Unveiling novel genetic structures associated with ´high-risk´ *bla*$_{VIM-2}$-producing *P. aeruginosa* clones in Portuguese hospitals

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Introduction and purpose

- *Pseudomonas aeruginosa* is an opportunistic pathogen commonly associated with nosocomial and community infections. [1,2] Intrinsic features and ability to acquire resistance to antibiotics, including to the ones with broaden activity, as carbapenems, threatens the success of antibiotherapy.[1,2] Currently, carbapenem resistance rates among this species range from 10-50% worldwide.[3,4] Carbapenem resistance mechanisms may include decreased permeability, multidrug efflux and/or production of carbapenemases, with the latter being increasingly documented.[1,2,5,6]

- The most widespread carbapenemases in *P. aeruginosa* are metallo-β-lactamases (MBLs), particularly VIM and IMP types, which are frequently associated with class I integrons containing additional antibiotic resistance genes.[2] Moreover, in *P. aeruginosa*, MBLs have been frequently associated with 'high-risk' clones belonging to Sequence Types (ST's) 111, 175, 235 and 244.[1,2,4,7-9]

- In Portugal, different carbapenemases have been reported among *P. aeruginosa*, with infrequent reports of IMP-5 and GES-6, in contrast with the dominant VIM-2.[6,10] Since the first worldwide report of *bla*$_{VIM-2}$-producing *P. aeruginosa,* in 1995 in Portugal, the spread of this enzyme have involved different *Pseudomonas* species and settings. [10-14] Nevertheless, we lack a comprehensive study allowing to understand the drivers (clonal and mobile genetic elements background) for their occurrence in the clinical setting.

- **This study aimed to understand the clones and genetic structures that might be responsible for dissemination of carbapenemase producing *P. aeruginosa* in the past fourteen years in different Portuguese hospitals.**

Methods

❑ A collection of carbapenem resistant *Pseudomonas aeruginosa* (CRPA) isolates (n=261) obtained from different clinical products from inpatients of six geographically distinct hospitals located at North (Hospitals A, n=248; B, n=2; C, n=1 and D, n=1) and Center (Hospitals E, n=2 and F, n=7) of Portugal between 2000 and 2014 were included (Table 1).

❑ Antimicrobial susceptibility to β–lactams, aminoglycosides, fluoroquinolones and colistin was performed by disc diffusion or broth microdilution methods according with EUCAST (www.eucast.org) and CLSI guidelines.

❑ Carbapenemase production was confirmed by Blue-Carba.[15] PCR's for the most prevalent carbapenemases (VIM, IMP, NDM and KPC) and ESBLs (GES, PER, VEB and BEL) were performed.[16] Association of carbapenemase genes with class I integrons was searched by PCR and confirmed by sequencing.[10] In*2*, In*4* and In*5*-Tn*402* integron conserved sequences were searched by PCR mapping in selected isolates.[17]

❑ The genetic location (chromosomal or plasmid) of carbapenemase genes was assessed by I-CeuI/S1 PFGE, followed by hybridization with probes specific for *bla* genes and 16S rDNA.[10]

❑ Conjugation transfer of carbapenemase genes was attempted with rifampicin-resistant *P. aeruginosa* PAO or rifampicin-resistant *Escherichia coli* K802N. Transconjugant selection was performed in MH agar plates containing rifampicin (100 mg/ml) and amoxicillin (30 mg/ml) or imipenem (2 mg/ml). Additionally, plasmid DNA was prepared for *E. coli* DH5α and *P. aeruginosa* PAO1 electro-transformation with *bla*$_{VIM-2}$-carrying plasmids. Transformants were selected in MH agar plates containing imipenem (0.5 µg/mL for *E. coli* DH5α and 4 µg/mL for *P. aeruginosa* PAO1).[18] Transformants were confirmed by PCR and antimicrobial susceptibility testing.

❑ Clonality analysis was conducted by SpeI-PFGE and MLST for all isolates. PFGE image analysis was conducted with InfoQuestTM FP software version 4.5 (Bio-Rad). Isolates clustering together with a ≥80% Dice coefficient (corresponding to a maximum 6 band difference) were considered to belong to the same PFGE type.[19] MLST was performed according with the *P. aeruginosa* database (pubmlst.org/paeruginosa).

Results

❖ Carbapenemase production was verified in n=25/261 (9,6%) isolates. All CPPA isolates produced *bla*$_{VIM-2}$, which is frequently found among *P. aeruginosa* isolates worldwide and associated with 'high-risk clones' exhibiting XDR and MDR phenotypes.

❖ The isolates were non-susceptible to imipenem, ceftazidime and cefepime, but presented variable susceptibility to meropenem, aztreonam, piperacillin/tazobactam, ciprofloxacin, amikacin, tobramycin and gentamicin (Table 1). Five isolates were resistant to colistin (MIC values ranging from 8 to 16 mg/L). Twenty isolates were defined as XDR and five as MDR (Table 1).[20]

❖ The *bla*$_{VIM-2}$ was located in a variety of class I integrons: In*58*, In*100*, In*796*, In*56* and three novel structures (In*102*, In*103* and In*1220*) (Figure 1). In*58* was the most frequently found (n=18). In*796* (n=1), In*103* (n=1) and In*1220* (n=1) present a similar structure to In*58*. Since these integrons were associated with an In*4*-Tn*402*-like structure, where orf5, orf6 and/or IS*6100* are commonly found downstream the 3'-CS region, genes excision and/or IS*6100*-mediated deletions into the genes cassettes may have occurred.

❖ The *bla*$_{VIM-2}$ gene was chromosomally located in most isolates (n=17), which seems to be common in *P. aeruginosa*. Eight isolates presented *bla*$_{VIM-2}$-carrying plasmids and four isolates presented both chromosomal and plasmid location for the *bla*$_{VIM-2}$ gene (Table 1). All plasmids were associated with In*58* integron. Conjugation experiments were unsuccessful, but we were able to transfer the 30kbp plasmid to *P. aeruginosa* PAO1, with transformants acquiring resistance to β-lactams and aminoglycosides (Table 1).

Table 1. Molecular characterization of *bla*$_{VIM-2}$ encoding *P. aeruginosa* isolates.

Integron	No. of isolates	Hospital/ Geographical location	Sources	Date	Sequence type (ST)[a] and no. of isolates	PFGE type	Genetic location (plasmid size [kb])	Antibiotic resistance profile[b]	MDR/XDR profile
In*58*	18	A/North; B/North; C/North; D/North; F/Center	Urine, bronchial secretions, respiratory secretions, bronchoalveolar lavage, catheter, exudate, unknown	2001, 2003, 2011-2013	179 (n=7)	A, B	C	IMP, (MEM), CAZ, FEP, (ATM), PIP+TAZ, GEN, AMK, TOB, (CIP), (COL)	MDR/XDR
				2001, 2003, 2006	175 (n=4)	D	C/P (30)		
				2008, 2010	253 (n=3)	E	C/P (460)		
				2013	111 (n=1)	C	C		
				2010	244 (n=1)	F	P (350)		
				2004	282 (n=1)	J	C/P (30)		
				2001	260 (n=1)	L	C		
In*1220*	1	A/North	Catheter	2011	1284	I	C	IMP, MEM, CAZ, FEP, ATM, PIP+TAZ, GEN, AMK, TOB, CIP	XDR
In*796*	1	A/North	Urine	2013	244	G	C	IMP, MEM, CAZ, FEP, PIP+TAZ, GEN, AMK, TOB, CIP	XDR
In*103*	1	A/North	Urine	2002	179	A	C	IMP, MEM, CAZ, FEP, ATM, PIP+TAZ, GEN, AMK, TOB, CIP	XDR
In*56*	1	E/Center	Bronchial aspirate	2002	235	H	C	IMP, MEM, CAZ, FEP, ATM, PIP+TAZ, CIP	XDR
In*100*	2	A/North; F/Center	Urine, blood	2012, 2014	111	C	C	IMP, MEM, CAZ, FEP, (ATM), PIP+TAZ, GEN, AMK, TOB, CIP, (COL)	XDR
In*102*	1	E/Center	Blood	2000	815	K	C	IMP, CAZ, FEP, PIP+TAZ, GEN, AMK, TOB	MDR

C, chromosome; P, plasmid; AMK, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; COL, colistin; FEP, cefepime; GEN, gentamicin; IPM, imipenem; MEM, meropenem; PIP+TAZ, piperacillin+tazobactam, TOB, tobramycin.
[a]The combination of alleles corresponding to each ST identified is as follows: ST111 (*acsA*-17, *aroE*-5, *guaA*-5, *mutL*-4, *nuoD*-4, *ppsA*-4, *trpE*-3) ST175 (*acsA*-28, *aroE*-22, *guaA*-5, *mutL*-3, *nuoD*-3, *ppsA*-14, *trpE*-19), ST179 (*acsA*-36, *aroE*-27, *guaA*-28, *mutL*-3, *nuoD*-4, *ppsA*-13, *trpE*-7), ST244 (*acsA*-17, *aroE*-5, *guaA*-12, *mutL*-3, *nuoD*-14, *ppsA*-4, *trpE*-7), ST1284 (*acsA*-32, *aroE*-8, *guaA*-5, *mutL*-3, *nuoD*-5, *ppsA*-6, *trpE*-26)
[b]Variability among isolates is indicated by parentheses.

❖ A high genetic diversity was observed (10 ST profiles and 12 PFGE-types), which is common in *P. aeruginosa*, [1, 8] with ST179 being the most frequently found (n=7) (Table 1). ST's 111, 175, 179 were isolated in different years and in distinct geographical locations, supporting the hypothesis of clonal dissemination of some representative lineages that are more prone to carry carbapenemase genes in different, but related, integron structures (Figure 1). Moreover, the ´high-risk' clones (ST's 175, 111, 244 and 235) were always associated with a XDR phenotype.

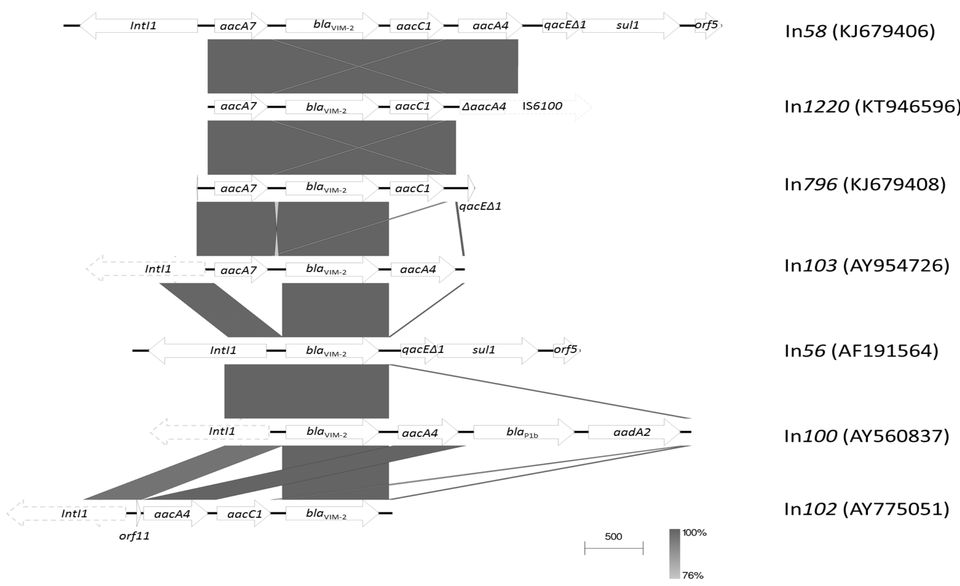

Figure 1. Schematic representation of *P. aeruginosa bla*$_{VIM-2}$ associated integrons described in this study. The arrows indicate the translation orientation of the coding genes. Dotted lines represent genes that are not fully sequenced.

Conclusions

- VIM-2 represents for more than a decade the main carbapenemase associated with *P. aeruginosa* in Portugal.
- *bla*$_{VIM-2}$ plamidic dissemination throughout In*58* with further chromosomal fixation, preceded or followed by recombinations, is associated with the emergence of this carbapenemase in different lineages circulating in Portuguese hospitals.
- The integron structures carrying *bla*$_{VIM-2}$ were highly related to previously described ones in *Pseudomonas* spp. from different Portuguese settings**,** suggesting an interconnection between them and/or a great stability of genetic structures encoding this carbapenemase.

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