Metallo-β-lactamase-production in meropenem-susceptible *Pseudomonas aeruginosa* isolates: risk for silent spread

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The aim of this study was to characterize two metallo-β-lactamases (MBLs)-producing Pseudomonas aeruginosa *clinical isolates showing meropenem susceptibility. Antimicrobial susceptibility was assessed by automated testing and Clinical and Laboratory Standards Institute agar dilution method. MBL production was investigated by phenotypic tests. Molecular typing was determined by pulsed field gel electrophoresis (PFGE). MBL-encoding genes, as well as their genetic context, were identified by polymerase chain reaction (PCR) and sequencing. The location of* bla_{IMP-16} *was determined by plasmid electrophoresis, Southern blot and hybridization. Transcriptional levels of* $bla_{IMP-16,}$ mexB, mexD, mexF, mexY, ampC *and* oprD *were determined by semi-quantitative real time PCR. The* P. aeruginosa *isolates studied, Pa30 and Pa43, showed imipenem and meropenem susceptibility by automated testing. Agar dilution assays confirmed meropenem susceptibility whereas both isolates showed low level of imipenem resistance. Pa30 and Pa43 were phenotypically detected as MBL producers. PFGE revealed their clonal relatedness.* bla_{IMP-16} *was identified in both isolates, carried as a single cassette in a class 1 integron that was embedded in a plasmid of about 60-Kb. Pa30 and Pa43 overexpressed MexAB-OprM, MexCD-OprJ and MexXY-OprM efflux systems and showed basal transcriptional levels of* ampC *and* oprD. *MBL-producing* P. aeruginosa *that are not resistant to meropenem may represent a risk for therapeutic failure and act as silent reservoirs of MBL-encoding genes.*

Key words: β-lactam resistance - β-lactamase - meropenem - efflux pumps - Gram-negative non-fermenting

The carbapenems represent important therapeutic options for serious infections caused by *Pseudomonas aeruginosa*. However, *P. aeruginosa* that are resistant to these antimicrobials have emerged and spread within hospital settings worldwide. This pathogen may become resistant to the carbapenems by modulating and combining different intrinsic mechanisms such as down regulation or loss of porins, especially OprD, overexpression of efflux pumps, substantial production of its chromosomal AmpC β-lactamase and target alterations (Lister et al. 2009). Alternatively, *P. aeruginosa* may acquire genes encoding metallo-β-lactamases (MBL), enzymes that are able to inactivate carbapenems efficiently and thus are considered the most clinically significant mechanism of carbapenem resistance in this species (Walsh 2008).

Over the last decades, different MBLs have been identified in *P. aeruginosa* from numerous geographic regions (Sacha et al. 2008). Noteworthy, carbapenem-susceptible *P. aeruginosa* clinical isolates that carried MBL-encoding genes have been reported (Martins et al. 2007, Pellegrino et al. 2008, Naas et al. 2011). Clinical isolates showing either diminished or no expression of MBL-encoding genes may present the carbapenem-susceptible phenotype. In the above referenced reports, however, the expression of MBL-encoding genes was not determined (Martins et al. 2007, Pellegrino et al. 2008, Naas et al. 2011). Nevertheless, these findings are worrisome since carbapenem-susceptible MBL producers may act as reservoirs of such resistance determinants with potential risk for silent spread. In the present paper, we report the occurrence of meropenem-susceptible *P. aeruginosa* clinical isolates producers of the MBL IMP-16. The expression of genes associated with carbapenem resistance was investigated, as well as the genetic context and location of the bla_{IMP-16} gene.

SUBJECTS, MATERIALS AND METHODS

Bacterial isolates - During 2003, two *P. aeruginosa* clinical isolates, namely Pa30 and Pa43, were recovered from different patients hospitalized in a teaching hospital located in the city of Londrina, southern Brazil. Pa30 was isolated from a necrotic tissue culture of a 24 year-old male patient presenting a surgical wound infection and Pa43 was recovered from the urine of a 54 year-old male patient with urinary tract infection. The genetic relatedness of Pa30 and Pa43 was evaluated by pulsed field gel electrophoresis (PFGE) of SpeI-digested DNA samples, as previously described (Picão et al. 2009).

Financial support: Fundação Araucária (375/2006)
ACG is a researcher from CNPq (307816/2009-5).
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Received 8 November 2011
Accepted 20 March 2012

Species identification, susceptibility testing and screening for carbapenemase production - Species identification and routine antibiogram of Pa30 and Pa43 isolates were performed using the MicroScan Walkaway automated system (Dade Behring, West Sacramento, CA) with Combo negative CN30 Panels. Antimicrobials tested included piperacillin, piperacillin-tazobactam, ceftazidime, cefepime, aztreonam, imipenem, meropenem, amikacin, gentamicin, tobramycin and ciprofloxacin. Susceptibility profiles of isolates were confirmed by Clinical and Laboratory Standards Institute (CLSI) agar dilution for amikacin, ciprofloxacin, ceftazidime, cefepime, imipenem and meropenem. *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 strains were used as quality control in susceptibility testing assays (CLSI 2009, 2012).

MBL phenotypic detection, identification of MBL-encoding genes and analysis of their genetic environment - Isolates Pa30 and Pa43 were phenotypically screened for MBL production using both modified Hodge and double disk synergy tests, as previously described (Lee et al. 2003, Picão et al. 2008). A multiplex polymerase chain reaction (PCR) targeting the MBL-encoding genes bla_{IMP}, bla_{VIM}, bla_{SPM}, bla_{GIM} and bla_{SIM} was performed, using previous published primers and cycling conditions (Mendes et al. 2007). The genetic environment of bla_{IMP} was determined by PCR using the specific primers to anneal at the 5' and 3' conserved sequences of class 1 integrons, followed by amplicon sequencing (Naas et al. 2011). Sequencing reactions were performed using specific primers with automated ABI 3130 sequencer (Applied Biosystems, Foster City, CA). The nucleotide and deduced protein sequences were analyzed with online software available at the National Center for Biotechnology Information website (ncbi.nlm.nih.gov).

Genetic location of bla_{IMP-16} - Plasmid extraction was performed by the Kieser technique (Kieser 1984). *E. coli* NCTC 50192 harbouring four plasmids of 154, 66, 38 and 7-kb was used as molecular size marker in gel electrophoresis assays. Hybridization of plasmid extracts was performed after gel electrophoresis and Southern blotting, which was performed by capillary transfer onto Hybond-N^+ nylon membrane (GE Healthcare, UK). Labelling of bla_{IMP-16} specific probe and signal detection were carried out using a nonradioactive labelling and detection kit according to the manufacturer's instructions (GE Healthcare, UK).

Relative quantification of mRNA by real time PCR (RT-PCR) - Transcriptional levels of bla_{IMP-16} and also of genes encoding components of efflux pumps (*mexB*, *mexD*, *mexF*, *mexY*), chromosomal cephalosporinase (*ampC*) and OprD porin (*oprD*) were determined with a Mastercycler Realplex apparatus (Eppendorf, Germany). Total RNA was isolated from logarithmic cultures of each strain using the RNase Mini Kit, following the manufacturer's recommendations (Qiagen, Hilden, Germany). Five micrograms of total RNA was submitted to cDNA conversion using random primers by High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, USA). Semi-quantitative RT-PCR was performed with Platinum SYBR Green Supermix (Invitrogen, Carlsbad, USA), using specific primers for the chromosomal genes *mexB*, *mexD*, *mexF*, *mexY*, *ampC* and *oprD,* as previously described (Xavier et al. 2010), and also for the acquired MBL-encoding gene, bla_{IMP-16} (Mendes et al. 2007). Amplification was carried out in triplicate from cDNA preparations. The relative transcriptional levels were calculated according to $\Delta\Delta C_T$ method by Realplex4 software following the manufacture's recommendations (Eppendorf, Germany). Briefly, the ΔΔCt method provides the relative gene expression levels by averaging cycle threshold (Ct) values from triplicate RT-PCR reactions for the target and housekeeping genes. The range of expression levels obtained for the triplicates, which incorporates the standard deviation (SD) of the ΔΔCt value into the fold-difference, was used to calculate the confidence interval (CI) considering a confidence level of 95%. Additionally, in order to minimize the error and preserve the accuracy and robustness of this test, we have excluded and repeated those triplicate reactions whose average of Ct values had presented a SD value higher than 0.20. The *rps*L endogenous gene was used as the housekeeping gene and the wild-type *P. aeruginosa* PA01 was used as the reference strain to determine the relative expression levels of those chromosomal genes, while, for the analysis of the acquired MBL-encoding gene expression, we have included as the reference strain a previously characterized IMP-16-producing *P. aeruginosa* (isolate 101-4704) which is fully resistant to carbapenems [imipenem and meropenem minimum inhibitory concentration (MICs), 256 μg/mL and 128 μg/mL, respectively], ceftazidime (MIC > 256 μg/mL) and cefepime (MIC 64 μg/mL) (Mendes et al. 2004).

The efflux systems MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM were considered overexpressed when the transcriptional levels of *mexB*, *mexD*, *mexE* and *mexY* were at least two, 100, 100 and 4 fold higher than those of the wild-type reference strain PAO1, respectively (Xavier et al. 2010). Reduced *oprD* expression and overexpression of *ampC* were considered relevant when their transcriptional levels were ≤ 70% and ≥ 10-fold, respectively, compared to that of the PAO1 reference strain (Xavier et al. 2010).

RESULTS

For surveillance purposes, during 2003, all ceftazidime-resistant *P. aeruginosa* isolates recovered in a tertiary teaching hospital located in Londrina were screened for carbapenemase production. Isolates Pa30 and Pa43 recovered in this period were initially subjected to automated susceptibility testing. Both isolates showed susceptibility to aztreonam, piperacillin-tazobactam, imipenem and meropenem but resistance to ceftazidime, cefepime, ciprofloxacin, gentamicin and tobramycin. Surprisingly, despite of their initial susceptibility to imipenem (MIC ≤ 4 μg/mL - by the time these isolate were tested, imipenem breakpoint for susceptibility was ≤ 4 μg/mL), isolates Pa30 and Pa43 were phenotypically detected as MBL producers. Susceptibility testing determined by CLSI agar dilution confirmed resistance

TABLE

Antimicrobials minimal inhibitory concentrations (MICs) and transcriptional levels of selected genes for *Pseudomonas aeruginosa* clinical isolates and reference strain

Bacterial isolates	Antimicrobial agents MICs (µg/mL)						Relative transcription levels						
	Ceftazidime	Cefepime	Imipenem	Meropenem	Amikacin	Ciprofloxacin	*mexB*	*mexD*	*mexF*	*mexY*	*oprD*	*ampC*	bla_{IMP-16}
101-4704[a]	> 256	64	256	128	4	> 8	174	6.25	0.9	64.2	1.4	634	1[a]
Pa30	> 256	32	8	1	> 128	> 8	4.5	1651	5.4	249	0.8	1.2	1.15
Pa43	> 256	32	8	1	> 128	> 8	1.9	2017	0.2	441	1.7	3.7	1.07
PA01[a]	1	1	1	0.5	2	0.125	1	1	1	1	1	1	ND

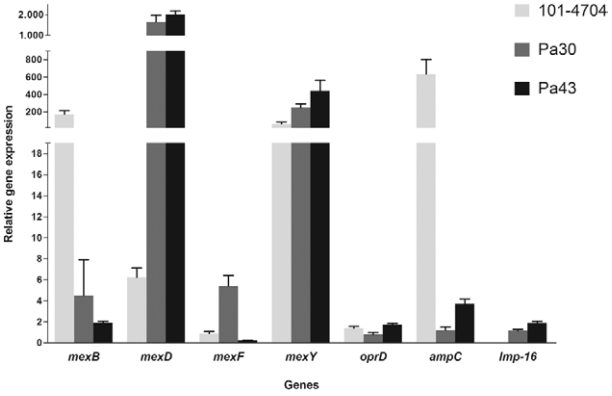
a: wild-type PA01 reference strain; *b*: IMP-16-producing *P. aeruginosa* used as calibrator for bla_{IMP-16} relative quantification; ND: not determined.

to amikacin, ciprofloxacin, ceftazidime and cefepime (Table). In addition, both isolates showed susceptibility to meropenem, but resistance to imipenem (MIC 8 µg/mL), level of resistance that was significantly lower than that shown by isolate 101-4704 (MIC 256 µg/mL). Pa30 and Pa43 shared the same PFGE restriction pattern, indicating their clonal origin (data not shown).

Pa30 and Pa43 isolates presented an amplicon consistent with the bla_{IMP} gene. Further sequencing identified the bla_{IMP-16} allele in both isolates. Analysis of bla_{IMP-16} genetic context revealed that both isolates carried this gene as a single cassette into the variable region of a class 1 integron. The integrase gene showed a GGG deletion between -10 and -35 boxes of P2 compared to that of isolate 101-4704, possibly inactivating this promoter in isolates Pa30 and Pa43. In contrast, Pa30 and Pa43 harboured the putative weak P_c version (TGGACA-N17-TAAGCT), which was identical to the one identified in isolate 101-4704.

Hybridization experiments revealed that bla_{IMP-16} was carried by a plasmid of about 60-Kb in both Pa30 and Pa43. However, repetitive attempts to transfer pPa30 and pPa43 by transformation performed as previously described were unsuccessful, regardless of the recipient strains used, either electrocompetent *E. coli* TOP10 or *P. aeruginosa* PA01 (data not shown) (Picão et al. 2009).

Semi-quantitative RT-PCR analysis showed that transcriptional levels of bla_{IMP-16} were similar in Pa30, Pa43 and 101-4704 isolates. In addition, Pa30, Pa43 and 101-4704 showed transcriptional levels of *oprD* comparable to that of the reference strain PA01, which might indicate normal expression of the OprD porin in these clinical isolates (Table). However, OMP analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed the absence of a 49 kDa band in the protein pattern of the 101-4704 isolate, while this structure was preserved in Pa30, Pa43 and PA01 strain (data not shown), indicating possible OprD loss in the carbapenem-resistant isolate 101-4704. Analysis of *ampC* transcription suggested that Pa30 and Pa43 produced basal amounts of AmpC, whereas isolate 101-4704 overexpressed this cephalosporinase (Table). Regarding efflux pumps constituents, *mexB*, *mexD* and *mexY* transcriptional levels were higher in Pa30 than in PA01, suggesting MexAB-OprM, MexCD-OprJ and MexXY-OprM overexpression, respectively. Similarly, Pa43 showed *mexD* and *mexY* transcriptional levels significantly higher than those of PA01, but its *mexB* expression was near the cut-off value for overexpression. The carbapenem-resistant *P. aeruginosa* producing IMP-16, isolate 101-4704, also showed increased transcriptional levels of *mexB* and *mexY* compared to PA01, indicating that the MexAB-OprM and MexXY-OprM efflux systems were overexpressed in this strain. Nevertheless, Pa30 and Pa43 showed transcriptional levels of *mexD* and *mexY* significantly higher than those of isolate 101-4704 which, in turn, showed *mexB* levels much higher than those of the former isolates (Table). The magnitude of transcriptional levels differences for the analyzed genes considering an 95% CI is depicted in Figure.



Relative expression of efflux pumps genes, *opr*D, *amp*C and bla_{IMP-16}. Data is represented as mean (n-fold) and 95% confidence interval.

DISCUSSION

In this study, we have identified two MBL-producing *P. aeruginosa* clinical isolates that exhibited low level of imipenem resistance but susceptibility to meropenem. PFGE analysis revealed that those isolates were genetically related to each other. However, previous report about hypersusceptible phenotype in *P. aeruginosa* suggested that this phenomenon is not inherent to one particular type of strain, once it has been observed among genetically unrelated clinical isolates (Wolter et al. 2009). Fortunately, the patients from whom the strains Pa30 and Pa43 were isolated were not seriously ill and showed clinical resolution of signs and symptoms after receiving ciprofloxacin and levofloxacin, respectively, for 14 days.

To date, IMP-16 production has only been identified in carbapenem-resistant *Pseudomonas* spp clinical isolates from Brazil (Mendes et al. 2004, Marra et al. 2006, Carvalho-Assef et al. 2010, Scheffer et al. 2010). In this study, we have observed that bla_{IMP-16} transcriptional level was similar among Pa30, Pa43 and 101-4704, despite of the probable impaired P2 promoter in Pa30 and Pa43. Nevertheless, bla_{IMP-16} expression was not enough to confer high level of carbapenem resistance in isolates Pa30 and Pa43.

High level carbapenem resistance in strain 101-4704 could be attributed not only to IMP-16 activity, but also to the OprD loss and the concomitant overexpression of AmpC and MexAB-OprM efflux system. It has been previously observed that mutants hyperexpressing the MexCD-OprJ efflux system usually show imipenem hypersusceptibility (Masuda et al. 1996, Wolter et al. 2005). Although the mechanism involved in such phenotype is unknown, it has been proposed that it is not related to OprD loss or AmpC repression (Wolter et al. 2005). One hypothesis is that the hyperexpression of *mex*CD-*opr*J operon may be associated with downregulation of an uncharacterized efflux system that extrudes imipenem, since the *P. aeruginosa* genome contains several uncharacterized efflux pumps belonging to multiple families (Angus et al. 1982, Wolter et al. 2005). It is also possible that MexCD-OprJ hyperexpression could alter the composition or architecture of the outer membrane, increasing its permeability to carbapenems (Angus et al. 1982, Wolter et al. 2005). Therefore, the hyperexpression of MexCD-OprJ might have contributed to limit levels of carbapenem resistance in the isolates Pa30 and Pa43, although further analyses are necessary to confirm this hypothesis.

MBL detection has not been routinely performed in most clinical microbiology laboratories. Consequently, the occurrence of MBL production in *P. aeruginosa* that remain susceptible to meropenem might be unrecognized by the clinical laboratories. It might lead to underestimation of MBL prevalence in the clinical setting. In this manner, these MBL producers might act as silent reservoirs of such resistance determinants, with ability to spread, since MBL-encoding genes are often carried by mobile genetic elements. In addition, failing in detecting MBL production among meropenem-susceptible isolates may lead to inadequate prescription of this drug and possible therapeutic failure in seriously ill patients.

In 2010, the CLSI revised the Enterobacteriaceae clinical breakpoints for carbapenems in order to overcome the problematic emergence of KPC producers that might be categorized as susceptible to these antimicrobial agents (CLSI 2010). More recently, the carbapenems clinical breakpoints for *P. aeruginosa* were also revised (CLSI 2012). Based on the findings reported here, although the CLSI initiative avoided the miscategorization of the MBL producers Pa30 and Pa43 as susceptible to imipenem, these isolates were still categorized as meropenem-susceptible. It might be difficult to establish the correct breakpoint that would certainly detect complex isolates like Pa30 and Pa43, or others that might appear even more susceptible to carbapenems due to other factors. Identification of carbapenemase producers could also rely upon phenotypic detection, especially in regions where MBL-producing isolates are prevalent. However, the implementation of such recommendation has been jeopardized by the lack of a consensus regarding the criterion to select isolates for screening, the method, the β-lactam substrates and the best inhibitor combinations to be employed for precise MBL phenotypic detection. Although much progress has been achieved in comprehending the mechanisms of antimicrobial resistance, it is imperative that the scientific community concentrates efforts in developing means to assess the real prevalence of carbapenemase producers. It is also very important to evaluate its clinical impact and establish whether carbapenems would be appropriate to treat infections due to carbapenemase producers that are still categorized as susceptible to these antimicrobials.

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