Prevalence of carbapenemase genes in extreme drug resistant *Pseudomonas aeruginosa* isolated from ICU in Egypt

Nermin M.K. Mohamed¹, Khaled Z. El-Baghdady¹, Eman. El-Kholy² and Gihan M. Fahmy²

1- Microbiology department, Faculty of Science, Ain Shams University, Cairo, Egypt
2- Microbiology department, Ain Shams Specialized hospital, Cairo, Egypt

**ABSTRACT**

The emergence of extreme drug resistant (XDR) *Pseudomonas aeruginosa* represents a major problem in health care settings. This work aimed to study the prevalence of some *OXA* genes and biofilm formation among *P. aeruginosa* clinical isolates collected from Intensive Care Units (ICUs), Ain-Shams University, Cairo, Egypt. Among 70 Gram negative clinical isolates, 25 isolates were phenotypically identified as *P. aeruginosa*. Antibiotic sensitivity and minimum inhibitory concentration (MIC) for 18 different antibiotics (Ticarcillin, Ticarcillin /Clavulanic acid, Piperacillin, Piperacillin-Tazobactam, Ceftazidime, Cefepime, Meropenem, Amikacin, Gentamicin, Aztreonam, Imipenem, Meropenem, Tobramycin, Ciprofloxacin, Pefloxacin, Minocyclin, Rifampcin, Trimethothoprim/Sulfamethoxazole and colistin) were carried out against *P. aeruginosa* isolates using VITEK 2. All isolates were resistant to all antibiotics except colistin that only 12% were resistant. Real time polymerase chain reaction with specific primers was used to detect the presence of selected *OXA* genes. *OXA group I* and *OXA group II* were detected in 44 and 52%, respectively, while *OXA group III*, *OXA51*, *OXA23* and *OXA 58* were totally absent. Biofilm formation assay showed strong, weak and moderate biofilm formation in 44, 28 and 12% of the isolates, respectively, while 16% were non biofilm forming isolates.

**Keywords:** *OXA*, multidrug resistant, *Pseudomonas aeruginosa* and colistin

**INTRODUCTION**

*Pseudomonas aeruginosa* is a Gram-negative bacillus which belongs to the pseudomonadaceae family (Alhazmi, 2015). *P. aeruginosa* infections are the most common cases treated in intensive care units (ICU) including haematological, surgical and burn units. Clinical forms of *P. aeruginosa* infections are hospital acquired pneumonia (HAP) including ventilator associated pneumonia (VAP), urinary tract infection (UTI), bloodstream infections (BSI), including central line associated bloodstream infections (CLABSI), burn wound infections, skin and soft tissue infections, surgical site infections, decubitus ulcers, ocular infections, central nervous system infection, bone and joint infections and otitis interna (Moore and Flaws, 2011).

Carbapenems are broad-spectrum beta-lactam antibiotic agents. They are usually considered the last choice for antibiotic therapy, especially in combating Extended-Spectrumβ-Lactamase (ESBL) producing microorganisms. The presence of carbapenem-resistant bacteria can be quite considerable because they enjoy the chance to shift to MDR strains commonly (Amini and Namvar, 2019). The recent emergence of extensively drug-resistant *P. aeruginosa* (XDR-PA), which is defined as those strains that remain susceptible to only one or two classes of antipseudomonal agents, has become a serious concern due to the lack of an effective antimicrobial therapy (Falagas
and Bliziotis, 2007). *P. aeruginosa* isolates resistant to carbapenems, or resistant to all antibiotics available for clinical use called pan drug resistance (PDRPA), have been reported to cause nosocomial infections and outbreaks among patients hospitalised in ICUs or burn units (Hsueh et al., 1998; Tacconelli et al., 2002).

*P. aeruginosa* can become resistant to carbapenem through various mechanisms (Lari et al., 2015; Bijari et al., 2016). The most important mechanism is the potential to produce carbapenemase because most of the carbapenemase genes can be found on the transferable genetic elements and they spread rapidly among bacteria (Azimi et al., 2015). Different classes of carbapenemase can be detected in Gram-negative bacteria including Ambler classes A, B, and D β-lactamases (Karbasi zad et al., 2015). According to Ambler molecular classification, β-lactamases are divided into four groups naming class A, B, C and D according to their amino acid sequence (Medeiros et al., 1995). Of these classes, class D β-lactamases or *OXA*-types are of great concern as they are encoded by genes which are transmissible and account for most of the resistance to β-lactams (Paterson et al., 2008). The *OXA*-type β-lactamases are also known as oxacillinases (*OXA* β-lactamases) due to their ability to hydrolyze oxacillin much faster than benzylpenicillin (Rondinaud et al., 2013). *OXA* type-β-lactamase falls into five groups (I–V). The *OXA* group I includes *OXA*-5, 7, 10, 13 and its extended spectrum derivatives (*OXA*-11, 14, 16, 17, and 19). *Group II* includes *OXA*-2, 3, 15 and 20. *Group III* includes *OXA*-1, 4, 30 and 31.

Biofilms are structurally complex surface connected populations in which bacterial cells are enclosed by extra cellular polymeric substances (EPS) produced by their own self. These EPS are mostly exopolysaccharides, extracellular deoxyribonucleic acid and proteins (Ryder et al., 2007). Biofilm formation contributes to pathogenesis of *P. aeruginosa* both in acute as well as chronic infection in clinical settings (Schaber et al., 2007). The biofilm production retards the antimicrobial therapy against bacteria because the biofilm develops a barrier which reduces the drug penetration leading to treatment failure as well as hindering the recognition of the microorganisms by immune system (Gil-Perotin et al., 2012). Colistin became the last-line therapy for treatment of serious infections caused by such XDR pathogens (Breilh et al., 2013). Unfortunately, resistance to colistin has been reported all over the world (Mammina et al., 2012; Lesho et al., 2013; Azimi, and Lari, 2019) which should be a reason for rationalization of using colistin to reduce the rate of emergence of such resistant superbugs (Gould, 2008; Rai et al., 2013).

**MATERIALS AND METHODS**  
**Collection and identification of clinical isolates**

Gram negative clinical isolates (70) were kindly provided from ICUs, Ain Shams University, Egypt. All isolates were subcultured on blood and MacConkey agar plates then incubated at 37°C for 24 h. Cultural characteristics, Gram stain and oxidase test were carried out for all isolates as preliminary identification (Cheesbrough, 2000). Confirmation of isolates identification and antibiotic susceptibility were carried out by Vitek2 (bioMérieux, Marcy l’E´toile, France). The AST-N 22 card was used to detect the sensitivity and MIC for 18 antibiotics (Ticarcillin, Ticarcillin /clavulanic acid, Piperacillin, Piperacillin-tazobactam, Ceftazidime, Cefepime, Meropenem, Amikacin, Gentamicin, Aztreonam, Imipenem, Tobramycin, Ciprofloxacin, Pefloxacin, Minocyclin, Rifampcin, Trimethothoprim/sulfamethoxazole and Colistin).
Prevalence of carbapenemase genes in extreme drug resistant *Pseudomonas aeruginosa* isolated from ICU in Egypt

Genotypic detection of OXA genes

DNA was prepared by picking up 3 pure colonies of fresh cultures on blood and MacConkey plates which incubated for 18-20 h at 37°C. The colonies were suspended in 50 μl sterile distilled water then boiled at 100°C for 10 min. Real time amplification reaction was performed by thermal cycler (Applied Bioline System) to amplify OXA genes (Qin et al., 2003). The reaction mixture was 10 μl SensiFAST SYBR Lo-Rox Master Mix (Bioline Scientific), 1.5 μl of each OXA gene primer (forward and reverse), 1 μl DNA template, and 7.5 μl distilled water. The amplification was performed using the following temperature profile: initial denaturation (94°C for 5 min); 25 cycles of denaturation (95°C for 45sec), annealing temperature 60-65°C for 10 sec and extension (72°C for 5-20 sec). The primers used to amplify; group I (OXA-10 group), OXA group II (OXA-2 group), OXA group III (OXA-1 group) OXA 51, OXA 24, OXA 23 and OXA 58, were listed in Table (1).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequences (5’→3’)</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXA group I (OXA-10 group)</td>
<td>5’TCAACAAATCGCCAGAGAAG 3’ 5’TCCCCACACCAGAAAACCAG 3’</td>
<td>276</td>
<td>Rasheed et al., 1997</td>
</tr>
<tr>
<td>OXA group II (OXA-2 group)</td>
<td>5’AGAAACGCTACTCGCTG 3’ 5’CCACTCAACCCATCTCTACCC 3’</td>
<td>478</td>
<td>Rasheed et al., 1997</td>
</tr>
<tr>
<td>OXA group III (OXA-1 group)</td>
<td>5’TITTCGTTTGGGT 3’ 5’TTCCTTCGCTTTATGCTG 3’</td>
<td>427</td>
<td>Rasheed et al., 1997</td>
</tr>
<tr>
<td>OXA-51</td>
<td>5’TATGTCCTTGGATCGGCTTGG 3’ 5’TGATGTCATTCATCTCTG 3’</td>
<td>353</td>
<td>Turton et al., 2006</td>
</tr>
<tr>
<td>OXA 23</td>
<td>5’GATCGGATTGGAACCCAGA 3’ 5’ATTCTGACCAGCTTCCAT 3’</td>
<td>501</td>
<td>Turton et al., 2006</td>
</tr>
<tr>
<td>OXA-58</td>
<td>5’AGTATTGGGGCCTTGCTG 3’ 5’AATCTTGTGCCTATTTG 3’</td>
<td>453</td>
<td>Ruiz et al., 2007</td>
</tr>
<tr>
<td>OXA-24</td>
<td>5’GGTTAGTGTGCGCCCTTAAA 3’ 5’GGTTAGGCGAAAGGGGATT 3’</td>
<td>345</td>
<td>Ruiz et al., 2007</td>
</tr>
<tr>
<td>OXA group I (OXA-10 group)</td>
<td>5’TCAACAAATCGCCAGAGAAG 3’ 5’TCCCCACACCAGAAAACCAG 3’</td>
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<td>Rasheed et al., 1997</td>
</tr>
</tbody>
</table>

Biofilm formation assay

Biofilm formation was assessed as modified from Stepanovic et al. (2000). Overnight cultures of isolates were prepared in tryptone soya broth (TSB). The turbidity was adjusted to 10^9 CFU/ml. Wells of sterile 96-well polystyrene micro plates with rounded bottom were inoculated with 100 μl of the bacterial suspension then incubated for 24 h at 37°C. After incubation period, bacterial suspension was gently aspirated from each well. Wells were washed three times with sterile phosphate buffered saline (pH 7.2) to remove any non-adherent cells. The adherent cells were fixed with 100 μl of 99% methanol for 20 min. The adherent cells were stained with 100 μl of crystal violet (1% ethanol) for 20 min. (after removing methanol), then the excess dye was removed, and the plate was air dried. The optical densities of the stained
adherent biofilms were measured at 490 nm using microplate reader. The test was made in triplicates and the average optical densities were calculated. The cut-off OD (ODc) was calculated. ODc is equivalent to mean OD of the negative control + 3 SD and the biofilm formation capacity was assessed as non-biofilm forming (OD ≤ ODc), weak biofilm forming (OD >ODc, but ≤ 2x ODc), moderate biofilm forming (OD>2x ODc, but ≤ 4x ODc), or strong biofilm forming (OD> 4x ODc).

- MIC of colistin

Two methods were adopted to measure the MIC of colistin for all isolates. The first one was performed by the VITEK 2 automated system as previously described (bioMérieux, Marcy l’Étoile, France). The second method was determined by broth microdilution method according to guidelines of CLSI (2021) recommendations and interpreted using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints for enterobacteriaceae (susceptible ≤2 mg/l; resistant=2 mg/l).

RESULTS

1- Collection and identification of isolates

Among 70 collected Gram-negative clinical isolates, only 25 isolates were identified as *P. aeruginosa*. Among these isolates, 72% and 28% were males and females, respectively (Fig. 1). According to age, the isolates were divided into 3 groups: 1-14 year, 14-30 and > 45 years where the high percentage were 48% in > 45 years demonstrated in Figure 2. As for the site of clinical isolates, the highest number of *P. Aeruginosa* isolates were recovered from wound (52%) followed by blood (16%). Lower percentages were detected in urine, sputum, CSF and plural fluid samples, 12, 12, 4 and 4%, respectively (Fig. 3).

![Fig. 1: Frequency of *P. aeruginosa* isolates according to patients' gender.](image1)

![Fig. 2: Frequency of *P. aeruginosa* isolates according to patients' age.](image2)
Prevalence of carbapenemase genes in extreme drug resistant *Pseudomonas aeruginosa* isolated from ICU in Egypt

**Fig. 3:** Percentage of clinical isolates distribution among different isolation sites.

### 2- Detection of OXA genes
Different OXA genes were detected in *P. aeruginosa*. OXA group I and group II were detected in 44% and 52%, respectively, and they were coexisted in 8 isolates however, OXA group III, OXA51, OXA23, OXA24 and OXA58 were absent (Fig. 4).

### 3- Biofilm formation
Only 44% of *P. aeruginosa* isolates showed strong biofilm formation, while moderate and weak biofilm formation was detected in 28 and 12% of the isolates, respectively. No biofilm formation was detected in 16% of *P. aeruginosa* isolates (Fig. 4). Strong biofilm formation was observed in isolates with or without OXA genes.

**Fig. 4:** Biofilm formation of *P. aeruginosa*.

### 5- MIC for colistin
All isolates were sensitive to colistin at ≥0.5 mg according to Vitek results while the MIC results using the Microtiter plate confirmed that three *P. aeruginosa* isolates were resistant to colistin at ≤ 4 mg, respectively. In *P. aeruginosa* the 3 resistant isolates were isolated from wound with moderate biofilm forming, only one of these wound isolates was negative for OXA1 and OXA11 (Fig. 5).
DISCUSSION

Pseudomonas aeruginosa has become an important cause of healthcare-associated infections (Sievert et al., 2013). Carbapenem has been widely used for empirical or directed therapy when a P. aeruginosa infection is suspected due to its natural resistance against several antibiotics (Siempos et al., 2007). Carbapenemase production is the most well-described resistance mechanism to carbapenems (Poirel et al., 2007). The present study showed that all clinical isolates of P. aeruginosa were resistant to carbapenem class (imipenem and meropenem). XDR and PDR P. aeruginosa were 88 and 12%, respectively, and the PDR isolates were resistant to colistin. These results were compatible with Nasirmoghadas et al. (2019) study that showed that P. aeruginosa isolates were almost resistant to all tested antibiotics, except polymixin B (2%). Saleem et al. (2018) and Preze et al. (2019) work was congruent with our study where 35.8 and 18.1% were XDR P. aeruginosa isolates. Parallel to our study, Palavutitotai et al. (2018) showed that 22% of P. aeruginosa exhibited an XDR phenotype.

In the present study, P. aeruginosa isolates were recovered from males higher than female isolates. Regarding the age, the high percentage of isolates age were >45 years old followed by 1-14 and 14-30. Wound showed the highest source of isolates compared with other sources followed by blood source. This was agreed with Khosravi et al. (2019) who reported that the percentage of male isolates were higher than female in 50 P. aeruginosa isolates with mean age 38.3 years, where all of them obtained from burn wound infections. Moreover, the results demonstrated by Rouhi and Ramazanzadeh (2018) agreed with our results, where they showed that male percentage was higher than female in both non nosocomial and nosocomial P. aeruginosa isolated with the mean age of 50.35±20.19 years, but the high percentage of isolates were obtained from urine, followed wound. Consistent with our results, Zafer et al. (2014) showed that the high source percentage of isolates was obtained from wound and blood. Contradictory, Sorour et al. (2008), Aghazadeh et al. (2016) and Mohamed et al. (2019) mentioned that urine was the major source of P. aeruginosa isolates compared with wound. Palavutitotai et al. (2018) study was compatible with our results where most patients (57.3%) were male, with a mean age of 64.9 (±17.6) years.

According to our OXA genes P. aeruginosa results Lee et al. (2005) Sorour et al. (2008) and Aghazadeh et al. (2016), were in consistent with this result because OXA group III was present in their studies in addition to OXA group I and OXA group II. Moreover, in Lee et al. (2005) and
Prevalence of carbapenemase genes in extreme drug resistant *Pseudomonas aeruginosa* isolated from ICU in Egypt

Sorour et al. (2008) studies OXA group III not only presented but also was coexisted with OXA group I and OXA group II. The great concern was that absence of OXA 51, OXA 23, OXA 24 and OXA 58 in recent tested *P. aeruginosa* isolates this inconsistent with Rouhi et al. (2018) due to not only some *P. aeruginosa* isolates had OXA-23 and OXA24/40 but also, they were coexisted in these isolates, in addition, OXA-23, OXA-24, OXA-51 and OXA-58 also present in Chaudhary and Payasi (2014) tested isolates.

Biofilms demonstrated greater protection against antibiotics, host immune defence, and adverse environmental conditions than the free-living cells (Gunn et al., 2016). It is estimated that 65–80% of human infectious are caused by biofilm-forming bacteria (Sun et al., 2013). Biofilm-associated infections are chronic infections; hence, they require higher doses of antibiotics for treatment than planktonic cells acute infection, with the resulting antimicrobial resistance having led to increased death, prolonged hospital stays, considerable economic loss, and loss of protection for patients (Yang et al., 2017). In this study, strong biofilm formation was high in *P. aeruginosa* isolates compared with moderate, weak and non-biofilm forming. These were compatible with many studies (Lima et al., 2017; Haji, 2018; Abdulhaq et al., 2019; Eladawy et al., 2020; Kamali et al., 2020) which demonstrated that biofilm formation in *P. aeruginosa* isolates showed different categories. Devaraj and Sajjan. (2015) described maximum biofilm production in *P. aeruginosa* 100% among Gram negative bacilli. In contrast to our study, Saxena et al. (2014) reported that out of 80 *P. aeruginosa* isolates the high percentage were weak biofilm forming then non biofilm forming where the lower percentage were strong and moderate.

Yung et al. (2017) found that most carbapenem-resistant transformants had decreased biofilm-forming capacity. The energy necessary for expressing the carbapenemase genes may decrease the biofilm formation. According to our results this relation was in consistent with above studied since some isolates showed weak and non-biofilm formation and had no any carbapenemase gene.

When the administration of a β-lactam, aminoglycoside, or quinolone is ineffective, the polymyxins, particularly colistin, remain as the antimicrobials drugs of last option. Furthermore, resistance to colistin is infrequently observed in spite of a daily selective pressure in patients receiving colistin by inhalation (Bialvaei and Kafil, 2015). Colistin, also called polymyxin E, and polymyxin B have been considered two of the last-resort treatments for such infections. Although the small use in human medicine in the past due to neurotoxicity and nephrotoxicity, colistin has been widely used in veterinary medicine to promote animal growth in the livestock and seafood industry (Lima et al., 2019). Colistin has anti-pseudomonal activity and has been used previously, either intravenously or in an aerosol form, for treatment of pneumonia caused by multidrug resistant *P. aeruginosa* (MDRPA) (Levin et al., 1999; Hamer, 2000). In this study, 12% of tested isolates were resistant to colistin, this was incompatible with Khosravi et al. (2019) who reported that *P. aeruginosa* isolates were sensitive to colistin. Zafer et al. (2014) was parallel with our results, where only 2.5% out of 122 *P. aeruginosa* isolates was resistant to polymyxin B. However, Aghazadeh et al. (2016) reported that colistin and polymyxin B had lowest level resistance (2.5% *P. aeruginosa* isolates). Low bacterial resistance (7%) was noted only with colistin by Mohamedet al. (2019) study. Also, Somily et al. (2012) and Afifi et al. (2013) reported 3 and 6% resistance rate of *P. aeruginosa* to colistin, respectively. Incompatible with the above-mentioned studies, Pokharel et al. (2019) reported 0%
resistance to colistin in \textit{P. aeruginosa} isolates.

In the present study one isolates out of 3 \textit{P. aeruginosa} colistin-resistant isolates was isolated from blood this result was lower than Balkhair \textit{et al.} (2019) who showed 9.1\% of carbapenem-resistant \textit{P. aeruginosa} isolated from blood. Colistin-resistant \textit{P. aeruginosa} can be related to two chromosomal mutations: 1) modification of lipid A and 2) loss of LPS showed a loss of 47,969 bp genomic regions containing some genes like \textit{ppk} and \textit{modA}, which have been previously related to biofilm production in enterobacteriaceae and pseudomonas by whole genome mapping (Dafopoulou \textit{et al.}, 2015). Thus, resistance to colistin can be associated with defective biofilm formation in \textit{Pseudomonas}. Biofilm formation is one of the antibiotic-resistance mechanisms in \textit{P. aeruginosa} and can lead to cross resistance based on the low penetration of antibiotics into the bacterial community after biofilm formation, and the appearance of MDR strains (Rashid \textit{et al.}, 2000; Owlia \textit{et al.}, 2014). This hypothesis may explain our study where 12\% \textit{P. aeruginosa} colistin-resistant isolates were moderate forming biofilm, while 88\% colistin-susceptible \textit{P. aeruginosa} 44\% were strong biofilm forming. Moreover, Azimi, and Lari (2019) supported our results, who showed biofilm formation was weak and moderate in 84 and 16\% of \textit{P. aeruginosa} colistin-resistant strains, respectively. In contrast, biofilm formation was strong and moderate in 52 and 32\% of colistin-susceptible \textit{P. aeruginosa} where \textit{modA} and \textit{ppk} genes were absent in colistin-resistant strains. On the other hand, all colistin-susceptible strains harbored at least one of these two biofilm formation genes, except two isolates.

\textbf{Conclusion:}

Prevalence of carbapenemase resistant genes and biofilm formation explains why high percentage isolates were XDR and other PDR also may give an explanation for colistin resistant isolates.

\textbf{REFERENCE}


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Nermin M.K. Mohamed et al.


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The prevalence of carbapenemases among Acinetobacter baumannii isolates from ICU in Egypt

Analysis of multidrug resistant Pseudomonas aeruginosa bloodstream infections in Egypt

Prevalence of carbapenemase genes in extreme drug resistant Pseudomonas aeruginosa isolated from ICU in Egypt
