Carbapenem-resistant *Pseudomonas aeruginosa* bloodstream infection: *in vitro* synergy, virulence and clinical outcome.

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Abstract

In the current study carbapenem-resistant *P. aeruginosa* bloodstream infection (CRPA-BSI) was associated with high 14-day mortality. Although, highly resistant to meropenem, half of the isolates achieved *in vitro* synergy with the combination of meropenem with colistin, and the patients that received this combination therapy showed a tendency towards lower mortality. Our strains did not carry *exoU* gene, on the other hand, patients with CRPA-BSI caused by strains harboring gene *lasB* evolved more frequent to death.

Introduction

Carbapenem-resistant *Pseudomonas aeruginosa* bloodstream infection (CRPA-BSI) has been associated with high morbidity and mortality in hematopoietic stem cell transplantation (HSCT) patients. Nowadays, it is a challenge with limited therapeutic options (1-2). Thus, *in vitro* synergy studies are important to define combination therapy that can be useful to treat these infections. To date, few studies evaluated antimicrobial synergy against CRPA (3 5) and the role of virulence on the high mortality associated with CRPA-BSI is controversial as well (2, 3).

Therefore, we aimed to describe the clinical data of 30 patients with CRPA-BSI from January 2012 to December 2014, at the Hospital das Clínicas of University of Sao Paulo, Brazil. *In vitro* synergy, pulsed field gel electrophoresis (PFGE) and PCR for carbapenemases (SPM; KPC, VIM, NDM) and virulence genes (*lasA* – protease *lasB* – elastase; *exoS* – exoenzyme S; *toxA* – exotoxin A; *phzM* – phenazine-specific methyltransferase) of the 30 strains, including those identified during the outbreak that occurred in our hospital in 2012,² were performed as described elsewhere. Based on PFGE profile,

whole genome sequencing (WGS) of five strains was carried out by Nextera XT, using Illumina MiSeq technology. *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. The annotation of genome was performed by Prokka v.1:11. *P. aeruginosa* PAO1 was used as reference. The antibiotic resistance and virulence genes analysis was performed using the following tools: Resfinder (<https://cge.cbs.dtu.dk/services/ResFinder/>), Blast2Seq (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and MAFFT (<http://mafft.cbrc.jp/alignment/server/>). Subsequently, the presence of the genes were confirmed by manual curation of CDS and by Artemis program v.16.0.0. The Multilocus Sequence Typing (MLST) analysis was performed by MLSTfinder tool (<https://cge.cbs.dtu.dk/services/MLST/>) and analyzed by software eBURST (<http://eburst.mlst.net/>).

BSI definition was based on Centres for Diseases Control and Prevention (CDC) criteria (6) and Pitt bacteraemia score was calculated (7). All patients received levofloxacin as prophylaxis during neutropenia. Initial antibiotic therapy was considered appropriate when colistin (COL) was administered within 24 hours after obtain blood culture. All patients received 2g of meropenem (MERO) tid, prolonged infusions (3 hours) adjusted for kidney function when indicated. Amikacin (AMK) was added to therapy with colistin and meropenem whenever CRPA was susceptible.

Minimum inhibitory concentrations (MIC) of COL (USP Reference Standard, Rockville, MD, USA), AMK (Sigma-Aldrich, St Louis, MO, USA), and MERO (Astra Zeneca, Cotia, SP, Brazil) were determined using the broth

85 microdilution method according to the Clinical and Laboratory Standards
86 Institute (8). *Time-kill* assay was performed in duplicate with drugs alone and
87 combined at 1x MIC and 0.5x MIC as previously reported (9-10). Aliquots
88 were removed at time 0 and 2, 4, 6, and 24 hours and serially diluted in 0.85%
89 sodium chloride solution. Diluted samples of 0.01 mL were plated on Müller-
90 Hinton agar, and the colonies were counted ($\log_{10}$ cfu/mL) after 20 hours of
91 incubation at 37°C. Synergy was interpreted as a $\geq 2 \log_{10}$ decrease in colony
92 count with the combination compared to the most active single agent; was
93 considered antagonistic for a $\geq 2 \log_{10}$ increase in cfu/mL, and indifferent for a
94 $< 2 \log_{10}$ increase or decrease in count (9-10). Bivariate Cox regressions were
95 used to evaluated 14-day mortality using SPSS software version 21 (SPSS
96 Inc., USA), value of p less than 5% was considered significant.

97 The strains presented MERO MIC₉₀ > 512µg/mL; two thirds were also
98 resistant to AMK (MIC 2-512 µg/mL) and all were susceptible to COL (Table
99 1). The combination of COL plus MERO achieved synergy in 57% of isolates,
100 COL plus AMK in 2/30 isolates (6.6%), and of MERO plus AMK in 33%
101 (10/30), only in isolates susceptible to AMK (table 2). Although, highly
102 resistant to meropenem, half of the isolates achieved *in vitro* synergy with
103 combination of meropenem with colistin, and the group that received this
104 combination showed a tendency towards lower mortality (6 of 7 patients that
105 survived received meropenem plus colistin) (p=0.06). Zusman et al (2013)
106 performed a meta-analysis and found similar results for *in vitro* combination of
107 carbapenem and polymyxin B or colistin in 59% of 43 CRPA isolates (4).

108 Clinical and demographic data are shown in table 2. Mortality was higher
109 among allogeneic HSCT recipients compared to autologous recipients (79%

110 vs. 17% $p = 0,012$). Patients treated with two or three drugs did not present a
111 statistically significant difference in 14-day death (29 % x44% $p = 0,65$) (table
112 2).

113 Five clones named as A, B, C, D and E harboured SPM and belonged to
114 ST277 were found by PFGE during the study period (table 1). ST277 is
115 common in Brazil and has been described in several outbreaks including
116 patients who travelled to the country (13-16). Interestingly, the six strains that
117 co-harbored KPC were distributed in four clones, except the predominant
118 clone named as clone A. We did not find *exoU* in our strains, on the other
119 hand, patients with CRPA BSI caused by strains harboring gene *lasB* evolved
120 more frequent to death (88.9% vs. 57.1%). This virulence factor is associated
121 with bacterial enzyme production of elastase that degrades immunoglobulin
122 and complement factor (12). In Brazil, the gene *exoU* was described in CRPA
123 belonging to ST2237 in burn patients instead of ST277 (17).

124 The most frequent carbapenemase identified was *bla*_{SPM}, and six isolates co-
125 harbored *bla*_{KPC} as shown in table 1. The five clones sequenced by illumina
126 were assigned as ST277 and harbored the Tn4371 (Table 3). In all the strains,
127 mutations on outer membrane protein OprD (T103S, K115T, V118P, F170L)
128 OprM (A261T), OprJ (D68G, M69V) were found, as well as the *mexT* gene
129 that possessed a mutation resulting in a frameshift. The 16S rRNA
130 methyltransferase gene *rmtD1*, which confers high-level resistance to all
131 aminoglycosides and has been associated to ST277 was present only in
132 clones I, IV and V. Clone III did not harbor *fosA*, it carried *exoS* and *toxA* of
133 Type Three Secretion System (TTSS) and lacked the *exoY* gene. Genbank

access number are QHLR00000000, QHLU00000000, QHLW00000000, QJPG00000000, and QJPA00000000.

Although, the study was underpowered to recommended combination therapy with more than two drugs, it highlights the importance to test *in vitro* synergy to treat infections caused by CRPA.

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Competing interests: None

Ethical statement

The ethical committee of Hospital das Clinicas and CONEP (National Ethics Commission), Brazil, gave ethical approval.

Author contribution statements

JR collected and analysed data and written the article, GCL performed time kill and PFGE, CR performed PCR, RR analysed the whole genome sequence, SS performed de whole genome sequence, FR provides the strains, TG provides clinical data, ASL analysed data and reviewed the article, VR provide clinical data, SFC design the study, analysed data and written the article.

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Table 1 . Microbiological characteristics of thirty isolates of carbapenem-resistant *P. aeruginosa* of bloodstream infection.

Isolates	PFGE carbapenemase	MIC Results (mg/ml)						In vitro synergy by time-kill		
		AMK		COL		MERO		COL+MERO	COL+AMK	MERO+AMK
1	D SPM/ KPC	512	R	0,5	S	256	R	Yes	-	RG 24h
2	B SPM/ KPC	4	S	0,5	S	512	R	Yes	RG 6h	Yes
3	B SMP/ KPC	4	S	0,5	S	256	R	RG 24h	RG 6h	Yes
4	E SPM/ KPC	2	S	0,5	S	16	R	Yes	RG 6h	Yes
5	B SPM/ KPC	4	S	1	S	512	R	RG 24h	RG 6h	Yes
6	D SMP	4	S	1	S	512	R	RG 24h	RG 24h	Yes
7	A SPM	4	S	1	S	512	R	Yes	-	Yes
8	A SPM	8	S	0,5	S	512	R	Yes	RG 24h	Yes
9	A SPM	512	R	0,5	S	256	R	RG 24h	-	RG 24h
10	A SPM	512	R	1	S	256	R	RG 24h	-	RG 24h
11	A SPM	512	R	1	S	512	R	RG 24h	-	RG 24h
12	A SPM	512	R	1	S	256	R	RG 24h	-	RG 24h
13	C SMP/ KPC	512	R	0,5	S	256	R	RG 24h	-	RG 24h
14	D	512	R	0,5	S	256	R	Yes	-	RG 24h
15	D	512	R	1	S	512	R	RG 24h	-	RG 24h
16	D SPM	512	R	0,5	S	256	R	RG 24h	-	RG 24h
17	D SPM	512	R	0,5	S	>256	R	RG 24h	-	RG 24h
18	A SPM	512	R	1	S	256	R	Yes	-	RG 24h
19	A SPM	512	R	1	S	512	R	RG 24h	-	RG 24h
20	A SPM	512	R	1	S	256	R	Yes	-	RG 24h
21	A SPM	512	R	0,5	S	256	R	RG 24h	-	RG 24h
22	A SPM	8	S	0,5	S	512	R	Yes	Yes	Yes
23	A SPM	4	S	0,5	S	512	R	Yes	Yes	Yes
24	A	256	R	0,5	S	256	R	Yes	-	RG 24h
25	A SPM	256	R	0,5	S	256	R	Yes	-	RG 24h
26	A SPM	8	S	0,5	S	512	R	Yes	RG 24h	Yes
27	B	512	R	4	I	512	R	Yes	RG 24h	RG 24h
28	B SPM	512	R	4	I	512	R	Yes	RG 24h	RG 24h
29	B	512	R	4	I	512	R	Yes	RG 24h	RG 24h
30	B	512	R	4	I	512	R	Yes	RG 24h	RG 24h

MIC: Minimum inhibitory concentration, S: susceptible, R: resistant, I: intermediate,
COL: colistin,
MERO: meropenem, AMK: amikacin, RG: bacterial re-grow, (-) no synergyism

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Table 2. Comparison of clinical and laboratorial features of 25 patients submitted to HSCT with carbapenem-resistant *P. aeruginosa* BSI appropriated treated regarding outcome – São Paulo, Brazil.

	SURVIVED (n=6)	DIED (n=19)	
	n (%)	n (%)	p Value
Age (mean ± SD)	52,33 ± 11,55	41,05 ± 15,27	0.11
Male gender	1 (17)	7 (37)	0.63
Previous leukaemia	0	9 (47)	0.10
HSCT allogeneic	1 (17)	15 (78)	0.02
Neutropenia	6 (100)	18 (94)	1.00
Pitt score (mean ± SD)	0 ± 0 (n=6)	1,58 ± 3,15 (n=19)	0,23
Previous gut colonization	3 (50)	4 (23)	0.48
Treatment < 24h	3 (50)	13 (68)	0.15
<i>In vitro</i> synergy			
COL + MERO	6 (100)	9 (47)	0.06
COL + AMK	2 (33)	0	0.07
MERO + AMK	3 (50)	3 (16)	0.24
Carbapenamase genes by PCR			
<i>bla_{SPM}</i>	4 (66)	10 (53)	0.89
<i>bla_{SPM}</i> and <i>bla_{KPC}</i>	0 (0)	4 (21)	0.55
Virulence genes by PCR			
<i>lasB</i>	3 (50)	17 (89)	0.12
<i>ExoS</i> ; <i>phzM</i> and <i>toxA</i>	6 (100)	18 (96)	1.00
Treatment received			
2 antibiotics (COL plus MERO)	5 (71)	10 (56)	0,65
3 antibiotics (COL plus MERO plus AMK)	2 (29)	8 (44)	

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HSCT: Hematopoietic stem cell transplant , BSI: bloodstream infection, SD: standard deviation,
COL: colistin, MERO: meropenem, AMK: amikacin, PCR: polymerase chain reaction;
SPM-Sao Paulo Metallo-beta-lactamase; KPC- *Klebisella pneumoniae* Carbapanemase
lasA – protease *lasB* – elastase; *exoS* – exoenzyme S; *toxA* – exotoxin A;
phzM – phenazine-specific methyltransferase

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Table 3. Resistance and virulence genes by whole genome sequence of main clones of carbapenem-resistant *P. aeruginosa* causing bloodstream infection in HSCT patients.

Isolate	Year	Tn 4371	Resistance genes	Quorum-sensing	Biofilme	Adhesion	Secretion System Type III	Phenazine operons	ST
Clone I	2012	Positive	<i>aph(3')-IIB</i> , <i>aadA7</i> , <i>rmtD</i> , <i>aacA4</i> , SPM-1 , <i>aac(6')Ib-cr</i> , <i>fosA</i> , <i>catB7</i> , <i>cmx</i> , <i>sul1</i>	<i>bfmR-S</i> , <i>qscR</i> , <i>qteE</i>	<i>gacA</i> , <i>gacS</i> , <i>ladS</i> , <i>lasB</i> , ,	<i>lecB</i> ,	<i>exoS</i> , <i>exoYt</i> , <i>toxA</i>	<i>phzM</i>	277
Clone II	2012	Positive	<i>aph(3')-IIB</i> , <i>aadA7</i> , <i>aacA4</i> , <i>aac(6')Ib-cr</i> , <i>fosA</i> , <i>catB7</i> , <i>cmx</i> , <i>sul1</i> , <i>bcr-1</i> , SPM-1	<i>bfmR</i> , <i>bfmS</i> , <i>qscR</i> , <i>qteE</i>	<i>gacA</i> , <i>gacS</i> , <i>ladS</i> , <i>lasA</i> , <i>lasB</i> ,	<i>lecB</i> ,	<i>exoS</i> , <i>exoY</i> , <i>toxA</i>	<i>phzM</i>	277
Clone III	2012	Positive	<i>aph(3')-IIB</i> , <i>aadA7</i> , <i>aacA4</i> , SPM-1 , <i>aac(6')Ib-cr</i> , <i>catB7</i> , <i>cmx</i> , <i>sul1</i> , <i>bcr-1</i>	<i>bfmS</i> , <i>qscR</i>	<i>gacS</i> , <i>ladS</i> , <i>lasA</i> , <i>lasB</i> ,	<i>lecB</i>	<i>exoS</i> , <i>toxA</i>	<i>phzM</i>	277
Clone IV	2012	Positive	<i>aph(3')-IIB</i> , <i>aadA7</i> , <i>rmtD</i> , <i>aacA4</i> , SPM-1 , <i>aac(6')Ib-cr</i> , <i>fosA</i> , <i>catB7</i> , <i>cmx</i> , <i>sul1</i> , <i>bcr-1</i>	<i>bfmR</i> , <i>bfmS</i> , <i>qscR</i> , <i>qteE</i>	<i>gacA</i> , <i>gacS</i> , <i>ladS</i> , <i>lasA</i> , <i>lasB</i> ,	<i>lecB</i>	<i>exoS</i> , <i>exoY</i> , <i>toxA</i>	<i>phzM</i>	277
Clone V	2013	Positive	<i>aph(3')-IIB</i> , <i>aadA7</i> , <i>rmtD</i> , <i>aacA4</i> , SPM-1 , <i>aac(6')Ib-cr</i> , <i>fosA</i> , <i>catB7</i> , <i>sul1</i> , <i>bcr-1</i>	<i>bfmR</i> , <i>bfmS</i> , <i>qscR</i>	<i>gacA</i> , <i>gacS</i> , <i>ladS</i> , <i>lasA</i> , <i>lasB</i> ,	<i>lecB</i>	<i>exoS</i> , <i>exoY</i> , <i>toxA</i>	<i>phzM</i>	277
Clone VI	2014	Positive	<i>aph(3')-IIB</i> , <i>aadA7</i> , <i>aacA4</i> , SPM-1 , <i>aac(6')Ib-cr</i> , <i>fosA</i> , <i>bcr-1</i> , <i>catB7</i> , <i>cmx</i> , <i>sul1</i>	<i>bfmR</i> , <i>bfmS</i> , <i>qscR</i>	<i>gacA</i> , <i>gacS</i> , <i>ladS</i> , <i>lasA</i> , <i>lasB</i>	<i>lecB</i>	<i>exoS</i> , <i>exoT</i> , <i>exoY</i> , <i>toxA</i>	<i>phzM</i>	277

Aminoglycoside resistance genes: RmtD 16S RNA Methylase, chromosomal aminoglycoside phosphotransferase gene, *aph(3')-IIB*; gene *aac(6')*- Streptomycin 3"-adenylyltransferase. Gene. *aadA7*.
 Fluoroquinolone resistance gene: *aac(6')Ib-cr*.
 Phenicol resistance gene: chloramphenicol export protein gene, chloramphenicol acetyltransferase (*catB7*) and CMX.
 Metallo-beta-lactamase: SPM
 Sulphonamide resistance gene: Dihydropteroate synthase: *sul1* gene.
bfmR, *bfmS*, two-component sensor BfmS
qscR: quorum-sensing regulator; *qteE*: *qteE* quorum threshold expression protein QteE,
gacA: response regulator GacA; *gacS*: sensor protein GacS; *ladS*: lost adherence sensor LadS
lasA: protease LasA; *lasB*: Elastase LasB; *lecB*: lectin-inhibiting carbohydrates (alpha-methyl-fucoside)
exoS: exoenzymes S; *exoY*: exoenzyme Y; *toxA*: exotoxine A ST: sequencing type

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