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Carbapenem-resistant and cephalosporin-susceptible: a worrisome phenotype among *Pseudomonas aeruginosa* clinical isolates in Brazil

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ABSTRACT

The mechanisms involved in the uncommon resistance phenotype, carbapenem resistance and broad-spectrum cephalosporin susceptibility, were investigated in 25 *Pseudomonas aeruginosa* clinical isolates that exhibited this phenotype, which were recovered from three different hospitals located in São Paulo, Brazil. The antimicrobial susceptibility profile was determined by CLSI broth microdilution. β-lactamase-encoding genes were investigated by PCR followed by DNA sequencing. Carbapenem hydrolysis activity was investigated by spectrophotometer and MALDI-TOF assays. The mRNA transcription level of *oprD* was assessed by qRT-PCR and the outer membrane proteins profile was evaluated by SDS-PAGE. Genetic relationship among *P. aeruginosa* isolates was assessed by PFGE. Carbapenems hydrolysis was not detected by carbapenemase assay in the carbapenem-resistant and cephalosporin-susceptible *P. aueruginosa* clinical isolates. OprD decreased expression was observed in all *P. aeruginosa* isolates by qRT-PCR. The outer membrane protein profile by SDS-PAGE suggested a change in the expression of the 46 kDa porin that could correspond to OprD porin. The isolates were clustered into 17 genotypes without predominance of a specific PFGE pattern. These results emphasize the involvement of multiple chromosomal mechanisms in carbapenem-resistance among clinical isolates of *P. aeruginosa*, alert for adaptation of *P. aeruginosa* clinical isolates under antimicrobial selective pressure and make aware of the emergence of an uncommon phenotype among *P. aeruginosa* clinical isolates.

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Introduction

Pseudomonas aeruginosa is one of the most frequent pathogens associated to nosocomial infections, especially among immunocompromised patients[1] and exhibits notorious versatility and capacity to acquire resistance mechanisms to antimicrobial therapy.[2] Beta-lactam antimicrobial agents are the main option to treat serious infection caused by this pathogen. However, the production of β-lactamases, such as cephalosporinases and carbapenemases, has been intensely reported among clinical isolates of *P. aeruginosa* from Latin America and represents the most effective mechanism of β-lactams resistance reported among Gram-negative worldwide.[3]

Since the carbapenems molecules are more resistant to hydrolysis activity by a great number of spread serine-β-lactamases, these drugs have a particular value in the treatment of infections caused by cephalosporinases producer strains,[4] which remain susceptible to carbapenems. In *P. aeruginosa*, the carbapenems resistance is modulated by acquired carbapenemases in association with intrinsic mechanisms such as down-regulation or loss of OprD porin, efflux pumps hyperexpression, chromosomal AmpC β-lactamase production, and target alterations.[2,3] However, since carbapenemases have the ability to hydrolyze penicillins, cephalosporins, besides carbapenems, Gram-negative bacteria carrying a carbapenemase-encoding gene frequently exhibit resistance to virtually all β-lactams.[5]

Given the importance of carbapenem for the treatment of infections caused by *P. aeruginosa*, it is essential to clarify the mechanisms involved in unusual and/or poorly known phenotypes. Knowledge of these mechanisms alert for an adaptation to the selective pressure exerted by antimicrobial and drug resistance development, thus affecting the treatment of infections caused by these pathogens often restricted to only polymyxins.

The aim of this study was to analyze the possible mechanism of antimicrobial resistance involved in clinical isolates of *P. aeruginosa* that exhibited an uncommon phenotype of resistance: resistance to carbapenems but susceptibility to broad-spectrum cephalosporins (Carb-R/Ceph-S).

Methods

Bacterial isolates, identification and antimicrobial susceptibility testing

Between May, 2012 and March, 2013, a total of 25 *P. aeruginosa* clinical isolates exhibiting carbapenem resistance but broad-spectrum cephalosporin susceptibility (Carb-R/Ceph-S) were recovered from 18 different infected patients from three distinct hospitals located in São Paulo, Brazil (Table 1). The identification at the species level was confirmed by MALDI-TOF MS in a Bruker Daltonics Microflex LT MALDI-TOF using the Biotyper MALDI 2.0 (Bruker Daltonics, Bremen, Germany) as previously described.[6] The susceptibility profile was confirmed by broth microdilution to imipenem, meropenem, ceftazidime, cefepime, amikacin, gentamicin, and ciprofloxacin according the CLSI recommendations.[7,8] American Type Culture Collection (ATCC) *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 29213 strains were used as susceptibility testing quality control.

β-Lactamases-encoding genes detection by PCR and DNA sequencing

Acquired β-lactamases encoding genes were investigated by PCR and DNA sequencing, as previously described, using primers for: cephalosporinases (*bla*AmpC); (b) serino-β-lactamases (bla_{TEM}, bla_{SHV}, bla_{GES}, bla_{CTX-M}, bla_{BES}, bla_{PER}, bla_{KPC}, bla_{SME}); (c) oxacillinases (bla_{OXA-1}, bla_{OXA-2}, bla_{OXA-3}, bla_{OXA-5}, bla_{OXA-7}, bla_{OXA-18}, bla_{OXA-45}, bla_{OXA-46}, bla_{OXA-50}, bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-51}, bla_{OXA-58}, bla_{OXA-20}, bla_{OXA-48}, bla_{OXA-62}, $bla_{OXA-143}$, $bla_{OXA-198}$); (d) metallo-β-lactamases (bla_{IMP}, bla_{VIM}, bla_{SPM}, bla_{GIM}, bla_{SIM}, bla_{NDM}); and (e) class 1 integron.[9] Amplicons were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced in both strands using the Applied Biosystems 3500 genetic analyzer equipment (Applied Biosystems, PerkinElmer, USA). The nucleotide sequence and the respective deduced amino acid sequences were analyzed using the Lasergene software package (DNAStar, Madison, WI, USA) and compared with the sequences available on the Internet using the BLAST tool (http://www.ncbi.nlm.nih.gov/blast/).

Carbapenemase hydrolysis assay

Carbapenemase activity was investigated in bacterial cell crude extracts by UV spectrophotometric assays against 100 μM imipenem and 100 μM meropenem in 100 mM phosphate buffer (pH 7.0), as previously described.[10] In parallel, carbapenem hydrolysis inhibition was performed by incubating the whole-protein extract with 25 mM EDTA for 15 min, previously to the assay with imipenem and meropenem. Imipenem hydrolysis was also investigated by MALDI-TOF MS according to the method described by Carvalhaes and colleagues.[11]

Relative gene transcriptional level

Relative transcriptional levels of *mexB*, *mexD*, *mexF*, *mexY*, *ampC*, and *oprD* were determined and analyzed with Real Time 7500 (Applied Biosystems, Warrington, United Kingdom) as previously described.[12] Quantitative RT-PCR was performed with Platinum SYBR Green Supermix (Invitrogen, Carlsbad, USA). According to previous studies, reduced *oprD* and increased *ampC* transcription levels were considered significant when it was ≤70% and ≥10-fold, respectively, compared to their transcriptional levels in PAO1 strain.[13] MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY efflux systems were considered overexpressed when *mexB*, *mexC*, *mexE*, and *mexY* transcriptional level were at least 2-, 100-, 100-, and 4-fold higher than those in PAO1 strain, respectively.[14]

Assessment of the OMP profile and oprD analysis

Amplification of *oprD* gene was carried out by conventional PCR as previously described[15] and the amplicon size analyzed