Changing the epidemiology of carbapenem-resistant *Pseudomonas aeruginosa* in a Brazilian teaching hospital: the replacement of São Paulo metallo-β-lactamase-producing isolates

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In Brazil, carbapenem-resistant Pseudomonas aeruginosa *isolates are closely related to the São Paulo metallo-β-lactamase (SPM) Brazilian clone. In this study, imipenem-resistant isolates were divided in two sets, 2002/2003 and 2008/2009, analysed by pulsed field gel electrophoresis and tested for the Ambler class B metallo-β-lactamase (MBL) genes* bla_{SPM-1}, bla_{IMP} *and* bla_{VIM}. *The results show a prevalence of one clone related to the SPM Brazilian clone in 2002/2003. In 2008/2009,* P. aeruginosa *isolates were mostly MBL negative, genetically diverse and unrelated to those that had been detected earlier. These findings suggest that the resistance to carbapenems by these recent* P. aeruginosa *isolates was not due to the spread of MBL-positive SPM-related clones, as often observed in Brazilian hospitals.*

Key words: carbapenemases - metallo-β-lactamase - *Pseudomonas aeruginosa*

Carbapenems, including imipenem and meropenem, have been considered for the treatment of *Pseudomonas aeruginosa* infections because they are not hydrolysed by most of the serine β-lactamases (Hurst & Lamb 2000). However, the prevalence of carbapenem resistance in this bacterium has increased worldwide, particularly in Latin America (Sader et al. 2004). This resistance can be conferred by the absence of the outer membrane protein OprD with the associated over-expression of the efflux pump MexAB-OprM (as in the case of meropenem resistance), by increased action of chromosome-encoded AmpC cephalosporinase or by the production of Ambler class B metallo-β-lactamases (MBLs) (Zavascki et al. 2005, Rodríguez-Martínez et al. 2009, Strateva & Yordanov 2009). Among the MBL genes, bla_{SPM-1} has thus far only been detected in Brazil and Switzerland and the dissemination of this gene in various regions of the country seems to be caused by a single epidemic *P. aeruginosa* clone (Gales et al. 2003, Salabi et al. 2010). In the last few years, an increasing number of carbapenem-resistant *P. aeruginosa* isolates have been observed in a teaching hospital in Recife, Pernambuco, Brazil. In light of this, we decided to conduct a phenotypic and genetic analysis of those isolates to investigate the production of MBL and establish their relationships to the São Paulo metallo-β-lactamase (SPM) Brazilian clone.

Imipenem/ceftazidime-resistant isolates of *P. aeruginosa* were recovered from patients undergoing treatment in the Oswaldo Cruz University Hospital during the time frames 2002-2003 and 2008-2009; one isolate was used per patient for a total of 73 isolates. The bacterial species were identified by standard biochemical tests. Susceptibility testing was performed by means of the disk-diffusion method with the following antimicrobial agents: imipenem, ceftazidime, ciprofloxacin, amikacin, gentamicin, piperacillin/tazobactam, aztreonam and polymyxin B, in compliance with the Clinical and Laboratory Standards Institute guidelines (CLSI 2010). The minimal inhibitory concentration (MIC) for carbapenems of MBL-negative isolates was determined by the automated BD Phoenix system and interpreted in accordance with CLSI. Selected isolates were screened for MBL production by the disk approximation test as previously described (Arakawa et al. 2000).

Presumptive MBL producers were further tested for the bla_{SPM-1}, bla_{IMP} and bla_{VIM} genes. Bacterial DNA was extracted by using the Brazol kit (LGC Biotecnologia, Brazil) following the recommendations of the manufacturer and analysed by polymerase chain reaction (PCR) using the primer pairs bla_{SPM-1} (forward: 5'-CCTACAATCTAACGGCGACC-3', reverse: 5'-TCGCCGTGTCCAGGTATAAC-3'), bla_{IMP} (forward: 5'-GGAATAGAGTGGCTTAATTCTC-3', reverse: 5'-GTGATGCGTCYCCAAYTTCACT-3') and bla_{VIM} (forward: 5'-TGCGCATTCGACCGACAATC-3', reverse: 5'-GTCGAATGCGCAGCACCAGG-3') (Migliavacca et al. 2002, Gales et al. 2003, Toleman et al. 2005). Positive controls for the *P. aeruginosa* bla_{SPM-1}, bla_{IMP} and bla_{VIM} genes were kindly provided by Special Clinical Microbiology Laboratory and ALERTA Laboratory (São Paulo, Brazil). The amplicons were purified with the aid of a PCR purification kit (Promega Co, USA) and submitted to DNA sequencing by the platform of the Aggeu Magalhães Research Centre, Oswaldo Cruz Foundation, Recife. The nucleotide sequences were evaluated with the BioEdit™ program and analysed by on-line BLASTn at GenBank dataset (National Centre for Biotechnology

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TABLE
Microbiological and molecular characteristics of carbapenem-resistant *Pseudomonas aeruginosa* isolates

Isolate number	Resistance profile			MBL test[a]	MBL gene	PFGE pattern[b]
	Resistance	Intermediate resistance	Susceptibility			
2002/2003						
Ps 53	cip, ami, gen	-	azt, pol B	+	SPM-1	A
Ps 71	cip, ami, gen	azt	pol B	+	SPM-1	A
Ps 76	cip, ami, gen	-	azt, pol B	+	SPM-1	A1
Ps 85	cip, ami, gen	-	azt, pol B	+	SPM-1	A
Ps 91	cip, ami, gen	-	azt, pol B	+	SPM-1	A
Ps 144	cip, ami, gen	-	pip/taz, azt, pol B	+	SPM-1	A2
Ps 158	cip, ami, gen, pip/taz	azt	pol B	+	SPM-1	A
Ps 159	cip, ami, gen, pip/taz	azt	pol B	+	SPM-1	A2
Ps 169	cip, ami, gen	-	pip/taz, azt, pol B	+	SPM-1	A2
Ps 172	cip, ami, gen	azt	pip/taz, pol B	+	SPM-1	A
Ps 178	cip, ami, gen	azt	pip/taz, pol B	+	SPM-1	A
Ps 199	cip, ami, gen	azt	pip/taz, pol B	+	SPM-1	A
Ps 212	cip, ami, gen	azt	pip/taz, pol B	+	SPM-1	A
Ps 215	cip, ami, gen	azt	pip/taz, pol B	+	SPM-1	A
Ps 241	cip, ami, gen	-	pip/taz, azt, pol B	+	SPM-1	A
Ps 246	cip, ami, gen	-	pip/taz, azt, pol B	+	SPM-1	A
Ps 305	cip, ami, gen	azt	pip/taz, pol B	+	SPM-1	A
Ps 307	cip, ami, gen	azt	pip/taz, pol B	+	SPM-1	A
Ps 327	cip, ami, gen	azt	pip/taz, pol B	+	SPM-1	A
Ps 370	cip, ami, gen, pip/taz	azt	pol B	+	AT	A
Ps 408	cip, ami, gen, pip/taz	azt	pol B	+	AT	A
Ps 444	cip, ami, gen, pip/taz	azt	pol B	+	SPM-1	A
Ps 445	cip, ami, gen, azt	-	pip/taz, pol B	+	SPM-1	A
Ps 467	cip, ami, gen, pip/taz	-	azt, pol B	+	SPM-1	A
Ps 527	cip, ami, gen, pip/taz	-	azt, pol B	+	SPM-1	A
Ps 581	cip, ami, gen, pip/taz	azt	pol B	+	SPM-1	A
Ps 598	cip, ami, gen, pip/taz	-	azt, pol B	+	SPM-1	A
Ps 603	ami, gen	azt	pip/taz, pol B	+	SPM-1	A
Ps 614	ami, gen	-	pip/taz, azt, pol B	+	SPM-1	A
Ps 616	ami, gen	-	pip/taz, azt, pol B	+	SPM-1	A
Ps 645	ami, gen	-	pip/taz, azt, pol B	+	SPM-1	A
Ps 646	ami, gen	-	pip/taz, azt, pol B	+	SPM-1	A
Ps 647	ami, gen	azt	pip/taz, pol B	+	SPM-1	A
Ps 655	cip, ami, gen, pip/taz	azt	pol B	+	SPM-1	A
Ps 668	cip, ami, gen, pip/taz	azt	pol B	+	SPM-1	A
Ps 678	cip, ami, gen	azt	pip/taz, pol B	+	SPM-1	A
Ps 712	cip, ami, gen	-	pip/taz, azt, pol B	+	SPM-1	A
Ps 724	cip, ami, gen	azt	pip/taz, pol B	+	SPM-1	A
Ps 725	cip, ami, gen, pip/taz	-	azt, pol B	+	SPM-1	A
Ps 727	cip, ami, gen	azt	pip/taz, pol B	+	SPM-1	A
Ps 762	ami, gen, pip/taz	azt	pol B	+	SPM-1	A
Ps 767	cip, ami, gen	azt	pip/taz, pol B	+	SPM-1	A
Ps 773	ami, gen	azt	pip/taz, pol B	+	SPM-1	A
Ps 776	ami, gen	azt	pip/taz, pol B	+	SPM-1	A
Ps 780	cip, ami, gen	azt	pip/taz, pol B	+	SPM-1	A
Ps 783	cip, ami, gen, pip/taz	azt	pol B	+	SPM-1	A
Ps 790	cip, ami, gen, pip/taz	azt	pol B	+	SPM-1	A

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Isolate number	Resistance profile			MBL test[a]	MBL gene	PFGE pattern[b]
	Resistance	Intermediate resistance	Susceptibility			
Ps 791	cip, ami, gen, pip/taz	-	azt, pol B	+	SPM-1	A
Ps 793	cip, ami, gen, pip/taz	azt	pol B	+	SPM-1	A
Ps 800	cip, ami, gen	azt	pip/taz, pol B	+	SPM-1	A
Ps 810	cip, ami, gen, pip/taz	azt	pol B	+	SPM-1	A
Ps 822	cip, ami, gen, pip/taz	azt	pol B	+	SPM-1	A
Ps 830	cip, ami, gen, pip/taz	azt	pol B	+	SPM-1	A
Ps 832	cip, ami, gen	-	pip/taz, azt, pol B	+	SPM-1	A
Ps 842	cip, ami, gen, pip/taz	-	azt, pol B	+	SPM-1	A
Ps 851	cip, ami, gen, pip/taz	azt	pol B	+	SPM-1	A3
Ps 860	cip, ami, gen, pip/taz	azt	pol B	+	SPM-1	A
Ps 874	cip, ami, gen	azt	pol B	neg	AT	A4
Ps 883	cip, ami, gen, pip/taz	azt	pol B	+	SPM-1	A5
Ps 887	cip, ami, gen	-	azt, pol B	+	SPM-1	A6
Ps 900	cip, ami, gen	-	pip/taz, azt, pol B	+	SPM-1	A
2008/2009		-				
Ps 901	cip, ami, gen, pip/taz azt	-	pol B	neg	AT	B
Ps 902	cip, ami, gen, azt	-	pip/taz, pol B	neg	AT	C
Ps 904	cip, ami, gen, pip/taz, azt	-	pol B	neg	AT	D
Ps 905	cip, ami, gen, pip/taz	azt	pol B	+	SPM-1	A7
Ps 906	cip, ami, gen, azt	-	pip/taz, pol B	neg	AT	D
Ps 908	cip, ami, gen, pip/taz, azt	-	pol B	neg	AT	E
Ps 909	cip, gen, azt	ami	pol B	neg	AT	D
Ps 912	cip, ami, gen, pip/taz	azt	pol B	+	SPM-1	A7
Ps 918	cip, ami, gen, pip/taz, azt	-	pol B	neg	AT	F
Ps 919	cip, ami, gen, pip/taz, azt	-	pol B	neg	AT	G
Ps 920	cip, ami, gen, pip/taz, azt	-	pol B	neg	AT	F
Ps 922	cip, ami, gen, pip/taz, azt	-	pol B	+	SPM-1	A

a: results showed as positive (+) or negative (neg) for the phenotypic Ambler class B metallo-β-lactamases (MBL) test; *b*: according to Tenover et al. (1995); ami: amikacina; AT: absence of tested MBL genes; azt: aztreonam; cip: ciprofloxacin; gen: gentamicin; PFGE: pulsed-field gel electrophoresis; pip/taz: piperacilin/tazobactam; pol B: polymyxin B; SPM: São Paulo metallo-β-lactamase.

and Information). In addition, all of the isolates were genotyped by DNA macrorestriction followed by pulsed-field gel electrophoresis (PFGE) using the endonuclease SpeI. A representative isolate of the SPM Brazilian clone (from São Paulo Hospital) and the sequenced strain PA01 (kindly provided by the *Pseudomonas* Genome Project, Boston, MA, USA) were included as a reference point. Clonal relationships among the isolates were established using the criteria of Tenover et al. (1995).

The isolates from the present work revealed an important change in the epidemiology of carbapenem-resistant *P. aeruginosa* isolates between the periods 2002-2003 and 2008-2009, showing a decreasing prevalence of the epidemic SPM-1-producing clone in the final period of bacterial recovery (Table). Moreover, the antimicrobial susceptibility test revealed that these bacteria were co-resistant to many other anti-*Pseudomonas* drugs, particularly the most recent strains (2008/2009). On the other hand, all of the bacterial samples were susceptible to polymyxin B (Table).

A high prevalence of the MBL phenotype was observed among the 2002/2003 isolates (98.4%) (Table). This coincided with a higher incidence of $bla_{\text{SPM-1}}$ than those found in previous studies conducted on a national scale (Sader et al. 2004, Gräf et al. 2008). Nevertheless, this high incidence of the $bla_{\text{SPM-1}}$ gene decreased in the 2008/2009 isolates (Table), suggesting that carbapenem-resistance mechanisms other than MBL must be present and are being spread in the hospital under study. Moreover, these other mechanisms, such as efflux pump over expression (associated or not with porin down-regulation), may also be involved in the overall increased resistance to anti-*Pseudomonas* drugs observed in 2008/2009. None of the isolates showed amplification of the bla_{IMP} or bla_{VIM} gene (data not shown). Thus, two presumptive MBL producers, from 2002/2003, did not carry any of the MBL genes tested. As expected, none of the 10 MBL-negative isolates indicated the presence of MBL genes.

Molecular typing indicated the prevalence of bacterial isolates (herein designated as genotype A) closely re-

lated to the widespread SPM Brazilian clone 48-1997A, which was widely disseminated in the 2002/2003 group of isolates (Table). The existence of common PFGE types among carbapenem-resistant *P. aeruginosa* isolates from distinct geographical locations has been reported and indicates clonal dispersion (Zavascki et al. 2005, Fonseca et al. 2010). In Brazil, there have been previous reports of the spread of a unique SPM-type MBL-positive clone (Gales et al. 2003). As expected, MBL-negative isolates from 2008/2009 were unrelated to the epidemic Brazilian clone and showed six distinct PFGE types (Table). Thus, new clones could be responsible for the dissemination of other resistance mechanisms to carbapenems. The three 2008/2009 MBL-positive isolates that carried the bla_{SPM-1} gene belonged to the clonal pattern A. Interestingly, the increase in bacterial variation was also accompanied by an increase in bacterial resistance. It is noteworthy that the MIC values for imipenem and meropenem were not high among the more recent isolates (MIC > 8 μg/mL), which corroborates the hypothesis that there are alternative resistance mechanisms. This is supported by the fact that MBL enzymes increase antimicrobial MICs more effectively than does either efflux pump over-expression or porin down-regulation alone (Xavier et al. 2010).

In conclusion, the population of carbapenem-resistant *P. aeruginosa* in the hospital under study was replaced by MBL-negative, genetically unrelated bacterial isolates. This finding emphasises the need for continuous surveillance strategies and an improvement of the infection control measures in this institution.

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