs>1mg/L) were examined for metallo-beta-lactamases (MBL) production, using the Etest MBL assay (AB Biodisk).

Serotyping was performed by 16 monovalent antisera (Bio-Rad, Marne’s-la-Coquette, France) as previously described (8). Among the IMP-NS isolates, bla\textsubscript{VIM} gene was detected using the multiplex PCR –ELISA System (hyplex MBL ID PCR module Hyb-module, test system: BAG Health Care, Lish, Germany) (9). Types of bla\textsubscript{VIM} genes were identified by sequencing analysis among selected bla\textsubscript{VIM}-positive strains after comparison with data bank [http://blast.ncbi.nlm.nih.gov/Blast.cgi]. In all isolates, TTSS-III genes (exoS, exoT, exoU, exoY) were investigated by PCR (10). Clones were defined by pulsed-field gel electrophoresis (PFGE) of SpeI (Roche, Penzberg, Germany) DNA digests. Banding patterns were compared by Fingerprinting II Informatics Software (Bio-Rad, Berkeley, California) and clones were defined according to already established criteria (11). A dendrogram was computed comparing molecular weights of strains’ DNA
fragments to those from a previous collection by using FPQuest software (Bio-Rad, Cat.Num: 1709300). Clustering was based on ≥ 75% similarity. Selected strains of the main PFGE types were characterized by MLST [http://pubmlst.org/paeruginosa]. Pearson’s chi-squared test was used to evaluate the differences in the frequencies of variables in ICU and non-ICU wards, conducted by the PASW Statistics 18, Release Version 18.0.0 (SPSS, Inc., 2009, Chicago, IL, www.spss.com). Results were considered significant at a p-value of ≤ 0.05.

Comparison of non-susceptibility to antibiotics between ICU and non-ICU *P. aeruginosa* isolates is presented in Figure 1. All isolates were susceptible to colistin (MICs ≤ 2 mg/L). During the study period, a high frequency of MDRPA was detected (152/240, 63.33%), mainly in ICU (Table 1). There were no statistically significant differences in antibiotic resistance patterns between infecting and colonizing isolates. *bla*$_{VIM}$ gene was detected in all 49 MBL-positive isolates. No statistically significant difference was observed between ICU and non-ICU isolates (17.4% vs 22.3%) (Table 1). The majority of the isolates carried *bla*$_{VIM-2}$, while only three strains carried *bla*$_{VIM-1}$ gene.

Serogroup O11 predominated in ICU as compared to all other wards (Table 1) as well as among MDRPA [75% (63/84) in ICU and 57.4% (39/68) in non-ICU wards (p=0.021)]. The majority of isolates (227/240, 94.6%) carried one or more toxin genes, while, only 79 (33%) carried all four. Ninety-one isolates (91/240, 37.9%) carried both *exoU* and *exoS* genes. *exoU* was detected mainly among ICU patients, while, *exoS* from non-ICU in a statistically higher frequency (Table
PFGE exhibited five predominant pulsotypes (Table 1, Figure 2). PFGE type a strains were identified as ST235, type b strains carrying bla\textsubscript{VIM-1} gene belonged to ST111, while those carrying bla\textsubscript{VIM-2} were identified as ST235; type c strains were of ST253 and type d of ST235, while those of type s were classified as ST309 and ST639. The remaining isolates were classified into 78 PFGE types, including 1-3 strains each. Polyclonality was observed mainly among non-ICU strains (Table 1). Common clones among infecting and colonizing isolates were identified. The MDRPA, including MBL-positives, belonged mainly to pulsotypes a and d, both characterized as ST235. Surveillance studies have documented increases in the frequency of outbreaks especially in ICUs caused by strains resistant to multiple classes of antibiotics (12, 13). \textit{P. aeruginosa} was related to 8\% of total infections and carriage during the study period, while among ICU patients an outbreak occurred due to the spread of one main clone (ST235) of MDRPA. In our Hospital setting, MDRPA accounted for 49.5\% of \textit{P. aeruginosa} infections before the present study, during the studied period reached 63.3\%, dropping afterwards to 38.5\%.

Higher prevalence of MBL production was observed among IMP-NS \textit{P. aeruginosa} isolates (33\%) as compared to other studies from Greece and other European countries (14, 15). All MBL-positive isolates in the present study carried bla\textsubscript{VIM} gene and were spread in all hospital wards, especially among non-ICU patients (Table 1). VIM-type MBLs are predominant in Europe, particularly in the Mediterranean region and have been associated
with large outbreaks of MDRPA (3, 16, 17). More specifically, \textit{bla}VIM-2 is the most frequent in Southern European countries (3), while in Greece \textit{bla}VIM-17, a variant of \textit{bla}VIM-2, was identified in another outbreak (16).

Serotype O11 is common in hospital outbreaks and associated with multidrug resistance (2, 12), as is also shown in the present study. In our collection, 94.6\% of \textit{P. aeruginosa} isolates carried one or more TTSS-III genes, as reported elsewhere (18). \textit{exoS} was more frequent in isolates from urinary tract and wound infections from non-ICU patients, a finding that is in accordance with other investigators (18). \textit{exoU} was associated with serotype O11, as reported also by Faure et al (4), and detected mainly among ICU isolates (\textit{p}=0.032). Expression of \textit{exoU} correlates with acute cytotoxicity and accelerated lung injury playing a role in the development of septic shock in ICU high risk patients (18, 19).

MDRPA belonged mainly to PFGE types a and d of serotype O11 and ST235. Comparison of the recently identified clones with previous ones, revealed no relationship (Figure 2), (20). Studies have reported clonally related nosocomial outbreaks of MDRPA producing IMP-13 MBL (13) and panantibiotic-resistant \textit{P. aeruginosa} in ICUs (12). The observation that the majority of pulsotype d strains (ST235) carry \textit{bla}VIM-2 gene, reinforces that clonal spread may have played a role in the outbreak of IMP-NS \textit{P. aeruginosa}. \textit{bla}VIM gene spread was identified among clonally related strains in the last decade (1, 17, 21).

The observation that most carriage isolates belonged to the two predominant PFGE types a (38/83) and d (11/83) and to the same clone, ST235, indicates
that colonization during ICU hospitalization contributes to infection and spread
to other wards. Clinical isolates of ST235 (serotype O11) have been reported
worldwide harboring acquired beta-lactamases (22). *P. aeruginosa* ST235
(serotype O11) strains from bloodstream infections were among the three
predominant epidemic clones in Czech Republic (22).

This study describes a clonal outbreak of MDRPA serotype O11 of ST235
clon in a University Hospital which occurred mainly in ICU, during a two-year
period. *P. aeruginosa* clearly represents one of the most challenging
pathogenic bacteria since MDRPA isolates spread clonally quite frequently.
The monitoring of MBL and exotoxin genes carrying isolates has
epidemiological significance in the identification of drug-resistant and virulent
*P. aeruginosa* isolates, especially in high-risk patients. Our work shows the
need of clonal identification since MDRPA outbreaks require targeted infection
control measures.
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Transparency declaration

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References


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leads to increased bacterial virulence in a murine model of acute pneumonia and systemic spread. Infect Immun. 68:3998-4004.


Table 1: Characteristics of ICU and non-ICU *P. aeruginosa* isolates.

<table>
<thead>
<tr>
<th></th>
<th>ICU (N=92)</th>
<th>non-ICU (N=148)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDRPA</td>
<td>84 (91.3)</td>
<td>68 (46.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MBL positive</td>
<td>16 (17.4)</td>
<td>33 (22.3)</td>
<td>0.359</td>
</tr>
<tr>
<td>Serotype O11</td>
<td>68 (73.9)</td>
<td>50 (33.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;vim&lt;/sub&gt;</td>
<td>16 (17.4)</td>
<td>33 (22.3)</td>
<td>0.359</td>
</tr>
<tr>
<td><em>exoS</em></td>
<td>25 (27.2)</td>
<td>86 (58.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><em>exoT</em></td>
<td>76 (82.6)</td>
<td>116 (78.4)</td>
<td>0.427</td>
</tr>
<tr>
<td><em>exoU</em></td>
<td>83 (90.2)</td>
<td>118 (79.7)</td>
<td>0.032</td>
</tr>
<tr>
<td><em>exoY</em></td>
<td>81 (88.0)</td>
<td>125 (84.5)</td>
<td>0.438</td>
</tr>
<tr>
<td>Clone a (ST235)</td>
<td>59 (64.1)</td>
<td>22 (14.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Clone d (ST235)</td>
<td>15 (16.3)</td>
<td>18 (12.2)</td>
<td>0.365</td>
</tr>
<tr>
<td>Clone b (ST111;ST235)</td>
<td>3 (3.3)</td>
<td>7 (4.3)</td>
<td>0.745</td>
</tr>
<tr>
<td>Clone c (ST253)</td>
<td>1 (1.0)</td>
<td>5 (3.4)</td>
<td>0.410</td>
</tr>
<tr>
<td>Clone s (ST309;ST639)</td>
<td>0 (0.0)</td>
<td>6 (4.0)</td>
<td>0.084</td>
</tr>
<tr>
<td>All other clones</td>
<td>14 (15.2)</td>
<td>90 (60.8)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

ICU: intensive care unit; MDRPA: multi drug resistant *P. aeruginosa*; MBL: metallo-beta-lactamases.
Figure 1: Percentage of *P. aeruginosa* non-susceptible isolates to various classes of antibiotics among ICU and non ICU patients.

Antipseudomonas Penicillins: azlocillin, carbenicillin, piperacillin, ticarcillin, clavulanic acid

Aminoglycosides: amikacin, netilmicin, tobramycin
Figure 2: Dendrogram of P. aeruginosa isolates after digestion of DNA with SpeI and PFGE. Comparison of clonal types identified in the present study with previous ones, recovered from patients in the same Hospital. No relationship was detected between the recent and the older clones.

Lines 8,9,6,7,2,3,10,11,4,5: present study (clones d, d, a, a, b, b, s, s, c, c)

Lines 17,18,15,16,19,20,21,13,14: previous study (clones C,C,B,B,D,D,E,A,A)