ROLE OF PSEUDOMONAS AERUGINOSA EXTENDED SPECTRUM B LACTAMASE PRODUCERS IN HOSPITAL–ACQUIRED PNEUMONIA MORTALITY

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Abstract

Introduction: Hospital-acquired pneumonia (HAP), particularly ventilator-associated pneumonia (VAP), causes considerable morbidity and mortality despite antimicrobial therapy and advances in supportive care *Pseudomonas* (*P. aeruginosa*) is a leading cause of nosocomial infections all over the world, especially of HAP and VAP. The extended spectrum β-lactamase *P. aeruginosa* producers (ESBLs) have recently emerged as one of the most worrisome resistance that have been rapidly spreading through many countries. The aim of this study was to evaluate the mortality of the subset of patients with HAP due to *P. aeruginosa* in a setting of beta-lactamases such as ESBL (Extended spectrum beta-lactamase production). New media were used in their isolation to be tested for pseudomonas selectivity and enhancement of different pigment that are produced by *Pseudomonas aeruginosa*.

Methods: In this study, 76 samples were collected from nasocomial pneumonia cases. *P. aeruginosa* isolates were recovered. Conventional microbiology methods were used for *P. aeruginosa* identification, isolation, antibiotic susceptibility testing (using disk-diffusion methods) and β-lactamase production testing. *P. aeruginosa* isolates were recovered and grown on different types of media [basic, enriched and selective media e.g *P. aeruginosa* selective agar (PASA), Kings A and B]; to demarcate its colonies, isolate pigments producers and to isolate the extended spectrum *P. aeruginosa* β-lactamase (ESBL) producers. ESBL testing was done using disc diffusion testing susceptibility and Epsilon (E) test. A prospective observational study was performed and it was constructed to identify risk factors for 30-day mortality.

Results: All *P. aeruginosa* isolates in this study were grown (100%) on all media used. Gram positive isolates were inhibited (100%) on PASA, Kings A and Kings B. While Gram negative isolates were inhibited (100%) on PASA only and were not inhibited on other used media. The demarcation of *P. aeruginosa* colonies were detected in 100% on PASA, 22.58% on Kings A, 37.50% on Kings B, while nutrient and blood agar media could not demarcate colonies at all.

In this study, Kings B agar was the best medium for isolation of pigmented producers (68% fluorescein and 31.50% rubrin), pigments detected on other media with variable range: 67.50% on PASA (only fluorescein), 40.70% on nutrient and 32.80% on Kings A agar (only pyocyanin). The isolated *P. aeruginosa* gave 100% positivity for production of β-lactamase by the colorimetric method in detection of β-lactamase by nitrocefin method.

In this study, the tested isolates for ESBL revealed that the results were (28%) positive, (54%) negative and (18%) not detected. 76 patients with *P. aeruginosa* HAP were evaluated. The 30-day mortality was 36.80% (28 of 76): 57.10% (12 of 21) for patients with HAP by ESBL-producing *P. aeruginosa* and 29.60% (16 of 55) for non-ESBL-producing *P. aeruginosa*, indicating higher
mortality in ESBL producers than non producers especially in ventilated [8/11(72.70%)] patients despite appropriate treatment.

**Conclusions:** ESBL-producing P. aeruginosa HAP resulted in higher mortality rates, particularly in patients with ventilator-associated pneumonia, most probably related to the less frequent institution of appropriate antimicrobial therapy. Therapeutic approaches should be reviewed at institutions with a high prevalence of ESBL. This reflects the serious problem of impending resistance to all known sensitive drugs. In this study Kings B agar was the best medium for isolation of pigmented producers.

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**Introduction**

Nosocomial pneumonia is a major subgroup of all hospital-acquired infections and an important public health problem. Longitudinal surveillance studies, such as those carried out by the Centers for Disease Control and Prevention through the National Nosocomial Infection Surveillance (NNIS) system and others have suggested that aerobic Gram-negative bacilli, particularly Pseudomonas aeruginosa, are the major causative organisms[1]. Hospital-acquired pneumonia (HAP), particularly ventilator-associated pneumonia (VAP), causes considerable morbidity and mortality despite antimicrobial therapy and advances in supportive care [2,3].

It is the second most frequent nosocomial infection and is the major cause of death among hospital-acquired infections[2]. *Pseudomonas aeruginosa* is a leading cause of nosocomial infections all over the world, especially of HAP and VAP, when it usually ranks as the first or second causative pathogen[2-4]. This organism is uniquely problematic because of a combination of inherent resistance to many drug classes and its ability to acquire resistance to all relevant treatments[4]. Severe infections due to *P. aeruginosa* are associated with high mortality regardless of appropriate antimicrobial therapy [4].

*P. aeruginosa* is a frequent and prominent cause of nosocomial pneumonia especially in persons on assisted ventilation in the intensive care units[5]. Pseudomonads are resistant to antibiotics. They are naturally resistant to many antibiotics due to the permeability barrier afforded by their outer membrane lipopolysaccharides [6].

Extended spectrum [beta]-lactamase (ESBL)-producing organisms pose unique challenges to clinical microbiologists, clinicians, infection control professionals and antibacterial-discovery scientists. ESBLs are enzymes capable of hydrolysing penicillins, broad-spectrum cephalosporins and monobactams, and are generally derived from TEM and SHV-type enzymes. ESBLs are often located on plasmids that are transferable from strain to strain and between bacterial species. Although the prevalence of ESBLs is not known, it is clearly increasing, and in many parts of the world [7].

ESBL-producing Enterobacteriaceae have been responsible for numerous outbreaks of infection throughout the world and pose challenging infection control issues. Clinical outcomes data indicate that ESBLs are clinically significant and, when detected, indicate the need for the use of appropriate antibacterial agents. Unfortunately, the laboratory detection of ESBLs can be complex and, at times, misleading [7].
Antibacterial choice is often complicated by multi-resistance. Many ESBL-producing organisms also express AmpC [beta]-lactamases and may be co-transferred with plasmids mediating aminoglycoside resistance. In addition, there is an increasing association between ESBL production and fluoroquinolone resistance. Although in in vitro tests ESBLs are inhibited by [beta]-lactamase inhibitors such as clavulanic acid, the activity of [beta]-lactam/[beta]-lactamase inhibitor combination agents is influenced by the bacterial inoculum, dose administration regimen and specific type of ESBL present. Currently, carbapenems are regarded as the drugs of choice for treatment of infections caused by ESBL-producing organisms. Unfortunately, use of carbapenems has been associated with the emergence of carbapenem-resistant bacterial species such as Stenotrophomonas sp. or Pseudomonas sp [7].

Pseudomonads maintain antibiotic resistance plasmids, and they are able to transfer these genes by means of the bacterial processes of transduction and conjugation[8]. Only few antibiotics are effective against Pseudomonas, including fluoroquinolones, gentamicin and imipenem, and even these antibiotics are not effective against all strains [9].

The first hospital outbreak of an ESBL producing gram-negative organism was reported in Germany in 1983[10].Within one year, nosocomial outbreaks caused by a multidrug resistant Klebsiella clone carrying a TEM-3 gene were described in France[11]. Over the past decade, ESBL-producing Enterobacteriaceae have emerged as serious nosocomial pathogens throughout Europe[12]. Outbreaks have occurred among the most critically ill patients in intensive care units (ICUs) [13].

ESBLs in P. aeruginosa Middle Eastern hospitals may be underestimated because routine detection with a double disc synergy test may be difficult. Identification of ESBLs is of interest since they confer resistance to all extended-spectrum cephalosporins and aztreonam, whatever their MICs[14].

The extended spectrum β-lactamases (ESBLs) have recently emerged as one of the most worrisome resistance that have been rapidly spreading through many countries. The emergence of these enzymes drastically compromises effective treatments of nosocomial infections by this organism, bringing us closer to the much feared 'end of antibiotics' [15-16].

The aim of this study was to evaluate the mortality of the subset of patients with Hospital Acquired Pneumonia(HAP) due to Pseudomonas Aeruginosa (PA) in a setting of beta-lactamase such as Extended Spectrum Beta-Lactamase(ESBL) production. New media were used in Pseudomonas isolation to be tested for their selectivity and enhancement of different pigments that are produced by Pseudomonas aeruginosa.

Subjects and methods

Study Design and patients A contemporary cohort observational prospective study of consecutive patients with P. aeruginosa nosocomial infections was performed at a teaching hospital in the United Arab Emirates, Abu- Dahabi. The study period was from February 2004 to December 2006 at Sheikh Zayed Military Hospital, a 300 bed hospital. The ethics review board of the hospital approved the study and informed consent was taken after full explanation of the nature and aim of the study.
A total of 236 hospitalized patients with hospital-acquired infection, presented the isolation of P. aeruginosa after more than 48 hours of hospital admission. Of these, 85 presented the isolation of P. aeruginosa in their respiratory secretions. 9 patients were excluded because they did not fulfill the criteria for HAP.

A total of 76 patients were analyzed. Twenty-one (21/76) patients presented Extended Spectrum Beta-Lactamase producing Pseudomonas Aeruginosa Hospital Acquired Pneumonia (ESBL-PA HAP) and all received appropriate medical treatment and the antimicrobial agents used were at the discretion of the patient's physicians, not the investigators. Eleven (11/21) ESBL-PA HAP patients needed assisted mechanical ventilation. Patients were followed from the first isolation of P. aeruginosa to discharge from hospital or to death.

This study was performed on 76 samples from 76 patients with P. aeruginosa hospital acquired pneumonia. They were 24 females and 52 males with a mean age of 62.40±18.20 years for males and 59.40±17.90 years for females.

**Patients Inclusion Criteria:** In the current study, we analyzed patients who are ≥ 18 years, who did not have cystic fibrosis, who had been diagnosed with HAP defined as follows. First, the presence of positive cultures for P. aeruginosa either recovered from respiratory secretions (>10^{6} cfu/ml from endotracheal aspirates or >10^{4} cfu/ml from bronchoalveolar lavage) after 48 hours of hospital admission, or within 48 hours if the patient had been hospitalized in the past 60 days, or recovered from blood without the presence of any other pathogen in respiratory secretions. Second, the presence of a radiographic infiltrate that was new or progressive, along with the presence of two or more of the following criteria: fever (temperature >38°C) or hypothermia (temperature <36°C), purulent sputum, leukocytosis (>10,000 cells/mm^{3}) or leukopenia (<4,000 cells/mm^{3}), and a decline in oxygenation. Sputum was considered purulent if >25 neutrophiles and <10 epithelial cells per high power field were present[2].

**Patients Exclusion Criteria:** Patients were excluded if they did not fulfill these criteria for HAP[2].

**Data collection:** Data were collected from medical charts and/or hospital computer system databases, both during and after the patients' hospitalization. The researchers were blinded for the status of P. aeruginosa isolates during data collection.

**Microbiology:**
Conventional microbiology methods were used for P. aeruginosa identification, and susceptibility tests were performed by disk-diffusion methods according to Clinical and Laboratory Standards Institute, (formerly National Committee for Clinical Laboratory Standards), guidelines [17].

The media used for culture were as follows:

**1-Basic media:**
Ordinary nutrient agar.
2- **Enriched media:**
Blood Agar.

3- **Selective media:**

A) *Pseudomonas aeruginosa* selective agar (PASA) *(Biorad):*
The selectivity of the medium is due to the presence of irgasan, a quaternary ammonium compound, which has detergent-like qualities and is inhibitory to most bacterial species, except for *P. aeruginosa.***

B) *Kings A agar* *(Pseudomonas P agar)* *(Biorad):*
Magnesium chloride and potassium sulfate in this medium are cationic salts, which act as activators for pyocyanin production. Pyocyanin is a blue, water soluble pigment that diffuses into the medium surrounding the colonies. Pseudomonas P agar should be used in conjunction with Pseudomonas F agar.

C) *Kings B agar* *(Pseudomonas F agar)* *(Biorad):*
Kings B agar is used for the differentiating *Pseudomonas aeruginosa* from other *Pseudomonads* based on fluorescein production.
Magnesium sulfate is a cationic salt that acts as an activator for pigment production, but the presence of dipotassium phosphate stimulates fluorescein production while inhibiting pyocyanin production by *Pseudomonas.* Fluorescein is a greenish-yellow pigment that diffuses into the medium surrounding the colonies.

**Identification of *Pseudomonas aeruginosa:***

*Colony morphology:* They are characterized by sweety odor. Different pigment production was assessed, and reported from ordinary media and from King’s A and B media.

Diagnosis of *P. aeruginosa* was assured by positive oxidase test and their characteristic biochemical reactions (lack of fermentation of different sugars), growth at 42°C.

Different isolated *P. aeruginosa* strains were submitted for the following:

A- **Antibiotic susceptibility testing by:**

1- **Disk diffusion test *(Kirby Baur):***
By using Mueller Hinton’s agar and antibiotic discs supplied from Oxoid. The interpretation as sensitive, intermediate, or resistant was based on criteria according to National Committee for Clinical Laboratory Standards[17].

2- **Stoke’s disc diffusion sensitivity testing technique:**
P. aeruginosa isolates were subjected to antimicrobial sensitivity by Stock’s disc diffusion technique, where both the test and control organisms were inoculated on the same plate[18].
An uninoculated gap 2-3 mm wide was left to separate the test and control areas. The control was inoculated to the peripheral of the plate leaving a 15 mm band around the plate edge. After the inoculum was dried, antibiotic discs were applied[19].

3- **Beta lactamase testing** using colorimetric hydrolysis of nitrocefin[20]:
 Beta-lactamase sticks (Oxoid) were used. The tips of the sticks are impregnated with nitrocefin, a chromogenic cephalosporin. The opposite end is coloured black to identify the correct end for handling. One stick was removed from the container. The stick was rotated to pick off a small
mass of cells. The reagent-impregnated tip of the stick was examined for up to 5 minutes, and, if negative, re-examined after 15 minutes. A positive reaction is shown by the development of a pink-red color.

4- **Tests for Extended Spectrum Beta Lactamase (ESBL)** were done using the following techniques:

A- **Simple disc diffusion:**
By using Cephpodoxim (Biorad) sensitivity test as shown earlier in disk diffusion to test ESBL production.

B- **Double disc diffusion:**
Any strain that is resistant to Ceftazidime or Cephpodoxim but becomes sensitive in the presence of Clavulanic acid, is likely to contain extended spectrum beta lactamase. We used Cephpodoxim and Amoxicillin/Clavulanic acid disks near to each other as in disk diffusion for testing ESBL production. If widening of the zone of Cephpodoxim disk near the Amoxicillin/Clavulanic acid disk occurs, this indicates positive production of ESBL. If no change in diameter, this indicates no ESBL production[19].

C- **Epsilon test (E-test)** (AB , Biodisk):
The E-test TZ/TZL strip consist of a thin, plastic carrier (5 X 60 mm) calibrated with MIC reading scales in μg/ml on one side while the reverse surface carries two predefined gradients. TZ codes for the ceftazidime (0.5-32 μg/ml) and TZL the ceftazidime with clavulanic acid (0.064-4 μg/ml). Negative result if MIC ratio of both TZ/TZL less than 8 and positive result if MIC ratio of both TZ/TZL more than or equal to 8. ND: not detected (MIC out of test range for all components or one result negative and the other ND)[20].

**Variables and definitions:**
The main outcome(*primary endpoint*) was 30-day mortality. Other *secondary outcomes* were the length of need for vasoactive drugs and the length of mechanical ventilation (both were assessed in survivors).
The *variable* in the study was ESBL production. Other *independent variables* analyzed included the following: age; sex; Charlson comorbidity score[21](assessed at the moment of HAP diagnosis); baseline diseases; iatrogenic immunosuppression, such as chemotherapy-induced neutropenia (neutrophile count ≤ 1,000 mm³), and/or receipt of corticoid drugs (prednisone ≥ 10 mg daily or equivalent doses) or other immunosuppressive agents for >14 days; the presence of other concomitant infections (infections by other organisms at a site other than the lung, excluding coagulase-negative staphylococci in a single blood culture); a previous surgical procedure during the hospital stay; the length of hospital stay (before the diagnosis of HAP); presentation of HAP with severe sepsis or septic shock [22]; infection by *P. aeruginosa* at more than one site (not including patients with HAP and bacteremia); polymicrobial infection (isolation of another organism from the respiratory secretions at the moment of *P. aeruginosa* HAP diagnosis); associated bacteremia (isolation of *P. aeruginosa* from one or more blood samples); VAP; receiving appropriate empirical therapy (defined as the administration of an antimicrobial agent to which the isolate was susceptible *in vitro* in ≤ 24 hours of sample collection); receiving appropriate definitive therapy (defined as the use for at least 48 hours of an antimicrobial agent to which the isolate was susceptible *in vitro*); time to receiving appropriate
definitive therapy (only for those who have not received appropriate empirical therapy; time in days from the sample collection to the first dose of appropriate therapy); and combination antibiotic treatment (treatment with more that one agent with \textit{in vitro} susceptibility)\cite{4}.

Aminoglycosides in monotherapy were not considered appropriate treatment therapy despite \textit{in vitro} susceptibility\cite{4}.

\textbf{Statistical analysis:}
All statistical analyses were carried out using SPSS for Windows, version 13.0. The relative risk (RR) and the 95\% confidence interval (CI) were calculated for 30-day mortality of patients with MBL-PA HAP and of patients with non-MBL-PA HAP. \(P\) values were calculated using the chi-squared test or Fischer exact test for categorical variables, and using Student's \(t\) test or the Wilcoxon rank-sum test for continuous variables.

A logistic regression model was constructed to identify independent factors associated with 30-day mortality using a forward stepwise approach. Variables for which the \(P\) value was < 0.20 in univariate analysis were included in the model. \(P = 0.05\) was set as the limit for acceptance or removal of the terms in the model. ESBL production remained in the model independent of the \(P\) value. All tests were two-tailed and \(P \leq 0.05\) was considered significant.

\textbf{Results}
A total of 236 patients presented the isolation of \textit{P. aeruginosa} after >48 hours of hospital admission. Of these, 85 presented the isolation of \textit{P. aeruginosa} in respiratory secretions. 9 patients were excluded because they did not fulfill the criteria for HAP. A total of 76 patients were analyzed. twenty-one (27.6\%) patients presented EBL-PA HAP.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
 & \textit{Female} & \textit{Male} \\
\hline
\textit{No} & 42 & 52 \\
\hline
\% & 32\% & 68\% \\
\hline
\end{tabular}
\caption{Classification of cases according to sex:}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
 & \textit{Female} & \textit{Male} \\
\hline
\textit{age} & 59.40 & 62.40 \\
\hline
\textit{SD} & \pm 17.90 & \pm 18.20 \\
\hline
\end{tabular}
\caption{Classification of cases. According to age:}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Isolates & \textit{N&Blood agar} & \textit{PASA} & \textit{Kings A agar} & \textit{Kings B agar} \\
\hline
\textit{P. aeruginosa} & 100\% & 100\% & 100\% & 100\% \\
\hline
\textit{Gram negative isolates other than P. aeruginosa} & 100\% & 00\% & 66\% & 66\% \\
\hline
\end{tabular}
\caption{Percentage of growth of all bacteria on different examiend media:}
\end{table}
Gram positive isolates

100% 0% 0% 0%

N:nutrient agar.

Regarding colonies demarcation and separation: Colonies demarcation of *P. aeruginosa* isolates was best detected on PASA medium, and with variable percentage on Kings A and Kings B. Colonies separation was very poor on nutrient and blood media (Table 4).

Table (4): Ability of different media to localize the colonies of *P. aeruginosa* isolates (inhibition of mucoid secretion and/or motility):

<table>
<thead>
<tr>
<th></th>
<th>Blood agar</th>
<th>PASA</th>
<th>Kings A agar</th>
<th>Kings B agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of plates with isolates of <em>P. aeruginosa</em></td>
<td>76</td>
<td>76</td>
<td>31</td>
<td>32</td>
</tr>
<tr>
<td>Colonies localization</td>
<td>0</td>
<td>76</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>% of localization</td>
<td>0%</td>
<td>100%</td>
<td>22.58%</td>
<td>37.50%</td>
</tr>
</tbody>
</table>

Regarding pigment production: 67% of *P. aeruginosa* isolates produced pigment (only fluorescin) on PASA, 32% of *P. aeruginosa* isolates produced pigment (only pyocyanin) on Kings A, and 100% of *Pseudomonas aeruginosa* isolates produced pigment (68% fluorocien and 31% rubrin) on Kings B medium (Tables 5 & 6).

Table (5): Pigments production by *P. aeruginosa* on different media:

<table>
<thead>
<tr>
<th></th>
<th>PASA</th>
<th>Kings A agar</th>
<th>Kings B agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of <em>P. aeruginosa</em> isolates</td>
<td>76</td>
<td>76</td>
<td>76</td>
</tr>
<tr>
<td>No of pigmented isolates</td>
<td>51</td>
<td>25</td>
<td>76</td>
</tr>
<tr>
<td>Percentage of pigment production</td>
<td>67%</td>
<td>32.89</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table (6): Types of pigment production on different media:

<table>
<thead>
<tr>
<th>Type of pigment</th>
<th>Nutrient</th>
<th>PASA</th>
<th>Kings A agar</th>
<th>Kings B agar</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pyocyanin</em></td>
<td>4</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td><em>Rubrin</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td><em>Fluorescin</em></td>
<td>27</td>
<td>51</td>
<td>0</td>
<td>52</td>
</tr>
<tr>
<td><em>Melanin</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>51</td>
<td>10</td>
<td>76</td>
</tr>
</tbody>
</table>

Table (7): Antibiotic resistance profiles of 21 extended spectrum β-lactamase (ESBL) producing *Pseudomonas aeruginosa*:

<table>
<thead>
<tr>
<th>Profile</th>
<th>Antibiotic</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymyxin B</td>
<td>Susceptible</td>
<td>8</td>
<td>38</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>Susceptible</td>
<td>7</td>
<td>33</td>
</tr>
<tr>
<td>Meropenem</td>
<td>Susceptible</td>
<td>3</td>
<td>14.8</td>
</tr>
<tr>
<td>Piperacillin tazobactam</td>
<td>Susceptible</td>
<td>1</td>
<td>4.7</td>
</tr>
<tr>
<td>Amikacin</td>
<td>Resistant</td>
<td>1</td>
<td>4.7</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Susceptible</td>
<td>1</td>
<td>4.7</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>Resistant</td>
<td>1</td>
<td>4.7</td>
</tr>
<tr>
<td>Cefepime</td>
<td>Resistant</td>
<td>1</td>
<td>4.7</td>
</tr>
</tbody>
</table>
Table 8: Characteristics of patients according to 30-day mortality:

<table>
<thead>
<tr>
<th>Variables</th>
<th>30-day mortality</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes (n =28)</td>
<td>No (n =48)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>65.40 ±17.40</td>
<td>59.40 ±16.90</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>Sex (male)</td>
<td>21(75.00)</td>
<td>31 (64.50)</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>Charlson score</td>
<td>4 (2–6)</td>
<td>3 (2–6)</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Comorbidities:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neurological</td>
<td>7(25)</td>
<td>17(35.40)</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>Cardiac</td>
<td>10 (35.70)</td>
<td>27(56.30)</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>Pulmonary</td>
<td>6(21.40)</td>
<td>22(45.80)</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>Malignancy</td>
<td>3(10.70)</td>
<td>8(16.60)</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>5(17.90)</td>
<td>11(22.90)</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>Renal</td>
<td>2 (7.10)</td>
<td>111(22.90)</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>2(7.10)</td>
<td>5(10.40)</td>
<td>0.37</td>
<td></td>
</tr>
<tr>
<td>Immunosuppression</td>
<td>20(41.70)</td>
<td>17(35.40)</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Other infections</td>
<td>12(42.90)</td>
<td>12(25)</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>Previous surgery</td>
<td>8(28.60)</td>
<td>12(25)</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>Length of hospital stay (days)</td>
<td>16.5 (7.5–30)</td>
<td>15.5 (5–30)</td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td>Severe sepsis or septic shock</td>
<td>12(42.90)</td>
<td>16.(33.30)</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>Patients needed mechanical ventilation</td>
<td>11(39.30)</td>
<td>15(33.00)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>&gt;1 site</td>
<td>16 (57.10)</td>
<td>8(16.70)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Polymicrobial pneumonia</td>
<td>12(42.90)</td>
<td>15(31.30)</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>Bacteremia</td>
<td>11(39.30)</td>
<td>4(8.30)</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>Appropriate therapy:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At any moment</td>
<td>15(53.60)</td>
<td>39(81.30)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>≤ 24 hours</td>
<td>6(21.40)</td>
<td>18(37.50)</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Time to initiate appropriate therapy (days)</td>
<td>4.50 ± 2.10</td>
<td>5.10 ± 5.10</td>
<td>0.60</td>
<td></td>
</tr>
</tbody>
</table>

Presented as the mean ± standard deviation, as the median (interquartile range), or as n (%).
Table (9): Results of ESBL by E- Test and its percentage:

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No of samples (%)</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>POSITIVE</td>
<td>21(28%)</td>
<td></td>
</tr>
<tr>
<td>NEGATIVE</td>
<td>41(54%)</td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>14(18.%)</td>
<td></td>
</tr>
</tbody>
</table>

**ESBL Negative:** when ceftazidime(TZ) / ceftazidime with clavulenic acid (TZL) MIC was <8 ;
**ESBL Positive:** when TZ/TZL MIC was ≥8 and ESBL not detected(ND): when MIC was out of test range for all components or one result negative and the other not detected.

Table (10): Comparison between 30-day mortality in ESBL & non ESBL producers HAP-pseudomonas aeruginosa patients:

<table>
<thead>
<tr>
<th></th>
<th>Producers (n = 21)</th>
<th>Non producers (n = 55)</th>
<th>Total (n = 76)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 day mortality</td>
<td>12 (57%)</td>
<td>16 (29%)</td>
<td>28 (36%)</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.0001 (H.S)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

H.S: Highly significant.
The mortality rate was higher (12/21, 57%) in ESBL producers than in non ESBL (16/55, 29%) producers despite appropriate treatment for both groups of patients (28/76 cases died, 36%).

Table (11): Therapy and 30-day mortality of patients with Pseudomonas aeruginosa producing β-lactamase hospital-acquired pneumonia (ESBL- PA HAP):

<table>
<thead>
<tr>
<th>Treatment.</th>
<th>Medical treatment only (n = 10)</th>
<th>Medical treatment with mechanical ventilator (n = 11)</th>
<th>Total (n = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treated patients (n = 4)</td>
<td>30- day mortality</td>
<td>Treated patients (n = 8)</td>
</tr>
<tr>
<td>✧ Appropriate monotherapy</td>
<td>6</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>meropenin</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Piperacillin – tazobactam</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>✧ Appropriate combination therapy</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Polymyxin B + Aztreonam</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Aztreonam + amikacin</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>✧ Nonappropriate combination (aztreonam+ceftazidime+amikacin)</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

NB: ESBL non producers received the same lines of treatment.
Discussion

*Pseudomonas aeruginosa* is a leading cause of nosocomial infections. *P. aeruginosa* ranked second among all nosocomial pathogens related to pneumonia in intensive care units reported to the National Nosocomial Infection Surveillance System in the last decade[23]. *P. aeruginosa* is primarily a nosocomial pathogen[24]. The frequency with which it causes disease is reliably estimated from annual surveillance data collected by the National Nosocomial Infection Surveillance (NNIS) system of the Centers for Disease Control and Prevention (CDC). According to these data, collected between 1986 and 1998, *P. aeruginosa* was the second most common cause of nosocomial pneumonia[24].

Multidrug-resistant *P. aeruginosa* nosocomial infections are increasingly recognized worldwide. The existence of extended-spectrum ß-lactamase-producing isolates exhibiting resistance to most ß-lactam antimicrobial agents greatly complicates the clinical management of patients infected with such isolates[24].

In the present study, *P. aeruginosa* isolates were recovered from 76 samples collected from nosocomial hospital acquired pneumonia patients.

In the present work, 100% of isolated *P. aeruginosa* (table 3) were recovered from cultures on nutrient agar plates, Blood agar plates, *Pseudomonas aeruginosa* selective agar (PASA) plates, Kings A and kings B agar plates. The ability of *P. aeruginosa* (which is unlike many environmental bacteria) to grow easily on these media is due to its ability to adapt to and thrive in many ecological niches, from water and soil to plant and animal tissues. The bacterium is capable of utilizing a wide range of organic compounds as food sources, thus giving it an exceptional ability to colonize many ecological niches, even with limitation of nutrients[25].

Inspite of the relative easiness in cultivating *P. aeruginosa* on different media, the problem ensues when it is present in polymicrobial samples. So, selectivity of different media was studied (table 3), aiming at recommending the best medium used to get pure *P. aeruginosa* isolates from potentially mixed or contaminated samples. In this study, Gram positive bacteria were inhibited 100% on PASA, kings A, and Kings B media, while they were not inhibited on nutrient and blood agar media. Regarding Gram negative bacilli other than *P. aeruginosa*, they were inhibited only on the PASA; while kings A and kings B were not inhibitory to Gram negative bacilli. This result came in agreement with other study [26], who reported that PASA is the selective media for isolation of *P. aeruginosa* as it includes the selective agent irgasan (quaternary ammonium compound, which has detergent-like qualities and is inhibitory to most bacterial species, except for *P. aeruginosa*).

Again, in mixed or contaminated samples, it is sometimes difficult to localize separate *P. aeruginosa* colonies, owing to abundant mucous production, or extension of growth around colonies. PASA medium gave well distinct colonies in all studied *P. aeruginosa* isolates. Kings A agar medium gave separate colonies in only 22.58% of isolates. Kings B agar medium yielded separate colonies in 37.50% of isolates. Nutrient agar and blood agar media failed to localize colonies at all(table 4).From these results, it is evident that PASA is the best medium for demarcation of colonies of *P. aeruginosa*.
In this study, Kings B agar was the best medium for detection of pigments (100%) of isolates. Pigments were detected on other media with variable degrees (67% on PASA, 40.70 % on nutrient and only 32.89% on Kings A agar) (table 5).

Kings A and kings B are combined with a selective supplement to allow the isolation of all pigmented and non pigmented Pseudomonas (table 6). Kings B agar was enhancing medium for detection of rubrin and fluorescein pigments (68% of isolates produced fluorescein and 31% produced rubrin). Melanin pigment was not produced on this medium. Magnesium sulfate is a cationic salt that acts as an activator for pigment production, but the presence of dipotassium phosphate stimulates fluorescein production while inhibiting pyocyanin production. Kings A was selective for detection of pyocyanin pigment as kings A medium has low phosphate content of peptone which makes it an ideal choice since the presence of phosphate is inhibitory to pyocyanin production. Glycerol is added as an alternate carbon source. Magnesium chloride and potassium sulfate are cationic salts to enhance pyocyanin pigment production[26].

PASA medium enhanced pigment production in 67% of isolates (table6). Fluorescein was the only produced pigment while agar enhanced pigment production 40.70% (fluorescin 35.50 % and pyocin 5.20 % ) and this results was in agreement with other study[26], who stated that PASA agar enhances fluorescein pigment as it contains magnesium chloride and potassium sulfate.

The studied P. aeruginosa isolates revealed six antibiotic sensitivity patterns, they were shown in table(9): P. aeruginosa isolates ESBL producers were 100 % resistant to(aztreonam, ceftazidime and cefipime) and 100% sensitive to polymexin B with variable sensitivity to meropenim , pipracillin/ tazobactam, amikacin and ciprofloxacin.

These observations in this study were in agreement with the results of other studies[27]&[28] in which they reported that Gentamicin and Amikacin are considered as suitable aminoglycoside antibiotics against drug resistant P. aeruginosa. This fact reflects the importance of controlling the use of these antimicrobials in the hospitals, for preventing the emergence of aminoglycosides-resistant strains. In addition,one study[26], recommended to start restriction of aminoglycosides use, when aminoglycosides resistant strains are detected.

One of the published multi-centric study[29], reported that Imipenem is the most active compound against P. aeruginosa infections followed by Ciprofloxacin. Other study[30] considered Imipenem and Ciprofloxacin a potent agents in treatment of infections caused by multi-resistant P. aeruginosa.

The results of present study came to reveal a new point of view, as the resistance against meropenim started between some isolated strains. This is may be due to emergence of new enzymes e.g the extended spectrum beta lactamase produced by by P. aeruginosa. These results indicated the high levels of antimicrobial resistance detected in the isolated strains and reinforced the idea that the empirical treatment adopted in hospitals routine practice induces selective pressure of multi-drug resistant strains.

One study[29], stated that prolonged indiscriminate use (or abuse) of broad spectrum beta- lactam antimicrobials will induce actual changes in the susceptibility pattern, and enhances the
transmission of resistance genes from one bacterial population to another. Few antibiotic resistance profiles were observed among ESBL-PA HAP patients (table 9). Two isolates were unexpectedly susceptible to piperacillin-tazobactam. Such an interesting finding, however, has already been reported previously[23&31]. ESBLs have been a major determinant of aztreonam resistance [32]. However, other resistance mechanisms to these agents were also present in some isolates, such as the loss of OprD outer membrane protein in the case of imipenem, and this latter mechanism with an associated overexpression of the MexAB-OprM efflux pump, as is the case for meropenem [32& 33].

The observations in this study were in accordance with many published other studies[28,34,35 and 36], who stated that the extensive antimicrobial drugs use was a predisposing risk factor to high acquisition of resistance, because this extensive use can select multi-drug resistant microorganisms.

Other studies[35 and37], reported also that Multi-resistant P. aeruginosa strains with different resistance forms were resulted in treatment failure, due to selective pressure promoted by indiscriminate use of antimicrobials, principally broad-spectrum antimicrobials.

To reduce the emergence of multi resistant strains, the restrictive policies in which an ordered and systematic use of antimicrobial drugs might be of benefit in the general strategy of chemotherapy to reduce the proportion of resistant organisms in a community. Also the rotational policies in which periodic changes of antibiotics used in treatment might also help to avoid the emergence of resistant strains [38].

Previous experiences with penicillin-non susceptible pneumococci, methicillin-resistant Staphylococcus aureus, vancomycin-resistant Enterococcus faecium, and extended-spectrum β-lactamase–producing Klebsiella pneumoniae indicate that once resistant bacteria can become widespread they cannot be controlled[39].

**Characteristics of patients according to 30-day mortality(table 8), showed that, Both presentation with severe sepsis or septic shock and VAP had the strongest impact on 30-day mortality, supporting the importance of these factors in overall mortality as recognized in many studies [40– &41]. Higher comorbidity scores had also a significant impact on the outcome of patients(table 8).**

Extended spectrum beta-lactamase test was made(table 9) and it was noticed that the tested isolated strains were positive for Epsilon test in 21/76 (28 %) , 41/76 (54%) were negative and 14/76 (18%) were not detected (MIC out of test range for both components or one result negative and the other not detected). 76 patients with P. aeruginosa HAP were followed for 30-day mortality. The 30-day mortality was 36% (28 of 76) for all studied (76) cases: 57% (12 of 21) and 29% (16 of 55) for patients with HAP by ESBL-producing P. aeruginosa and by non-ESBL-producing P. aeruginosa respectively. Our study showed a higher mortality in patients with HAP due to ESBL producing P. aeruginosa isolates and ESBL production by these isolates significantly increased the mortality of these patients more than ESBL non producers ( P value <0.0001 i.e highly significant)(table 10).

Among the 21 patients with ESBL-PA HAP, 12 patients (57%) died although they received appropriate therapy. Antimicrobial therapies of patients with ESBL-PA HAP are presented in(table11). Among the 21 patients with pseudomonas HAP ESBL producers, 17 patients treated
appropriately, 12 of the 21 patients died (57%) despite appropriate treatment and 11/21 patients needed mechanical ventilation and the percentage of mortality among them was 72% (8/11) (table 1) inspite of appropriate treatment. These results were in agreement with another study[33], who reported that the Imipenem-resistance rate of P. aeruginosa has rapidly risen from 6% in 1996 to 19% in 2001, in Korea ESBL Study, and in a later study, they discovered that approximately 10 to 50% of Imipenem resistance in P. aeruginosa was due to multi-drug resistant pseudomonas aeruginosa producing a new enzyme called metallo-β-lactamase. Owing to the relatively small sample size in our study, no definitive conclusion about superiority of any antibiotic for the treatment of ESBL-PA HAP can be made.

A limitation of our study was that, patients who were discharged within 30 days were not followed-up after their hospitalizations, and it is possible that some of them could have died after hospital discharge within this period. This potential bias might not have influenced our results, however, since the length of follow-up of patients who have not presented the outcome did not differ between ESBL PA patients and non-ESBL-PA patients. Our study did not demonstrate a significant effect of early appropriate therapy on mortality, it may therefore be possible that our sample size lacks sufficient power to detect statistical differences within this period of time and these observations are in agreement with many other studies [2,40,41].

Conclusions: ESBL-producing P. aeruginosa HAP resulted in higher mortality rates, particularly in patients with mechanical ventilation, most probably related to the less frequent institution of appropriate antimicrobial therapy. Therapeutic approaches should be reviewed at institutions with a high prevalence of ESBL P. aeruginosa producers. This reflects the serious problem of impending resistance to all known sensitive drugs. In this study, Kings B agar was the best medium for isolation of pigmented ESBL-PA producers.

REFERENCES


