

High mortality of bloodstream infection outbreak caused by carbapenem-resistant *P. aeruginosa* producing SPM-1 in a bone marrow transplant unit

Lucas Chaves,¹ Lísia Moura Tomich,¹ Matias Salomão,¹ Gleice Cristina Leite,² Jessica Ramos,¹ Roberta Ruedas Martins,¹ Camila Rizek,² Patricia Neves,² Marjorie Vieira Batista,¹ Ulysses Amigo,³ Thais Guimaraes,⁴ Anna Sara Levin^{1,2} and Silvia Figueiredo Costa^{1,2,*}

Abstract

Purpose. Carbapenem resistance in *P. aeruginosa* is increasing worldwide. In Brazil, SPM-1 is the main *P. aeruginosa* carbapenemase identified. Little is known about the virulence factor in SPM-1 clones.

Methodology. We describe a carbapenem-resistant *P. aeruginosa* bloodstream infection (CRPa-BSI) outbreak in a bone marrow transplant Unit (BMT). Twenty-nine CRPa-BSI cases were compared to 58 controls. Microbiological characteristics of isolates, such as sensitivity, carbapenemase gene PCR for *P. aeruginosa*, and PFGE are described, as well as the whole-genome sequence (WGS) of three strains.

Results/Key findings. The cultures from environmental and healthcare workers were negative. Some isolates harboured KPC and SPM. The WGS showed that the 03 strains belonged to ST277, presented the same mutations in outer membrane protein, efflux pump, and virulence genes such as those involved in adhesion, biofilm, quorum-sensing and the type III secretion system, but differ regarding the carbapenemase profile. A predominant clone-producing SPM harbouring Tn 4371 was identified and showed cross-transmission; no common source was found. Overall mortality rate among cases was 79 %. The first multivariate analysis model showed that neutropenia ($P=0.018$), GVHD prophylaxis ($P=0.016$) and prior use of carbapenems ($P=0.0089$) were associated with CRPa-BSI. However, when MASCC ≥ 21 points and platelets were added in the final multivariate analysis, only prior use of carbapenems remained as an independent risk factor for CRPa-BSI ($P=0.043$).

Conclusions. The predominant clone belonging to ST277 showed high mortality. Carbapenem use was the only risk factor associated with CRPa-BSI. This finding is a wake-up call for the need to improve management in BMT units.

INTRODUCTION

Bloodstream infections (BSI) are related to increased morbidity and mortality in haematopoietic stem cell transplant (HSCT) patients [1, 2]. Since the last decade, an increasing incidence of BSI due to Gram-negative bacilli (GNB) has been described, including non-fermenting bacteria [2] relative to Gram-positive bacteria (GPB) [3, 4] in this population. Multidrug-resistant (MDR) bacterial infection contributes to higher rates of death and economic burden [2–4].

P. aeruginosa infections are usually related to high mortality rates in HSCT patients [5, 6], and this can be attributed to many virulent factors such as biofilm production, endotoxins, the type III secretion system and ‘quorum sensing’ [7]. Bloodstream infection caused by *P. aeruginosa* isolates harbouring the type III secretion system including three exotoxins (ExoU, ExoT and ExoS) has been associated with poor outcome [8–10]. Recently, Peña *et al.* [9] demonstrated a significant association of *exoU*-positive *P. aeruginosa* isolates with early mortality in patients with BSI.

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Author affiliations: ¹Department of Infectious Diseases, School of Medicine, University of São Paulo, São Paulo, Brazil; ²Laboratory of Bacteriology-LIM54, Hospital das Clínicas, Institute of Tropical Medicine, University of São Paulo, São Paulo, Brazil; ³Bone Marrow Transplantation Unit, Hospital das Clínicas, University of São Paulo, São Paulo, Brazil; ⁴Infection Control Committee, Hospital das Clínicas, University of São Paulo, São Paulo, Brazil.

*Correspondence: Silvia Figueiredo Costa, costasilviaf@ig.com.br

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Abbreviations: BMT, Bone Marrow Transplant; CRPa-BSI, carbapenems-resistant *P. aeruginosa* bloodstream infection; CVC, central venous catheter; GNB, Gram-negative bacilli; GPB, Gram-positive bacteria; GVHD, graft versus host disease; HSCT, Hematopoietic Stem Cell Transplant; KPC, *Klebsiella pneumoniae* carbapenemase; LTC, long-term catheter; MDR, multidrug-resistant; MLST, multilocus sequence typing; MASCC score, Multinational Association for Supportive Care in Cancer; PFGE, pulsed field gel electrophoresis; SNPs, single nucleotide polymorphisms; SPM, São Paulo metallo- β -lactamase; ST, sequence type; TBI, total body irradiation; Tn, Transposon; WGS, whole genome sequence.

Many types of metallo- β -lactamases (MBLs) have been described in carbapenem-resistant *P. aeruginosa* isolates, including Imipenemase (IMP), Verona integron-encoded metallo- β -lactamase (VIM) and São Paulo Metallo- β -lactamase (SPM) [11, 12]. In Brazil, SPM is the most frequent mechanism of resistance among carbapenem-resistant *P. aeruginosa*, since the endemic clone harbouring the blaSPM-1 MBL gene, sequence type 277 (ST277), has spread throughout the country [13]. In contrast, the STs described in isolates of *P. aeruginosa* causing outbreaks in hospitals worldwide are ST235, ST111 and ST175 [14].

In order to analyse the mortality and risk factors for developing BSI caused by carbapenem-resistant *P. aeruginosa* (CRPa), we conducted a retrospective case-control study during an outbreak at the bone marrow transplant (BMT) unit of a teaching hospital in São Paulo, Brazil, and the evaluated microbiological and molecular characteristics of the isolates.

METHODS

Setting

The study was conducted at the 12-room BMT unit of Hospital das Clínicas, São Paulo, Brazil, a 1000-bed tertiary-care unit affiliated with the University of São Paulo, from December 2011 to January 2013.

Case and case-control definitions

A case was defined as any patient in the BMT unit with a positive peripheral and/or central line blood culture for CRPa during this period and, for further analysis, the reference date used was the day of the first positive culture. Carbapenem resistance was defined as resistance to imipenem and/or meropenem. A control was defined as any patient that remained in the same unit, during the same period, for a minimum of seven days, with at least one negative surveillance culture for CRPa collected in the final seven days before hospital discharge, and who had not developed any infection due to CRPa during the study period. The reference date used for comparison was the day of the final negative surveillance culture.

Study design and data collection

We conducted a 1:2 case-control study to identify risk factors for CRPa-BSI. Data regarding demographic and clinical characteristics were collected from medical records. The following information was obtained for each patient: age, sex, underlying disease, status of the disease (initial diagnosis, complete remission, partial remission or refractory disease), admission date, time from admission, the reference date, data regarding prior HSCT, type of transplant (i.e. autologous or allogeneic, related and matched) and outcome (i.e. discharge or death). Additional data were collected regarding the 30 days prior to the reference dates for cases, and throughout the in-hospital stay for the controls, addressing the exposure to potential risk factors such as chemotherapy; total body irradiation (TBI); graft-versus-host disease (GVHD) prophylaxis; transplantation conditioning; recently administered

antibiotics (used 30 days before the reference date for cases, and for all admission periods for controls); dosage of corticosteroids (adjusted for milligrams of prednisone); presence and duration of neutropenia (defined as an absolute neutrophil count <500 cells mm^{-3}); and central venous catheter use (type and duration). Clinical data such as the presence and grade of mucositis (I–IV according to the Southwest Oncology Group grading system), presence of GVHD, severity of neutropenia (<100 cells mm^{-3}), MASCC score (Multinational Association for Supportive Care in Cancer), platelet count and creatinine value also were obtained. In addition to these data, information regarding the treatment of cases, such as antibiotic(s) used for the treatment of CRPa-BSI; time of introduction of appropriate antibiotic (polymyxins B or E or aminoglycoside based on susceptibility profile); source of infection (related to central catheter or not); removal of the device if catheter-related bloodstream infection (CRBSI); outcome (e.g. discharge or death); catheter insertion; and transplant until first positive culture for CRPa were collected. The timing of antibiotic was considered correct up to 6 h of positive culture. Also obtained was information from the reference date to death, and whether this was related to the BSI. Non-related death was considered if there was any another cause of death in the autopsy, or if it occurred after the resolution of the CRPa infection described.

Microbiological and molecular analysis

Isolate identification was performed using the automated Vitek system (bioMérieux, Hazelwood, MO).

Antibiotic sensitivity profile

Minimum inhibitory concentrations (MICs) for aminoglycosides, imipenem, meropenem and polymyxins B and E were determined by broth microdilution, according to the Clinical Laboratory Standards Institute (CLSI 2012; <https://clsi.org/>).

Molecular typing

Molecular profiles were assessed by pulsed field gel electrophoresis (PFGE), using SpeI restriction enzyme (Fermentas, USA) in chromosomal DNA Ultrapure Agarose (Invitrogen, Life Technologies). Patterns were interpreted according to Bionumerics version 7.1 (Applied-Maths, Sint-Martens-Latem, Belgium). A Dice coefficient >0.80 was considered the cut-off for potential clonal relatedness.

Carbapenemase genes

PCR for the following carbapenemase genes, bla_{IMP}, bla_{SPM}, bla_{VIM}, bla_{SIM}, bla_{NDM}, bla_{KPC} and bla_{GES}, was performed as described previously [15–17]. The following reference strains were used in this study: *P. aeruginosa* producing IMP-1, VIM-1, SIM-1, SPM-1 enzymes KPC ATCC, *E. coli* NDM. Nucleotide sequencing to confirm the enzyme gene types was performed by MegaBACE 1000. The sequences were analysed using Sequence Analyzer software with Base Caller Cimarron 3.12. The genetic sequence was compared to the database available on the Internet (BLAST-<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Whole-genome sequencing

Whole-genome sequencing (WGS) of the predominant clone of CRPa and two strains belonging to a clone with a different profile of resistance identified during the outbreak in the BMT unit, in São Paulo, Brazil, was performed to investigate the mechanism of resistance, multilocus sequence typing (MLST) and virulence genes. The libraries were prepared with the Nextera XT Illumina™ kit and submitted for WGS by MiSeq Illumina™ methodology. *De novo* assembly of reads was performed using VelvetOptimiser v. 2.2.5 (Victorian Bioinformatics Consortium, Australia), and contigs were ordered by Abacas v. 1.3.1 [18]. The genome annotation was performed by Prokka [19]. The study of resistance and virulence genes was performed using the tools Resfinder (<https://cge.cbs.dtu.dk/services/ResFinder/>) and Blast 2 Seq (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Clonal relatedness was investigated by MLST (<http://pubmlst.org/paeruginosa/>). Subsequently, the results generated by these tools and the presence of the gene were confirmed by manual curation in the program Artemis v. 16.0.0.

Single nucleotide polymorphisms (SNPs) were identified by mapping the sequencing reads of the isolates against *P. aeruginosa* PAOI (Genbank accession number AE004091.2) using BWA, SAMtools and Genome Analysis Toolkit (GATK). All SNPs were manually checked.

Statistical analysis

All statistical analyses were performed using STATA version 12.1. Unpaired *t*-test was performed for continuous variables, and Chi square or Fisher's exact test for categorical data. A multivariate analysis was assessed with stepwise logistic regression for variables with a *P* value of <0.10 in the bivariate analysis and biological plausibility. A *P* value of <0.05 was considered significant. In our study, to avoid bias, we used two models to perform the multivariate analysis, one with neutropenia, MASCC and use of corticoids, and another without. Establishing a reference date in order to analyse risk factors for infection can easily overestimate clinical characteristics related to severity, as these factors would point to more severe values in the infection group (cases) than in the group without infection (controls). A Kaplan–Meier survival curve was performed to compare treatment with two or three antibiotics.

RESULTS

Outbreak description

In December 2011, an outbreak of CRPa-BSI was identified at the BMT and up to January 2013, 29 patients were diagnosed with positive blood culture for CRPa; 176 HCSTs were performed in our hospital during the study period. When two cases in the same month were detected (in January 2012), the following measures were implemented: surveillance cultures (weekly rectal swab and faeces) from patients admitted to the unit; contact precaution for all cases during hospitalization; central line bundle; reinforcement of the cleaning regimens; and compliance with hand hygiene and contact precaution.

One hundred and twenty-one opportunities for compliance with contact precautions, including hand hygiene, were observed. Compliance with hand hygiene was 71 % among nurses and 79 % among physicians; in regard to wearing a gown the figures were 86 and 84 %, respectively; and for wearing gloves 81 and 84 %, respectively. The BMT unit baseline data on compliance with contact precaution varied from 66 to 70 % prior to the outbreak. In total, 132 samples from environmental (water sources, such as sink and shower; frequently touched equipment such as infusion pumps, stethoscopes and others) and healthcare workers (hand cultures) were collected, and all were negative for *P. aeruginosa*.

Case-control study

Over the study period, 58 patients were selected as controls. It was not possible to include patients with BSI caused by GNBs other than MRSA as controls, because during the outbreak only 11 *K. pneumoniae*, 2 *Enterobacter cloacae*, 2 *Serratia marscecens*, 1 *A. baumannii* and 1 MRSA BSI were identified. Demographic and clinical characteristics are shown in Table 1. Bivariate analysis showed that prior allogeneic transplantation (OR=5, 95 % CI: 1.6–16.8; *P*=0.002) was significantly associated with CRPa-BSI. Long-term catheter (LTC) use was associated with CRPa-BSI (OR=8.08, 95 % CI: 1.08–355; *P*=0.029), but the type of LTC (Permcath, Hickman and Portocath) (*P*=1.0) was not associated with CRPa-BSI. The mean interval between LTC insertion and HSCT and the identification of carbapenem-resistant *P. aeruginosa* in blood was 51.9±7.8 cases (Table 1).

Regarding the study cases, the average time between admission and first positive culture was 35.1 days, and from the time of culture collection to death was 7.2 days, with 14 cases (58 %) dying in three days or less from the time of blood assessment. Twenty-two of the 29 BSI cases were diagnosed as CRBSI. Twenty-three (79 %) culminated in death, with 19 cases (65 %) being directly associated with BSI; two patients died due to other complications (pulmonary embolism and cerebral hemorrhage), and two from a recurrent CRPa infection (one with pneumonia and the other with another BSI that occurred after the study period). Of all CRBSIs, only 10 had the LTC removed by the end of treatment or death; this strategy had no impact on death (*P*=0.2). Four cases died before the isolation of CRPa in the blood and were treated only with carbapenems. Polymyxin alone was used in one case, polymyxin plus carbapenems in 12 cases and finally, polymyxin plus aminoglycoside plus carbapenem in 12 patients (from 24 patients that used it, nine received polymyxin B and 15 received polymyxin E). However, there was no difference in prognosis regarding the antibiotic treatment used, since the Kaplan–Meier survival curve comparing the use of two and three drugs in treatment showed no impact on outcome (*P*=0.17). Data regarding the timing of antibiotic administration was available for 17 patients; 1/4 of patients that survived and 7/13 of patients that died received antibiotic within 6 h of the availability of microbiology results.

The first model with no severity score showed that neutropenia (OR 33.24; 95 % IC 1.82–607; *P*=0.018), GVHD

Table 1. Bivariate analysis of demographic and clinical variables in case-control study of risk factors for CRPa-BSI in HSCT patients

	Cases (n=29)	Controls (n=58)	OR (95 % IC)	P
Age	38.6±2.6	43.6±1.8	–	
Sex – Male	17 (59 %)	27 (46 %)	1.62 (0.66–4.00)	0.36
Length of hospital stay (days, mean)	35.1±4.9	43.6±1.8	–	0.12
Underlying disease				
Acute leukaemia	10 (34 %)	6 (10 %)	4.5 (1.1–17.2)	0.008
Chronic leukaemia	1 (2 %)	2 (3 %)	1.0 (0.08–11.05)	1.00
Lymphoma	4 (14 %)	22 (38 %)	0.26 (0.08–0.85)	0.016
Aplastic anaemia	5 (17 %)	11 (20 %)	0.89 (0.27–2.8)	0.8
Multiple myeloma	3 (10 %)	6 (10 %)	1.0 (0.23–2.45)	1.0
Others	6 (21 %)	17 (19 %)	0.62 (0.21–1.81)	0.54
Type of HSCT				
Allogeneic HSCT	16 (36 %)	20 (74 %)	5.00 (1.73–14.3)	0.020
Unrelated HSCT	2 (4.5 %)	(18.5 %)	4.72 (0.85–26.6)	0.067
Immunosuppressive drugs and therapy				
Conditioning for HSCT	23 (85 %)	38 (85 %)	(0.64–6.60)	0.89
GVHD Prophylaxis	22 (81 %)	13 (30 %)	10 (2.9–410)	<0.001
TBI	9 (33 %)	3 (7 %)	6.8 (1.4–42)	0.007
Medium corticoid dose, mean	1610±475	810±155	–	0.049
Antibiotic usage				
Previous use of carbapenem	24 (83 %)	19 (33 %)	9.8 (2.9–37)	<0.01
Days of carbapenem usage, mean	6.1±1.1	4.1±0.9	–	0.19
Levofloxacin usage	11 (38 %)	11 (19 %)	2.61 (0.86–7.96)	0.55
Days of levofloxacin usage, mean	4.2±0.6	3.5±0.5	–	0.52
Presence of neutropenia	27 (93 %)	35 (60 %)	8.8 (1.8–82)	0.001
Severe neutropenia (<100 cells)	25	11	31 (7.89–122)	0.029
Permcath	24	39	0.92 (0.23–2.61)	1.00
Hickman	3	5	1.22 (0.27–5.51)	0.80
Portocath	1	1	1.91 (0.11–32.0)	1.00
MASCC score, mean patient neutropenia	16.4±0.7	20.4±0.4	–	0.001
MASCC score >21 patient neutropenia	1 (4 %)	10 (34 %)	0.07 (0.009–0.67)	0.005
Platelet count, mean	25.600±2.700	97.741±13.915	–	0.01
Creatinine level (mean)	1.06±0.13	0.85±0.07	–	0.13
Mucositis	15 (52 %)	9 (17 %)	3.33 (0.77–14.31)	0.006
Grades I–II	6 (26 %)	6 (11 %)	2.26 (0.65–7.76)	0.20
Grades III–IV	9 (45 %)	3 (6 %)	8.25 (2.02–33.5)	0.001
GVHD	5	0	–	0.006

Bold indicates the variables that were significant in bivariate analysis or and in the multivariate model.

prophylaxis (OR 223.36; 95 % IC 2.73–1827.2; $P=0.016$) and prior use of carbapenems (OR 68.73; 95 % IC 3.14–1503.6; $P=0.0089$) were associated with CRPa-BSI; however, when MASCC ≥ 21 points and platelets were included in the final multivariate analysis, only prior use of carbapenems remained as an independent risk factor for CRPa-BSI (OR 22.06; 95 % IC 1.19–44.4; $P=0.043$) (Table 2).

Microbiological data

All isolates were resistant to carbapenems (Imipenem MIC ranged from 16 to 128 and meropenem MIC from 8 to

256 $\mu\text{g ml}^{-1}$) and susceptible to polymyxins B and E (MIC ranged from 0.5 to 1.0 $\mu\text{g ml}^{-1}$). Twelve isolates were susceptible to aminoglycosides.

Molecular analysis

Sixteen isolates were analysed, seven harbouring the SPM-1 gene, seven co-harbouring both KPC and SPM genes, and in one isolate no carbapenemases were detected. Six of 29 patients were co-infected by carbapenem-resistant Enterobacteria (5 *K. pneumoniae* and 1 *Serratia marscescens*), but KPC was detected by PCR in only 1

Table 2. Multivariate analysis of risk factors for CRPa-BSI in HSCT patients

	OR	95 % CI	P
Acute leukaemia	1.17	0.06–21.1	0.91
Lymphoma	0.18	0.01–2.16	0.18
Allogeneic HSCT	0.34	0.03–3.47	0.15
Mucositis III–IV	0.40	0.02–5.51	0.49
TBI	0.98	0.01–49.8	0.68
GVHD prophylaxis	15.19	0.52–440.0	0.11
Neutropenia	6823.5	0.001–1.01	0.49
Platelet count	1.00	0.99–1.00	0.77
MASCC >21 points	0.19	0.01–9.31	0.45
Previous use of carbapenems	22.06	1.19–44.4	0.043

K. pneumoniae strain identified from a patient with KPC-negative *P. aeruginosa* infection. The dendrogram showed that there was a predominant clone in our BMT unit, evidencing probable cross-transmission (Fig. 1). In total, three strains were sequenced, a representative of the predominant clone (strain 963) and two strains that belonged to another clone, 1266 (SPM-1 positive) and 1315 (carbapenemase negative). Strains 1266 and 1315 were chosen because they belong to the same clone but differ in their carbapenemase profile. The three strains sequenced were assigned as ST277, harbouring Tn4371 and several genes with resistance to aminoglycosides, beta-lactam agents, fluoroquinolone, phenicol and sulfonamide (Tables 3 and 4), and did not differ regarding either outer membrane protein, or efflux pumps. Virulence genes involved in adhesion, biofilm, quorum sensing and type III secretion systems were identified, but *exoU* was not present in the three strains sequenced (Table 3).

DISCUSSION

This is the first report of an outbreak due to *P. aeruginosa* harbouring *bla*_{KPC} and co-harbouring both *bla*_{KPC} and *bla*_{SPM} genes in HSCT patients. Our findings showed that prior carbapenem use was the only independent factor associated with CRPa-BSI and that combination therapy with two or three antibiotics had no impact on mortality. The overall mortality among our cases was very high (79%), but similar to prior reports showing that mortality from BSI caused by *P. aeruginosa* in HSCT patients ranged from 40 to 87.5% [5, 20]. Interestingly, the carbapenem-resistant *P. aeruginosa* in our study harboured different carbapenemases and two carbapenemase genes. Nonetheless, the predominant clone harboured SPM-1 gene and belonged to ST277, and four strains co-harboured KPC. One previous study showed that SPM was the most frequent carbapenemase identified in the BMT unit at our hospital, followed by KPC-2 [21]. KPC in *P. aeruginosa* is rare and occurs mainly on the American continent

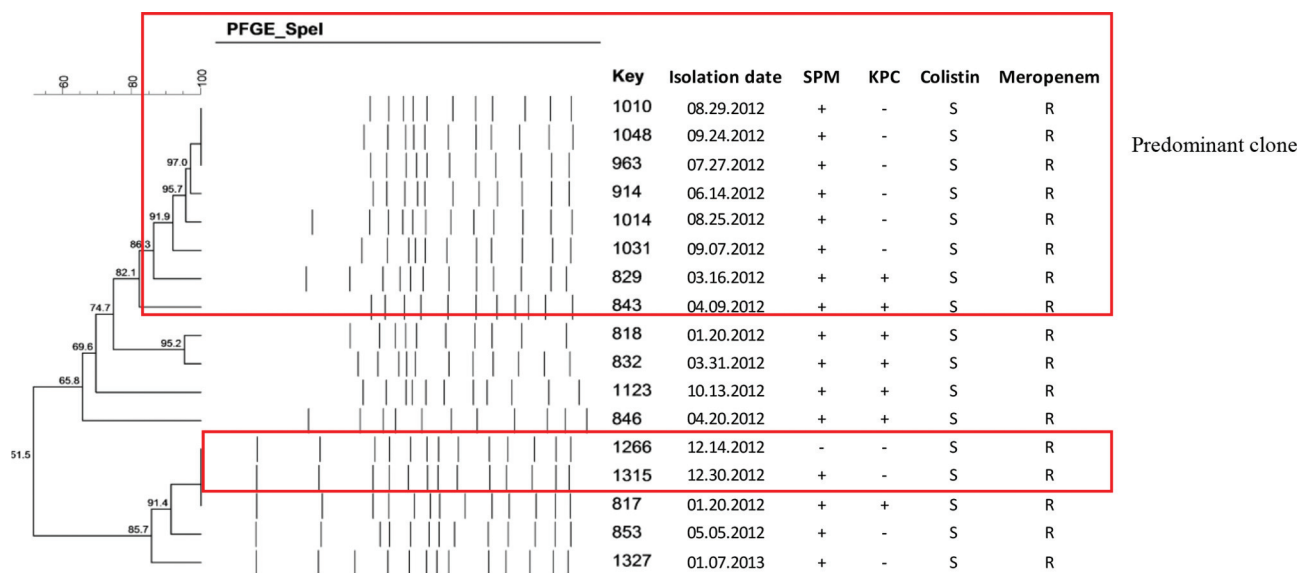
**Fig. 1.** Dendrogram of 17 carbapenem-resistant *P. aeruginosa* strains isolated from bloodstream infection in HSCT patients

Table 3. Whole-genome sequencing of predominant clone of *P. aeruginosa* harbouring SMP identified in a bone marrow unit in São Paulo, Brazil

Laboratory number	Isolation site	Resistance profile MIC ($\mu\text{g ml}^{-1}$)				Whole-genome sequence analysis					
		AK	MER	COL	Tn4371	Virulence genes					
Isolation year						Resistance genes	Quorum sensing	Biofilm	Adhesion	Cytotoxicity and invasion process SST III	ST
Predominant Clone Strain 963 2012	Blood	8	512	0.5	Present	aph(3')-Iib, aadA7, aacA4, bla _{SPM-1} , bla _{PAO} , bla _{OXA-56} , bla _{OXA-50} , aac(6')Ib-cr, fosA, catB7, cmx, sul1	bfmR-S, qscR, qteE	gacA, gacS, ladS, lasB	csuD lecB	exoS, exoY	277
Strain 1266 2012	Blood	512	256	0.5	Present	aph(3')-Iib, aadA7, rmtD, aacA4, bla _{PAO} , bla _{OXA-56} , bla _{OXA-50} , aac(6')Ib-cr, fosA, catB7, cmx, sul1	bfmR-S, qscR, qteE	gacA, gacS, ladS, lasB	csuD lecB	exoS, exoY	277
Strain 1315 2012	Blood	512	512	1	Present	aph(3')-Iib, aadA7, rmtD, aacA4, bla _{SPM-1} , bla _{PAO} , bla _{OXA-56} , bla _{OXA-50} , aac(6')Ib-cr, fosA, catB7, cmx, sul1	bfmR-S, qscR, qteE	gacA, gacS, ladS, lasB	csuD lecB	exoS, exoY	277

AK, amikacin; MER, meropenem; COL, colistin; MIC, minimal inhibitory concentration; ST, sequence type. Tn4371 – transposon. *aacA4* – aminoglycoside *N*(6')-acetyltransferase type 1; *aac(6')Ib-cr* – aminoglycoside 6'-*N*-acetyltransferase Ib-Cr type; *aadA7* – streptomycin 3'-adenylyltransferase; *aadA10* – streptomycin 3'-adenylyltransferase; *aph(3')-Iib* – aminoglycoside-phosphotransferase; *blaOXA-50* – oxacillinase; *blaOXA-56* – oxacillinase; *blaPAO* – beta-lactamase; *blaSPM-1* – beta-lactamase; *blaVIM-36* – beta-lactamase; *catB7* – chloramphenicol acetyltransferase; *cmx* – chloramphenicol resistance protein; *fosA* – glutathione transferase; *rmtD* – 16S rRNA methylase; *sul1* – sulfonamide-resistant dihydropteroate synthase; *gacA* – response regulator; *gacS* – sensor protein; *ladS* – lost adherence sensor; *lasA* – protease *lasB* – elastase; *plcB* – phospholipase C; *plcH* – haemolytic phospholipase C; *plcN* – non-haemolytic phospholipase C; *plcR* – phospholipase accessory protein; *qscR* – quorum-sensing control repressor; *qteE* – quorum threshold expression element; *bfmR* – response regulator; *bfmS* – sensor kinase; *lecB* – fucose-binding lectin PA-III; *exoT* – exoenzyme T; *exoY* – adenylate cyclase; *exoU* – phospholipase protein; *toxA* – exotoxin A.

[22–25]. Moreover, *P. aeruginosa* harbouring KPC was described recently in Iran, showing the potentially rapid dissemination of this resistance mechanism around the world [26]. Association between colonization and infection by KPC-positive Enterobacteria and the spread of KPC among *P. aeruginosa* has been described [27]. One previous study [27] observed that 43 % of *Pseudomonas* isolates obtained after an outbreak of KPC-producing *K. pneumoniae* harboured the blaKPC gene. In our study, although

co-infection by carbapenem-resistant Enterobacteria occurred in six patients, only one *K. pneumoniae* strain harboured KPC. This strain was identified from a patient with a KPC-negative *P. aeruginosa* infection.

The predominant clone identified in our hospital harboured several resistance genes, such as 16S rRNA methyltransferase gene *rmtD1*, which confers high-level resistance to all aminoglycosides and has been associated with ST277. It also

Table 4. Outer membrane protein and efflux pump gene mutations by whole-genome sequencing of 3 carbapenem-resistant *Pseudomonas aeruginosa* strains

Isolate	OprD	OprE	OprM	MexD	OprJ	MexE	MexI	OpmD	MexT
Predominant Clone 963	T103S , S213G, T377G, V378L, V380L, I388M, S391K, K394N, K115T , T397S, A398G, S410A, S434T, A438V, A439S, S443T, V118P, N444A, N445T, S441_S442insG, L106_D107_insGL F170L		A261T	E257Q, S845A	D68G, M69V	P397Q, A407V	A782E	L381R	F94I, I263F, D267E
1266*	T103S , S213G, T377G, V378L, V380L, I388M, S391K, K394N, K115T , T397S, A398G, S410A, S434T, A438V, A439S, S443T, V118P, N444A, N445T, S441_S442insG, L106_D107_insGL F170L		A261T	E257Q, S845A	D68G, M69V	P397Q, A407V	A782E	L381R	F94I, I263F, D267E
1315	T103S , S213G, T377G, V378L, V380L, I388M, S391K, K394N, K115T , T397S, A398G, S410A, S434T, A438V, A439S, S443T, V118P, N444A, N445T, S441_S442insG, L106_D107_insGL F170L		A261T	E257Q, S845A	D68G, M69V	P397Q, A407V	A782E	L381R	F94I, I263F, D267E

Bold indicates the OprD mutations already described in CRPa related to carbapenems-resistance.

*Strain 1266: SPM gene negative.

Porins – OprC, OprD, OprE, OprF; Efflux pump genes: MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexGHI-OpmD; MexR – repressor of operon MexAB-OprM; and MexT – post-transcriptional activator MexEF-OprN. Mutations T103S, K115T and F170L already described in CRPa, associated with carbapenem-resistance due to porin alterations.

carried other resistance genes for phenicol, fluoroquinolone and the carbapenemases SPM-1 and Tn4371. ST277 was recently reported in the UK from a patient who underwent surgery during travel to Brazil [28]. The authors demonstrated that the SPM-1 was located in Tn4371, flagging up the potential of spread of this lineage throughout the world. In this context, the dissemination of SPM is worthy of concern. An intriguing finding in our study is that the clone assigned as ST277 did not harbour carbapenemase and presented mutation of an outer membrane protein, which explains its resistance to carbapenems. The three strains analysed by WGS did not differ regarding virulence and mutations in OMP, efflux pump and PBP. Mutations in OprD and efflux pump could, alternatively, explain carbapenem resistance in the strain that did not harbour carbapenemase. The mutations T103S, K115T and F170L were found in the three strains analysed by WGS. These mutations have already been described in CRPa, associated with carbapenem resistance due to porin alterations [29–33]. The mutation V118P was found in all three strains, but as it has not yet been described as being associated with carbapenem resistance, further studies are required to verify its role in resistance.

The ST277 clone harboured important virulent genes involved in adhesion, quorum sensing, biofilm production and the type III secretion system [14], which explains the persistence of this clone over one year and the difficulty in controlling the outbreak. On the other hand, an important virulence gene, *exoU*, was not identified in any of the three strains sequenced. A recent study showed a similar result, demonstrating that among high-risk MDR clones ST111, ST235 and ST175, *exoU* was observed only in the epidemic clone ST235 [34].

P. aeruginosa is the second most prevalent pathogen among all infections related to nosocomial outbreaks, and a frequent cause of infection among HSCT patients [35]. Outbreaks due to *P. aeruginosa* are mostly related to environmental contamination, cross-transmission, medical equipment and water source [36, 37]. However, in 2011, Vonberg *et al.* [35], showed that in 39.7% of reports no environmental source was identified and almost half (47.5%) were linked to cross-transmission. As soon as an outbreak was identified, molecular analysis showed that there was a predominant clone, evidencing cross-transmission. Thus, surveillance cultures of patients, staff and the environment were performed, but despite extensive investigation, no environmental source was identified. The outbreak was controlled after one year of reinforcement of all infection control measures such as hand hygiene, environmental cleaning, contact precaution and CVC care.

In our study, in order to avoid bias, we used two models to perform the multivariate analysis, with and without all the clinical factors described above. The model without a severity score showed that neutropenia ($P=0.018$), GVHD prophylaxis ($P=0.016$) and prior use of carbapenems

($P=0.0089$) were associated with CRPa-BSI. However, when MASCC ≥ 21 points and platelets were included in the final multivariate analysis, only prior use of carbapenems remained as an independent risk factor for CRPa-BSI ($P=0.043$).

A possible explanation for the observed high mortality is the low number of antibiotics available to treat MDR bacteria, especially carbapenem-resistant *P. aeruginosa*. In this scenario, combined therapy has been used as an option to treat MDR pathogens [38–40]. All the cases described here received carbapenems in combination with polymyxins, and some also received aminoglycoside. Combination therapy with two or three drugs, however, did not have any impact on outcome, probably because of the size of population analysed.

An important limitation of our study is that we did not address the expression of the phenotypic characteristics of virulence genes. Thus, we cannot infer the role of virulence of the predominant clone on mortality. The controls used in our study represent another important limitation. Because of the scarcity infections caused by other bacteria during the outbreak and the number of patients submitted for HSCT, it was not possible to include BSIs caused by GNB other than MRSA as controls.

In conclusion, CRPa-BSI harbouring SPM-1 in HSCT patients resulted in high mortality, and combination therapy with two or three drugs did not have any impact on outcomes. The predominant clone producing SPM-1 belonged to ST277 and harboured Tn 4371. Prior exposure to carbapenems was the only independent risk factor associated with CRPa-BSI, and showed that the rational use of carbapenems in this setting should be reconsidered.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

These experiments were approved by the Ethical Committee of Hospital das Clinicas of University of Sao Paulo, Brazil.

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