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# Carbapenem resistance in non-carbapenemase-producing *Pseudomonas aeruginosa* strains: the role importance of OprD and AmpC

**Resistência aos carbapenêmicos em cepas de *Pseudomonas aeruginosa* não produtoras de carbapenemases: a importância do papel de OprD e AmpC**

**Resistencia a carbapenémicos en cepas de Pseudomonas aeruginosa no productoras de carbapenemasas: la importancia del papel de OprD y AmpC**

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**Abstract**

Carbapenem-resistance is a great challenge for antimicrobial therapy in *Pseudomonas aeruginosa* multidrug-resistant infections, as it reduces therapeutic options. This study investigated carbapenem-resistance mechanisms in six strains of non-carbapenemase-producing *P. aeruginosa*. Minimal inhibitory concentrations for imipenem and meropenem were determined by epsilometric test and broth microdilution. Mutations in the *oprD* gene were investigated by PCR, followed by sequencing. Transcriptional levels of *oprD*, *ampC*, and efflux pumps genes were analysed through RT-qPCR. Detection of efflux and AmpC activity was assessed by MIC reduction in the presence of the inhibitors: PAβN and cloxacillin, respectively. Resistant strains showed moderate levels of resistance for the evaluated carbapenems. Sequencing of *oprD* gene revealed similar mutation patterns in strains of the same Sequence Type -ST. A premature stop codon was detected only in the resistant strains of ST2236. Indel mutations were found in *the oprD* gene of ST2237 strains. Failure to detect *oprD* transcripts by RT-qPCR further confirms the absence of porin on ST2237 strains. ST2236 strains showed low transcriptional levels for *oprD*. MexXY-OprM was the only efflux system overexpressed in resistant strains. However, no efflux and AmpC activity was detected. High transcriptional levels of

*ampC* were found in 50% of non-induced resistant strains. All imipem-induced resistant strains showed an increase in *ampC* expression (>$10^2$ - $10^3$ X). It was concluded that the reduction and/or loss of OprD associated with AmpC overexpression is probably the main carbapenem-resistance mechanism in the evaluated strains.
**Keywords:** *Pseudomonas aeruginosa*; Carbapenems; Resistance; OprD; AmpC.

**Resumo**
A resistência aos carbapenêmicos é um grande desafio para a terapia antimicrobiana em infecções causadas por *Pseudomonas aeruginosa* multirresistentes, pois reduz as opções terapêuticas. Este estudo investigou os mecanismos de resistência aos carbapenêmicos em seis cepas de *P. aeruginosa* não produtores de carbapenemases. Concentrações inibitórias mínimas para imipenem e meropenem foram determinadas por teste epsilométrico e microdiluição em caldo. Mutações no gene *oprD* foram investigadas por PCR e sequenciamento. Os níveis transcricionais dos genes *oprD, ampC* e genes de bombas de efluxo foram analisados por RT-qPCR. A detecção de atividade de efluxo e AmpC foi avaliada pela redução da CIM na presença dos inibidores: PAβN e cloxacilina, respectivamente. Cepas resistentes apresentaram níveis moderados de resistência para os carbapenêmicos avaliados. O sequenciamento do gene *oprD* revelou padrões de mutação semelhantes em cepas do mesmo Tipo de Sequência -ST. Um códon de parada prematuro foi detectado apenas nas cepas resistentes do ST2236. Mutações do tipo inserções e deleções, foram encontradas no gene *oprD* das cepas ST2237. A falha em detectar transcritos de *oprD* confirma a ausência desta porina nas cepas ST2237. As cepas ST2236 apresentaram baixos níveis de transcrição para *oprD*. MexXY-OprM foi o único sistema de efluxo superexpresso em cepas resistentes. No entanto, nenhuma atividade de efluxo e AmpC foi detectada. Altos níveis transcricionais de *ampC* foram encontrados em 50% das cepas resistentes não induzidas. Todas as cepas resistentes induzidas por imipem mostraram um aumento na expressão de *ampC* (>$10^2$ - $10^3$ X). Concluiu-se que a redução e/ou perda de OprD associada à superexpressão de AmpC é provavelmente o principal mecanismo de resistência aos carbapenêmicos nas cepas avaliadas.
**Palavras-chave:** *Pseudomonas aeruginosa*; Carbapenêmicos; Resistência; OprD; AmpC.

**Resumen**
La resistencia a los carbapenémicos es un desafío importante para la terapia antimicrobiana en infecciones causadas por *Pseudomonas aeruginosa* multirresistente, ya que reduce las opciones terapéuticas. Este estudio investigó los mecanismos de resistencia a los carbapenemes en seis cepas de *P. aeruginosa* no productoras de carbapenemasas. Las concentraciones inhibitorias mínimas para imipenem y meropenem se determinaron mediante prueba epsilométrica y microdilución en caldo. Las mutaciones en el gen *oprD* se investigaron mediante PCR y secuenciación. Los niveles transcripcionales de los genes *oprD, ampC* y eflujo se analizaron mediante RT-qPCR. La detección de la actividad de eflujo y AmpC se evaluó reduciendo la CIM en presencia de los inhibidores: PAβN y cloxacilina, respectivamente. Las cepas resistentes mostraron niveles moderados de resistencia a los carbapenémicos evaluados. La secuenciación del gen *oprD* reveló patrones de mutación similares en cepas del mismo Tipo de Secuencia -ST. Se detectó un codón de terminación prematuro solo en cepas resistentes de ST2236. Se encontraron mutaciones de inserciones y deleciones en el gen *oprD* de las cepas ST2237. La falta de detección de transcripciones de *oprD* por RT-qPCR confirma la ausencia de esta porina en las cepas ST2237. Las cepas ST2236 mostraron bajos niveles de transcripción para *oprD*. MexXY-OprM fue el único sistema de eflujo sobreexpresado en cepas resistentes. Sin embargo, no se detectó flujo de salida ni actividad de AmpC. Se encontraron altos niveles transcripcionales de *ampC* en el 50% de las cepas resistentes no inducidas. Todas las cepas resistentes inducidas por imipem mostraron un aumento en la expresión de *ampC* (>$10^2$ - $10^3$ X). Se concluyó que la reducción y/o pérdida de OprD asociada a la sobreexpresión de AmpC es probablemente el principal mecanismo de resistencia a carbapenémicos en las cepas evaluadas.
**Palabras clave:** *Pseudomonas aeruginosa*; Carbapenémicos; Resistencia; OprD; AmpC.

## 1. Introduction

*Pseudomonas aeruginosa* is the most frequent microorganism in nosocomial infections affecting mainly immunocompromised patients (Kaiser et al., 2017). High mortality rates are associated with the ability of this pathogen to develop antimicrobial resistance. In addition to being intrinsically resistant, this bacterium has a remarkable capacity to acquire multiple resistance mechanisms through mutations on chromosomal genes leading to efflux pumps overexpression, loss of outer membrane porin (OprD), and acquisition of genes encoding enzymes that hydrolyzes or modify antimicrobial agents, among other mechanisms (De Rosa et al., 2019).

The emergence of multidrug-resistant (MDR) isolates, including carbapenem-resistant *P. aeruginosa* (CRPA), is a great challenge for antimicrobial therapy as it reduces therapeutic options. CRPA is in the high priority category on the global list of pathogens reported by the World Health Organization, in 2017 (Nordmann & Poirel, 2019). Carbapenem resistance is

often associated with enzymatic hydrolysis by carbapenemases (Pacheco et al., 2019). However, in absence of carbapenemases, the loss of OprD is the most prevalent mechanism among CRPA isolates, followed by overexpression of efflux pumps, like MexAB-OprM, MexEF-OprN, MexCD-OprJ and MexXY-OprM; and/or by overexpression of chromosomal AmpC cephalosporinase (Castanheira et al., 2014; Chalhoub et al., 2016; Feng et al., 2021).

This study aims to investigate carbapenem-resistance mechanisms in six isolates of non-carbapenemase-producing *P. aeruginosa*.

## 2. Methodology

This is a descriptive study with a quantitative approach (Pereira et al., 2018), carried out with *P. aeruginosa* strains, which are part of strains collection recovered from burn patients and balneotherapy tanks in a previous work (Deutsch et al., 2016), which was approved by the Ethics Committee of the Universidade Federal Fluminense by the number 68538.

Six non-carbapenemase-producing *P. aeruginosa* clinical strains, of these, five were resistant to both carbapenems - imipenem and meropenem (CRPA) and one resistant only to imipenem (IRPA) and five carbapenem-susceptible *P. aeruginosa* (CSPA) strains were analyzed in this study, as presented in Table 1. All carbapenem-resistant strains (CRPA and IRPA), as well as one susceptible, were typified previously by MLST in two different STs, ST2236 and ST2237 (de Almeida Silva et al., 2017). *Pseudomonas aeruginosa* PAO1 and ATCC27853 were used as control strains.

**Table 1.** Antimicrobial resistance profile and molecular typing of *P. aeruginosa* strains, resistant and susceptible to carbapenems, collected from burned patients and balneotherapy tanks.

| | | Strains | Source | *Antimicrobial Resistance* | PFGE | MLST |
|---|---|---|---|---|---|---|
| ***MDR*** | **CRPA** | **2** | P | CAZ, ATM, IMP, MER, GEN, CIP | D | ST2237 |
| | | **3** | P | CAZ, ATM, IMP, MER, GEN, CIP | A2 | ST2236 |
| | | **4** | B | ATM, IMP, MER, GEN, CIP | E | ST2237 |
| | | **5** | P | ATM, IMP, MER, GEN, CIP | NT | ST2237 |
| | | **31** | P | ATM, IMP, MER, GEN, CIP | C | ST2237 |
| | **IRPA** | **24** | B | CAZ, IMP, GEN, CIP | A1 | ST2236 |
| ***Non-MDR*** | **CSPA** | **16** | P | GEN, CIP | I | * |
| | | **17** | P | GEN, CIP | A4 | * |
| | | **20** | P | CIP | A7 | * |
| | | **21** | P | GEN, CIP | A1 | * |
| | | **26** | B | GEN, CIP | A1 | ST2236 |

MDR: multidrug-resistant; CRPA: carbapenem-resistant *P. aeruginosa*; IRPA: imipenem-resistant *P. aeruginosa;* CSPA: carbapenem-susceptible *P. aeruginosa*; P= pacient; B= balneotherapy tank; CAZ= ceftazidime; ATM= aztreonam; GEN= gentamicin; IMP= imipenem; MER= meropenem; CIP= ciprofloxacin; NT= nontypeable; *: unperformed. Source: Authors.

Minimum inhibitory concentrations (MICs) for imipenem and meropenem were determined in all strains by epsilometric test using M.I.C.Evaluator™ strips (Oxoid Ltd.). For carbapenem-resistant strains, MICs for both antibiotics (imipenem and meropenem – Sigma-Aldrich) were also determined by broth microdilution method, using cation-adjusted Mueller-Hinton broth, according to Clinical and Laboratory Standards Institute (CLSI; 2018) guidelines. *Pseudomonas aeruginosa* ATCC 27853 was used as the control strain in all susceptibility tests.

To evaluate efflux pumps activity, MICs were determined in the presence and absence of 50 µg/mL of the efflux pump inhibitor phenylalanine arginine β-naphthylamide -PAβN (Sigma-Aldrich), added from 1mM $MgSO_4$ to strengthen the outer membrane (Chalhoub et al., 2016). Similarly, AmpC activity was achieved by MIC determination in the presence and absence of 200 µg/mL of AmpC inhibitor cloxacillin (Sigma-Aldrich). The presence of efflux pumps or AmpC activity was considered when MICs determined in the presence of inhibitors were at least four-fold lower than MICs in their absence (Goli et al., 2018; Rodríguez-Martínez et al., 2009).

Mutations in the *oprD* sequence were investigated in all carbapenem-resistant strains (CRPA and IRPA) and one CSPA (strain 26). The full-length *oprD* gene was amplified by polymerase chain reaction (PCR), according to previous work (Ocampo-Sosa et al., 2012) using specific primers as shown in Table 2.

**Table 2 -** Primers used for PCR and RT-qPCR.

| Gene | Primer | Sequence 5'-3' | Use | References |
|---|---|---|---|---|
| *oprD* | OprD_FlankF<br>OprD_FlankR | CGGCTGAGGGGAAAGTCGCC<br>TACGCGGTCATTCTCGGGCG | Amp. and Seq. | (Ocampo-Sosa et al., 2012) |
| | OprD- F<br>OprD-R | GGAACCTCAACTATCGCCAAG<br>GTTGCCTGTCGGTCGATTAC | Amp. and Seq. | (Rodríguez et al., 2010) |
| | OprD2-F<br>OprD2-R | ACTTCACCGAGGGCAAGG<br>CAGAGTTGGCGAGGAAAATC | Amp. and Seq. | (Rodríguez et al., 2010) |
| | OprDrt –F<br>OprDrt-R | CTACGGCTACGGCGAGGAT<br>GACCGGACTGGACCACGTACT | RT-qPCR | (Quale et al., 2006) |
| *mexA* | MexA-F<br>MexA-R | AACCCGAACAACGAGCTG<br>ATGGCCTTCTGCTTGACG | RT-qPCR | (Quale et al., 2006) |
| *mexX* | MexX-F<br>MexX-R | GGCTTGGTGGAAGACGTG<br>GGCTGATGATCCAGTCGC | RT-qPCR | (Quale et al., 2006) |
| *mexC* | MexC-F<br>MexC-R | GTACCGGCGTCATGCAGGGTTC<br>TTACTGTTGCGGCGCAGGTGACT | RT-qPCR | (Chalhoub et al., 2016) |
| *mexE* | MexE-F<br>MexE-R | TACTGGTCCTGAGCGCCT<br>TCAGCGGTTGTTCGATGA | RT-qPCR | (Quale et al., 2006) |
| *ampC* | AmpC-F<br>AmpC-R | CGCCGTACAACCGGTGAT<br>CGGCCGTCCTCTTTCGA | RT-qPCR | (Quale et al., 2006) |
| *rpoD* | RpoD-F<br>RpoD-R | GGGCTGTCTCGAATACGTTGA<br>ACCTGCCGGAGGATATTTCC | RT-qPCR | (Quale et al., 2006) |

Amp.= amplification; Seq.= sequencing; RT-qPCR= real time- quantitative PCR. Source: Authors.

PCR products were sequenced by 3130 Genetic Analyser® (Applied Biosystems). The nucleotide sequences obtained were analyzed using the software Lasergene (DNAstar) and BioEdit (Ibis Biosciences) and then compared to the sequence of the *oprD* gene from the reference strain PAO1 (GenBank Gene ID: 881970).

Amino acid sequence alignment and secondary structure depiction of OprD from different strains were carried out using ESPript 3.0 and OprD amino acid sequence from PAO1 as reference (GenBank Accession Number: CAA78448). Secondary structure was extracted from PAO1 OprD crystal structure deposited in the Protein Data Bank (PDB ID: 3SY7).

Three-dimensional models of OprD protein were generated for strain "26" (CSPA) and strain "3" (CRPA), from the respective amino acid sequences using the SWISS-MODEL web server - https://swissmodel.expasy.org/. The OprD models were generated using the three-dimensional structure of the *P. aeruginosa* OprD (code PDB 3SY7, also called OccD1) relative to the reference sequence (code UniProtKB P32722), experimentally determined (Eren et al., 2012).

Transcriptional levels of *mexA*, *mexX*, *mexC*, *mexE* (efflux genes), *oprD* and *ampC* genes, were determined by real-time quantitative PCR (RT qPCR), as previously described (Castanheira et al., 2014), with some modifications. Overnight cultures of all strains were diluted 1:100 in Tryptic Soy broth (TSB) and grown to mid-log phase (optical density at 600 nm [$OD_{600}$] ~ 0.3 to 0.5) at 37° C and 150 rpm. Resistant strains (CRPA and IRPA) were cultured in duplicate to evaluate the transcription of resistance genes in the presence and absence of imipenem as an inducing agent. Therefore, part of the carbapenem-resistant strains cultures was exposed to imipenem at 4 µg/mL after 5 h of growth (early-log phase). After growth to the mid-log phase (6 h), bacterial cells were harvested by centrifugation. Total RNA was extracted using the PureLink RNA Mini Kit (Ambion) and treated with RQ1 RNase-Free DNase (Promega) to eliminate residual DNA. Synthesis of cDNA was performed by reverse transcription of 500 ng of purified RNA, using High-Capacity RNA-to-cDNA Kit (Applied Biosystems). Quantification of the transcripts was performed in a Step One Real-Time PCR System (Applied Biosystems) using specific primers as described previously in Table 2, and Power SYBR Green PCR Master Mix (Applied Biosystems). The housekeeping gene *rpoD* was used to normalize the relative amount of mRNA. Relative gene expression was achieved by using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Strains were considered MexAB-OprM hyperproducers if the transcriptional levels of *mexA* were at least three-fold higher than that of the reference strain PAO1. If *mexA* relative transcription was lower than two-fold or between two- and three-fold the overexpression of MexAB-OprM was considered negative or borderline, respectively (Cabot et al., 2011). Strains were considered hyperproducers of MexEF-OprN, MexCD-OprJ, MexXY-OprM, or AmpC if the transcriptional levels of *mexE*, *mexC*, *mexX*, and *ampC* were at least 10-fold higher than that of PAO1, respectively. The overexpression of the same genes was considered negative if relative transcription was lower than 5-fold and borderline if between 5- and 10-fold (Cabot et al., 2011). Reduced *oprD* expression was considered relevant when its transcriptional levels were ≤30% compared with that of PAO1 (Wi et al., 2018).

## 3. Results

CRPA strains showed MICs of imipenem ranging from 16 to >32 µg/mL and of meropenem ≥32 µg/mL by using epsilometric test, as shown in Table 3. Except for the IRPA strain "24", which was susceptible to meropenem and presented MIC= 2 µg/mL for this antibiotic. All CSPA strains exhibited MIC values of 0.25 µg/mL and 1 µg/mL for meropenem and imipenem, respectively.

**Table 3.** Meropenem and Imipenem MICs for *P. aeruginosa* strains.

| | | MIC (µg/mL) | | | |
|---|---|---|---|---|---|
| | | ***Epsilometric test*** | | ***Broth microdilution*** | |
| | **Cepas** | **MER** | **IMP** | **MER** | **IMP** |
| **CRPA** | 2 | >32 | 32 | 16 | 16 |
| | 3 | >32 | >32 | 16 | 16 |
| | 4 | >32 | 16 | 16 | 16 |
| | 5 | >32 | 16 | 16 | 16 |
| | 31 | 32 | 32 | 16 | 16 |
| **IRPA** | 24 | 2 | >32 | 1 | 16 |
| **CSPA** | 16 | 0,25 | 1 | * | * |
| | 17 | 0,25 | 1 | * | * |
| | 20 | 0,25 | 1 | * | * |
| | 21 | 0,25 | 1 | * | * |
| | 26 | 0,25 | 1 | * | * |

CRPA: carbapenem-resistant *P. aeruginosa*; IRPA: imipenem-resistant *P. aeruginosa;* CSPA: carbapenem-susceptible *P. aeruginosa*; MER: meropenem; IMP: imipenem; *: unperformed. Source: Authors.

As presented in Table 3, MIC values were confirmed by broth microdilution for all carbapenem-resistant strains (CRPA and IRPA). These strains showed MIC=16 µg/mL for both carbapenems. Only the IRPA, strain "24", showed MIC=1 µg/mL for meropenem.

No efflux pumps and ampC activity was detected for both carbapenems in the strains evaluated. Except for strain "31", which presented efflux pumps activity for meropenem.

Sequencing of *oprD* gene revealed both indel and point mutations as described in Table 4, with similar mutation patterns in strains of the same ST.

**Table 4**. Mutations found in *oprD* gene among *P. aeruginosa* strains in this study.

| | Strains | |
|---|---|---|
| **Mutations** | **ST2237** | **ST2236** |
| $_{nt32}$T →C | | 26 |
| $_{nt122-123}$ΔpGC (frameshift) | 2, 4, 5 and 31 | |
| $_{nt243}$T →C; $_{nt276}$G →C; $_{nt279}$C →T; $_{nt300}$G →C; $_{nt308}$G →C; $_{nt312}$C →T; $_{nt344}$A →C; $_{nt348}$G →C; $_{nt351}$C →T; $_{nt354}$T →C | 2, 4, 5 and 31 | 3, 24 and 26 |
| $_{nt357}$C →T | | 3, 24 and 26 |
| $_{nt372}$C →T; $_{nt387}$G →A; $_{nt406}$C →T; $_{nt426}$A →G; $_{nt435}$C →T; $_{nt450}$T →C; $_{nt492}$G →A; $_{nt508}$T →C; $_{nt522}$C →T; $_{nt528}$G →A; | 2, 4, 5 and 31 | 3, 24 and 26 |
| $_{nt529-530}$GC →AG; $_{nt530-531}$*C | 31 | |
| $_{nt531}$A →G; $_{nt546}$G →A; $_{nt553}$G →C; $_{nt556-558}$CCG →GGC; $_{nt565-566}$GT →AC; $_{nt570}$A →G; $_{nt576}$T →C; $_{nt588}$T →C; $_{nt591}$C →A; $_{nt597}$C →T; | 2, 4, 5 and 31 | 3, 24 and 26 |
| $_{nt619-620}$GC →CG; $_{nt622-624}$GAT →CGA; $_{nt627-628}$CA →TC; $_{nt628-629}$*ATT; $_{nt629-630}$TT →GG | 31 | |
| $_{nt630-631}$*G | 2 | |
| $_{nt635}$G →C | 31 | |
| $_{nt678}$C →T; $_{nt681}$C →T; $_{nt702}$C →T; | 2, 4, 5 and 31 | 3, 24 and 26 |
| $_{nt756}$G →A | | 3, 24 and 26 |
| $_{nt928-930}$CGC →GAG; $_{nt936}$C →T; $_{nt939}$T →C; $_{nt944-945}$CA →GC; $_{nt948}$T →C; $_{nt951}$C →T; $_{nt975}$T →C; $_{nt978}$C →G; $_{nt1002}$T →C; $_{nt1023}$T →C; $_{nt1041}$A →C; $_{nt1161}$T →C; $_{nt1176}$A →G; $_{nt1185}$C →G; | 2, 4, 5 and 31 | 3, 24 and 26 |
| $_{nt1241}$G →T; $_{nt1244-1246}$AGG →CCC; | 2 and 4 | |
| $_{nt1257}$T →C | 2, 4, 5 and 31 | 3, 24 and 26 |
| $_{nt1270}$CAG →TAG (premature stop codon) | | 3 and 24 |
| $_{nt1274}$C →G; | 2, 4, 5 and 31 | 3, 24 and 26 |

Δp: base pair deletion; *: base insert. Source: Authors.

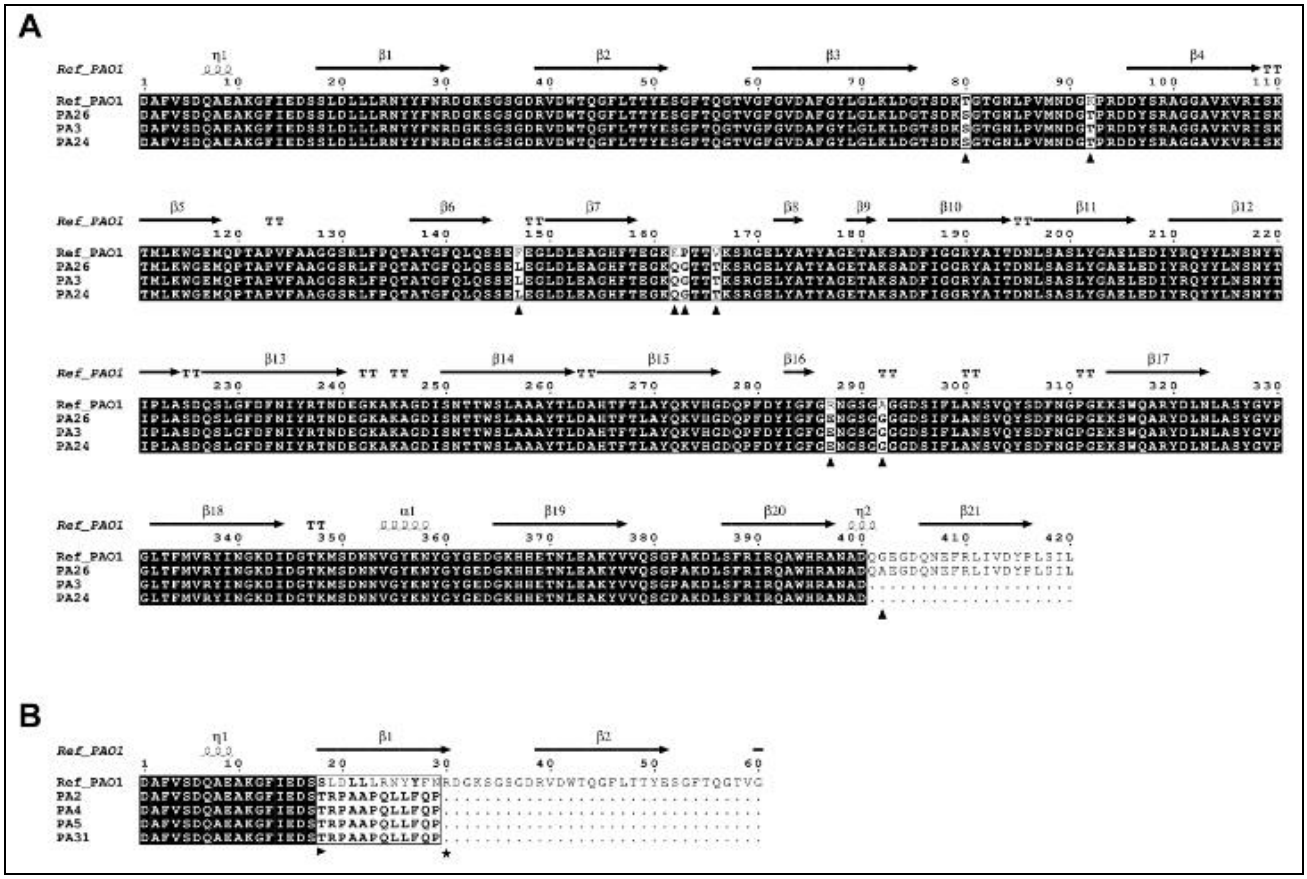
As shown in Table 4, no indel mutation was detected in the *oprD* gene of ST2236 strains (3, 24, and 26), only base pair substitutions. Among ST2236 strains, the major difference on the sequences was a point mutation present at nucleotide position 1270 in the CRPA (strain 3) and IRPA (strain 24), then absent in the CSPA strain (strain 26), that replaced cytosine (C) by thymine (T) and generated a TAG stop codon.

Amino acid sequence analysis showed that this premature stop codon shortens the resulting protein by 20 amino acids (Figure 1A) and eliminates the last β-sheet of the porin (Figure 2). Besides the shorter chain for IRPA and CRPA strains, further amino acid sequence analysis of ST2236 strains showed others amino acid substitutions ($T_{80}S$, $K_{92}T$, $F_{147}L$, $E_{162}Q$, $P_{163}G$, $V_{166}T$, $R_{287}E$, $A_{292}G$) which are also present in CSPA (strain 26) and are highlighted in Figure 1A.

On the other hand, ST2237 strains (2, 4, 5, and 31) *oprD* gene presented a common deletion of two base pairs (ΔpGC) at nucleotide positions 122-123 (Table 4). This indel mutation caused a frameshift in *oprD* sequence, leading to the formation

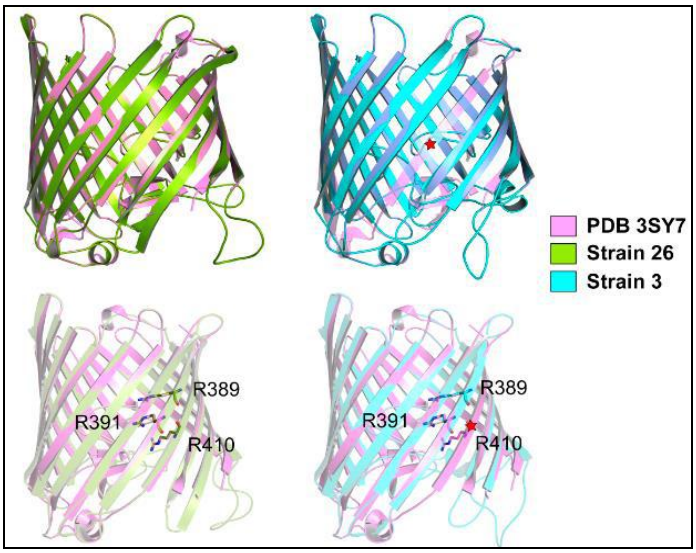
of a premature stop codon at nucleotide position 157. This stop codon interrupted the OprD chain at amino acid position 30, right at the end of the first β-sheet (Figure 1B), causing loss of the porin.

**Figure 1:** Multiple sequence alignment of OprD amino acid sequences of (A) ST2236 and (B) ST2237 strains.




OprD sequence and secondary structure from strain PAO1 was used as reference for the alignment. Up-arrowheads shows amino acids substitutions in that position; right-arrowhead signals frameshift in that position; star signals a premature stop codon at that position.
Source: Authors.

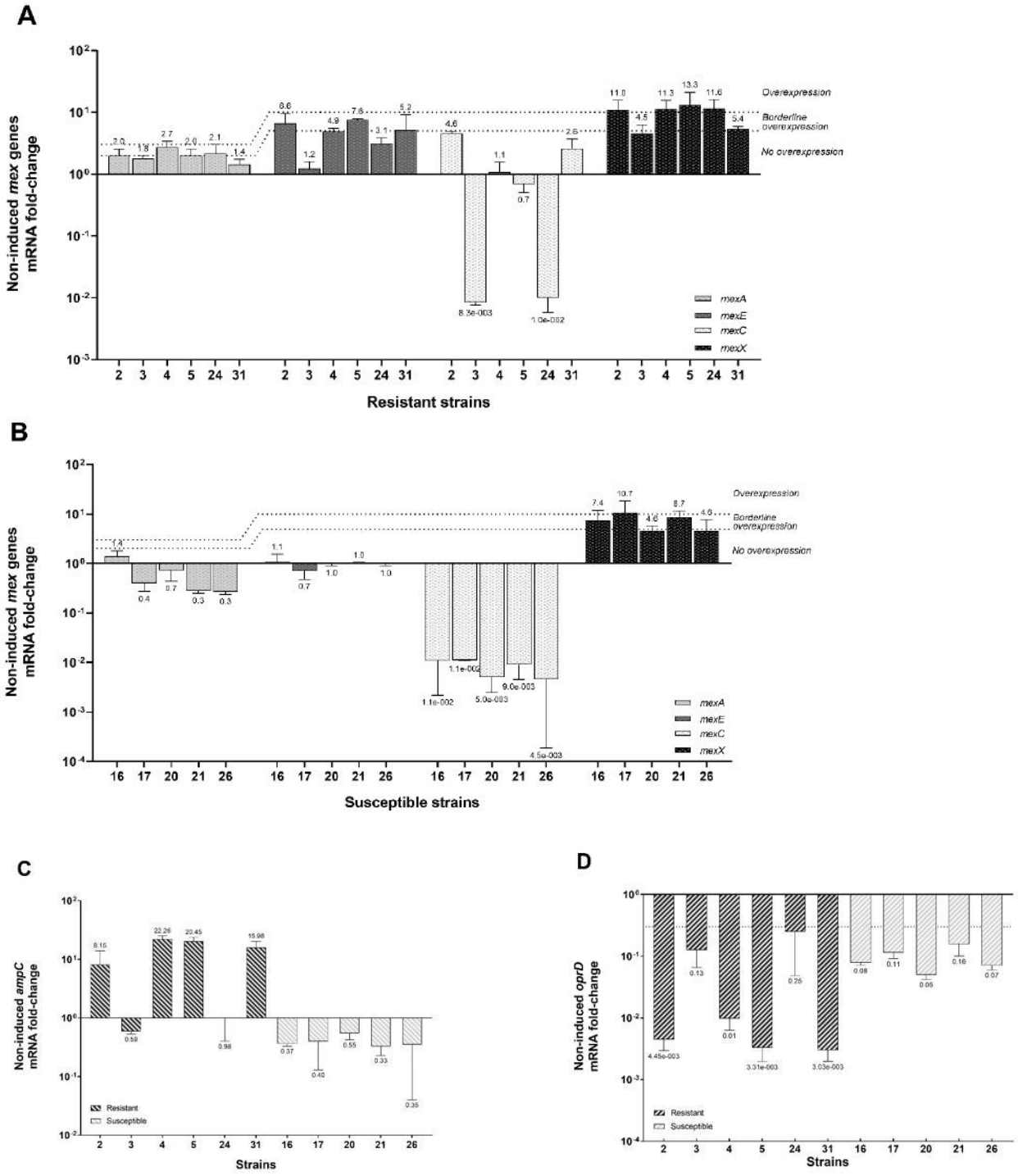
**Figure 2**. OprD three-dimensional models.




The missing β-sheet, due to the deletion of the 10 amino acid residues from strain 3, is highlighted with a red star. Residue numbering relative to the structure of OccD1, experimentally determined (code 3SY7). The peptide-signal sequence (MKVMKWSAIALAVSAGSTQFAVA) was not included in the sequence alignment and the modeling process. Source: Authors.

Efflux pumps overexpression was detected only for MexXY-OprM system evaluated in CRPA and IRPA strains not induced by carbapenem (Figure 3A).

**Figure 3.** Relative expression of resistance-related genes from *P. aeruginosa* clinical strains.



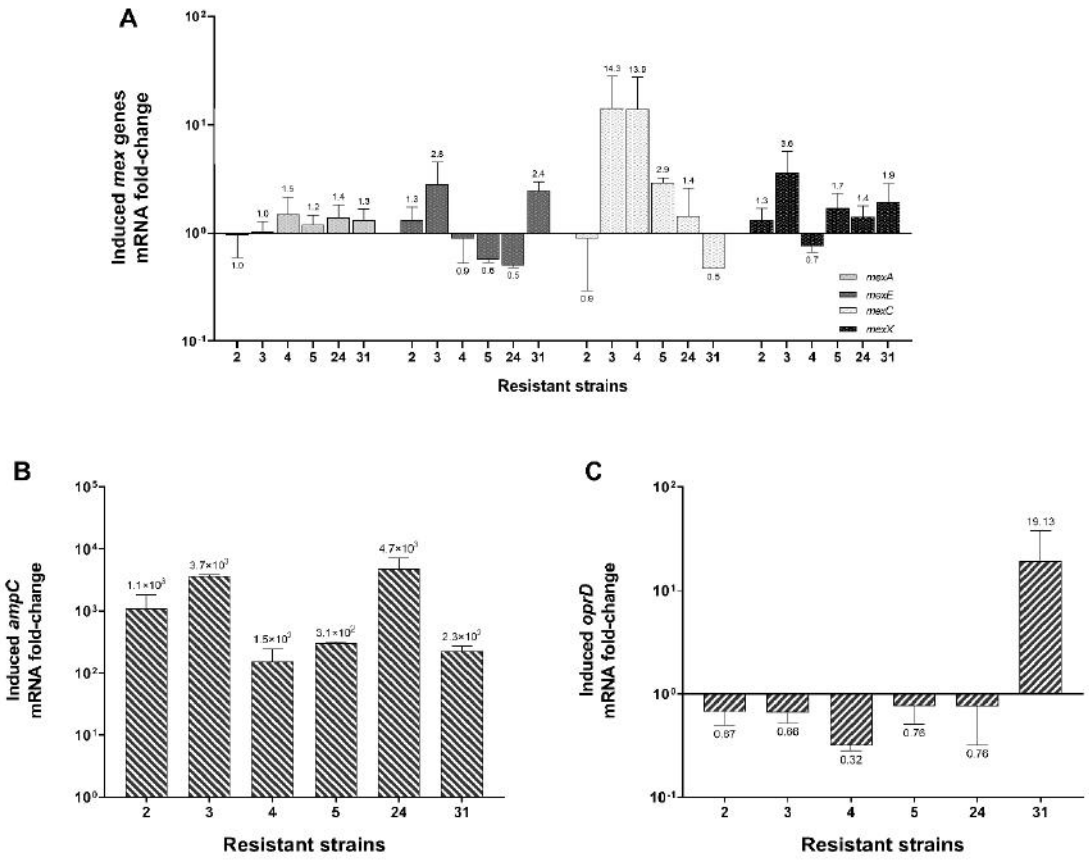

RT-qPCR transcription analysis of **(A, B)** efflux pumps genes, **(C)** *ampC*, and **(D)** *oprD* from non-induced carbapenem-resistant (2,3,4, 5, 24 and 31) and carbapenem-susceptible (16, 17, 20, 21 and 26) strains grown to mid-log phase. Results are expressed as fold change relative to expression levels of each gene in PAO1 strain. Source: Authors.

As shown in Figure 3A, overexpression of the *mexX* gene was present in 60% of CRPA and IRPA strain (strain 24). However, borderline expression was observed for the efflux genes *mexA* (60% of CRPA and in IRPA strain), *mexE* (60% of CRPA), and *mexX* (20% of CRPA). CSPA strains displayed no overexpression of any of the assessed efflux pumps, except strain "17", which showed *mexX* overexpression (Figure 3B). Three out of five non-induced CRPA strains (4, 5 and 31)

exhibited overexpression of *ampC* (Figure 3C). No *ampC* overexpression was detected in CSPA strains, which presented lower transcript quantification than PAO1 (Figure 3C). Low transcriptional levels for *oprD*, below 0.3 (<30%), were observed in non-induced CRPA and IRPA strains (Figure 3D), suggesting reduced OprD expression on these strains. Moreover, ST2237 strains (2, 4, 5, and 31) presented no detectable transcription of *oprD* (Figure 3D), implying loss of porin. CSPA strains also showed low transcriptional levels of *oprD* (Figure 3D), below 0.3 (<30%).

Compared to non-induced CRPA and IRPA strains, expression induction of at least 1.5-fold in the presence of imipenem was noted for the *mexA* (20% of CRPA), *mexE* (40% of CRPA), *mexC* and *mexX* (60% of CRPA) on induced strains (Figure 4A).

**Figure 4.** Relative expression of resistance-related genes from *P. aeruginosa* clinical strains, after exposure to imipenem.




RT-qPCR transcription analysis of **(A)** efflux pumps genes, **(B)** *ampC* and **(C)** *oprD* from carbapenem-resistant strains grown to mid-log phase, with addition of imipenem (4 µg/mL) after 5h of growth. Results are expressed as fold change relative to expression levels of non-induced gene from each strain. Source: Authors.

As presented in Figure 4B, all induced CRPA and IRPA strains showed an increase in *ampC* expression, on the order of $10^2$-$10^3$ times higher than non-induced strains. In the presence of the antibiotic, a 19.1-fold increase was detected in the transcriptional level of the *oprD* gene for the strain "31" (Figure 4C), compared to non-induced expression.

## 4. Discussion

Resistance to carbapenems is an emerging event of great concern in the world public health scenario. The clinical importance of carbapenems in combating infection by *P. aeruginosa* MDR is due to their broad-spectrum antibacterial activity and stability to most beta-lactamases, such as extended-spectrum beta-lactamases – ESBLs (Pragasam et al., 2016). However,

they can be hydrolyzed by carbapenemases, which leads to high MIC values for carbapenems (Codjoe & Donkor, 2017). Nevertheless, MexAB-OprM overexpression and OprD inactivation, associated with AmpC overexpression, drive to high-level resistance to meropenem (MIC ≥ 64 µg / mL) in non-carbapenemase-producing *P. aeruginosa* (Chalhoub et al., 2016). In the present study, moderate levels (MIC = 16 µg / mL) of resistance were found for both carbapenems in CRPA strains and only for imipenem in IRPA strain, confirmed by broth microdilution test. According to Li and collaborators, *P. aeruginosa* whose OprD expression is reduced and does not show efflux pumps overexpression exhibits moderate resistance to imipenem (Li et al., 2012).

Mutations in the *oprD* gene sequence were found across all CRPA and IRPA strains and were particularly impactful in ST2237 strains. The premature stop codon on these strains led to the loss of the porin, like premature stop codons previously described (Ocampo-Sosa et al., 2012). Failure to quantify *oprD* transcripts by RT-qPCR further confirms the absence of a functional porin on ST2237 strains. Mutations in CRPA and IRPA strains of ST2236 also resulted in premature stop codons, but with loss of only 20 amino acids instead of the 390 from ST2237. The exact nine amino acid substitutions found on CSPA strain "26" of ST2236 were previously detected in three strains from a study by Ocampo-Sosa and collaborators (2012), with *P. aeruginosa* strains susceptible to imipenem and meropenem. However, the same study did not detect the premature stop codon that shortened OprD in the resistant strains "3" and "24" of ST2236. Due to the absence of one of the 18 β-sheet in strain "3", it has no residue equivalent to the Arg410 from the reference framework (Figure 2). Studies of mutagenesis demonstrate that the substitution of any of the three arginines (Arg389, Arg391 e Arg410) significantly reduces the influx of substrates like carbapenems (Eren et al., 2013). These alterations in the OprD sequence may explain transcription analysis results found for *oprD*, in which ST2236 strains exhibited extremely low transcriptional levels for *oprD* (<0.3) and ST2237 strains did not express this gene at all. Very low transcriptional levels of *oprD* (<0.3) have also been found in CSPA strains, suggesting reduced porin expression in these strains. Other studies have also detected a significant reduction in *oprD* mRNA and frameshift mutations in carbapenem-susceptible clinical isolates (de Oliveira Santos et al., 2019; Liu et al., 2018). These data indicate that loss or reduction of OprD is not limited to CRPA strains.

Efflux pumps overexpression was noticed only for MexXY-OprM system in the resistant strains evaluated. According to Khalili and collaborators (Khalili et al., 2019), MexXY-OprM overexpression was detected in 68.8% of non-carbapenemase-producing *P. aeruginosa*, followed by MexAB-OprM overexpression (60.9%). Another study also reported the overexpression of MexXY-OprM in carbapenem-resistant strains (Petrova et al., 2019). As most efflux systems are not substrate-specific, MexXY-OprM system exports in addition to β-lactams, other antimicrobials such as macrolides, fluoroquinolones, chloramphenicol, tetracycline, and is described as the only efflux system for aminoglycosides (Kumari et al., 2014). For this reason, the detection of efflux activity was carried out to evaluate its role in meropenem and imipenem resistance. According to the result of this test, only CRPA strain "31" exhibited efflux activity, suggesting that efflux pumps overexpression is not the main mechanism of carbapenem resistance in the studied strains.

High transcriptional levels (> 10X) of *ampC* were found in 50% of non-induced resistant strains (CRPA and IRPA). The constitutive overexpression of AmpC detected in the non-induced CRPA strains of ST2237, probably originates from acquired mutations in genes involved in *ampC* transcription regulation, like *ampD*, *ampR* and being less frequent in *ampG* (Tamma et al., 2019). However, all induced CRPA and IRPA strains presented a remarkable increase ($10^2$-$10^3$-fold) in transcriptional levels of *ampC* compared to non-induced resistant strains. This data demonstrates that AmpC is strongly induced by imipenem in these strains.

Since CRPA and IRPA strains were also resistant to other β-lactams, the activity of AmpC was evaluated by using cloxacillin as an inhibitor of this enzyme. In this test, AmpC activity for imipenem and meropenem was not detected for any resistant strains. Although this test has not demonstrated the contribution of AmpC to the carbapenem-resistance phenotype

studied, the transcriptional levels of *ampC* demonstrated overexpression of this enzyme, mainly when induced by imipenem. According to another study, the use of cloxacillin as an AmpC inhibitor may fail to characterize AmpC-variants, such as extended-spectrum AmpCs – ESACs (Berrazeg et al., 2015). AmpC-producing isolates are of great concern since they become resistant during antibiotic therapy owing to AmpC overproduction (Ito et al., 2018).

These results highlighted the importance of AmpC overexpression in carbapenems resistance in the evaluated strains since *oprD* reduced expression was present in both CRPA/IRPA and CSPA strains. Consistent with Horner and collaborators, loss of OprD only confers imipenem resistance in *P. aeruginosa* if AmpC β-lactamase is expressed (Horner et al., 2019). Another study demonstrated that AmpC overexpression along with OprD deficiency altered the resistance phenotype of an isolate susceptible to imipenem, making it resistant, as well as reducing its susceptibility to meropenem and biapenem (Xu et al., 2020).

## 5. Conclusion

In conclusion, our findings demonstrated that the reduction and/or loss of OprD porin associated with AmpC overexpression seems likely to be the main determinants of resistance to carbapenems in the evaluated strains.

AmpC overexpression is probably the main cause of therapeutic failure during antimicrobial therapy with imipenem in the studied strains, due to its high induction by this antibiotic.

For future studies, we suggest the sequencing of the *ampC* genes of the evaluated strains, to determine whether they are ampC variants such as extended-spectrum AmpCs (ESACs), and thus promote the development of β-lactamases inhibitors that target AmpC cephalosporinase, and its variants.

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