Bloodstream Infections caused by *Klebsiella pneumoniae* and *Serratia marcescens* isolates co-harboring NDM-1 and KPC-2.

Taniela Bes (✉ taniela.bes@hms.harvard.edu)

University of Sao Paulo

Debora Nagano

Universidade de Sao Paulo

Roberta Martins

Universidade de Sao Paulo

Ana Paula Marchi

Universidade de Sao Paulo

Lauro Perdigão Neto

Universidade de Sao Paulo

Hermes Higashino

Universidade de Sao Paulo

Gladys Prado

Universidade de Sao Paulo

Thais Guimaraes

Universidade de Sao Paulo

Anna S Levin

Universidade de Sao Paulo

Silvia Costa

Universidade de Sao Paulo

Short report

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Abstract

Carbapenem-resistant Enterobacteriaceae is a worldwide health problem, however isolates carrying both blaKPC-2 and blaNDM-1 are unusual. Here we describe microbiological and clinical characteristics of five cases of bloodstream infection (BSI) caused by carbapenem-resistant *Klebsiella pneumoniae* and *Serratia marcescens* co-harboring blaKPC-2 and blaNDM-1. Of the five blood culture isolates, three from are from hematopoietic stem cell transplantation patients, one from a renal transplant patient, and one from a soft tissue surgical patient. All patients lived in low-income neighborhoods and had no travel history. Despite antibiotic treatment, four of five patients died. The phenotypic assays showed that Meropenem added with either EDTA, PA or both showed increased zone of inhibition in comparison to Meropenem alone. Molecular tests confirmed blaKPC-2 and blaNDM-1 genes, the *K. pneumoniae* were assigned as ST258 and ST340 by Whole Genome Sequencing.

Introduction

The increased of Carbapenem resistance in Enterobacteria represents a clinical challenge and a public health problem(1,2). Even though strains carrying multiple carbapenemases have emerged in last years(3), reports of isolates carrying *bla*_{KPC-2} and *bla*_{NDM-1} are unusual. Few cases have been described(4–9), including reports of a burn patient colonized by *Klebsiella pneumoniae* co-harboring KPC and NDM assigned as ST86(4), a urinary tract infection in a cranioencephalic traumatism patient(5) and three cases of no specify site of infection, in China, caused by *K. pneumoniae* assigned as ST1, ST147, ST1383(6).

Coproduction of carbapenemases are equally infrequent in *Serratia marcescens*. Silva et al, 2014(7) described six strains of *S. marcescens* isolated from urine or tracheal aspirate samples producing simultaneously KPC-2 and IMP-10 simultaneously in Brazil.

Routine susceptibility testing cannot precisely predict the presence of Metallo- β -lactamase (MBLs), and the co-existence of other carbapenemases further aggravates the issue. The aim of this study was to describe microbiological and clinical characteristics of five cases of BSI caused by *K. pneumoniae* (N 4) and *Serratia marcescens* (N 1) co-harboring KPC-2 and NDM-1.

Results

The five isolates used in this study were from blood cultures: three from Hematopoietic Stem Cell Transplantat (HSCT) patients, one from a renal transplant patient, and one from a soft tissue surgical patient. All patients lived in low-income neighborhoods in Sao Paulo, with less than eight years of schooling and no history of international travel. Mortality in 14 days of diagnosis of the BSI occurred in three of the five patients. Most of patients were treated with meropenem, tigecycline and colistin, except the *S. marcescens* case which received tigecycline and fosfomycin. Clinical data is shown in the table 1.

Microdilution susceptibility test by Sensititre demonstrated resistance to β -lactams including Aztreonam, by all isolates (Table 1). Phenotypic tests revealed probable presence of serine carbapenemase and metallo-beta-lactamase as well. Meropenem (MPM) added with either Ethylenediaminetetraacetic acid (EDTA), phenylboronic acid (PBA) or both showed increased zone of inhibition in comparison to Meropenem alone. Three of the five isolates had full growth around the MPM disk alone and the zone of inhibition was higher than 9mm, with double zone of inhibition, when submitted to MPM disks added with EDTA and/or PBA (Table 1). The remaining two isolates, the *S. marcescens* and a *K. pneumoniae* (ST340) did not show a difference higher than 4mm in the zone of inhibition in any of the combinations.

The PCR analysis detected *bla*_{KPC-2} and *bla*_{NDM-1} genes in all five strains, which were confirmed by Sanger Sequencing. The *K. pneumoniae* isolates were assigned as ST340 and ST258 (Figure 1). Virulence genes related with adhesion, efflux pump, iron acquisition, regulation and secretion system were present in the four *K. pneumoniae* samples (Table 1).

Discussion

We report, for the first time in Brazil, the identification of *K. pneumoniae* and *S. marcescens* isolates co-harboring *bla*_{KPC-2} and *bla*_{NDM-1}, including two *K. pneumoniae* STs (ST258 and ST340) that have not been previously described, coproducing KPC-2 and NDM-1. The ST258 emerged during the early 2000s as a hybrid clone created by recombination between ST11 and ST442 and the ST340 which is a single-locus variant of ST11 as well(10). These isolates tend to be pan-resistant, restricting the therapeutic options, particularly to high resistant *S. marcescens* isolates, since they are intrinsic resistance to polymyxins(11).

The dissemination of NDM-1 and KPC-2 in Brazil is of great concern since a *Providencia rettgeri* isolate carrying NDM-1 was described in the South region in 2013(12) and subsequently in 2014, Rozales *et al* published a study analyzing 1134 isolates of *Enterobacteriaceae* among which 11 of them harbored *bla*_{NDM-1} (0.97%)(13). The most alarming data was that none of these cases had a history of traveling outside Brazil, which might represent local acquisition. Our cases present common characteristics, such as low socioeconomic class, low educational background, severe clinical conditions and poor functional status, already described as risk factors for infection by co-resistant agents(6,13,14). Outside the hospital setting, another concern is the detection of KPC-2-producing *K. pneumoniae* isolates in Brazilian rivers in urban areas(15).

Our isolates display a high-level of resistance to β -lactams, aminoglycosides, and fluoroquinolones, but initially remained susceptible to tigecycline. In addition, three of them were susceptible to colistin, except the *S. marcescens* which was intrinsically resistant and the *K. pneumoniae* belonging to ST340. A possible reason for this finding is that carbapenem-resistant enterobacteria isolates carrying *bla*_{NDM-1} are more likely to be resistant to several antibiotics. They are often accompanied by other genes, which are resistant to β -lactams, fluoroquinolones and aminoglycosides(16).

The isolate which was colistin resistant did not harbor plasmidial mediated resistance genes nor chromosomal mutation on the WGS analyzes. We speculate that the resistance derived from virulence genes encoded at Lipopolysaccharide (LPS) *rfb* locus, *wzm* and *wzt* codify the proteins to form an ATP-binding cassette (ABC) transporter responsible for export O-antigenic polysaccharide (O-PS), a glycan necessary to synthesize cell surface LPS. The ABC transporters are formed to a specific O-PS. The Wzt protein dictates the specificity of the substrate and the glycan chain length, which serves as an export signal recognized by the ABC transporter. The *K. pneumoniae* isolate 1581 carries just the *wzm* gene therefore our hypothesis is that lacking *wzt* trapped the transporter in a state incapable to complete the O-PS export. It might explain the colistin resistance, specifically because in *K. pneumoniae* the cytosolic glycan synthesis and export are obligatorily coupled(17,18).

This study shows that BSI caused by carbapenem-resistant enterobacteria coproducing KPC-2 and NDM-1 results in high mortality. Based on the evidence presented here, significant effort is required to identify these isolates with both resistance genes in the routine and research laboratory. To complicate matters further, there may be a correlation between high-resistance bacteria found in urban rivers in Sao Paulo and those found in hospitals. The detection of samples co-harboring KPC-2 and NDM-1 remains unsatisfactory with phenotypic tests. As a result, routine microbiology laboratories must be on alert for samples co-harboring these mechanisms. Early detection, therefore, becomes extremely important for the implementation of infection control measures and epidemiological surveillance.

Methods

Four carbapenem-resistant KP and one carbapenem-resistant *S. marcescens* were obtained from blood cultures from patients hospitalized at Hospital das Clínicas, Faculdade de Medicina da Universidade de São Paulo a tertiary teaching hospital in Sao Paulo, Brazil comprised of 2,200 beds.

The five isolates were from the Microbiology Laboratory samples bank and were involved in previous studies, when informed consent term was obtained. This is a retrospective study and all methods were performed in accordance with the guidelines and regulations, approved by the Ethics Committee of Hospital das Clínicas of University of São Paulo, Brazil. Data was consolidated and analyzed, and patients' identity was confidential. The approval numbers are 1.310.231, 2.158.859 and 2.452.282.

Vitek II (BioMerieux-France) was used to identify the isolates species until February 2015, since then we have been using the Matrix-assisted laser desorption ionization-time of flight (MALD-TOF/Bruker) as well.

The minimum inhibitory concentration (MIC) was tested by Sensititre (ThermoFisher Diagnostics-) to Aztreonam, Meropenem, Imipenem, Colistin, Amikacin and Tigecycline. The susceptibility test results were interpreted according to the criteria recommended by CLSI. *Escherichia coli* isolate ATCC 25922 was used as the quality control of the test.

Additional phenotypic analysis was done to characterize the resistance using disk diffusion (DD) method, cutoff according to CLSI: Meropenem commercial disks containing 10µg were added with 0.05 M of Ethylenediaminetetraacetic acid (Sigma-Aldrich, St. 115 Louis, MO) and/or 20 µg ml⁻¹ of phenylboronic acid (Sigma-Aldrich, St. Louis, MO). According to Migliavacca *et al* (2002)(19), a difference of ≥4mm in the zone of inhibition diameter was used as criteria to determine whether the isolate produced serine carbapenemase, metallo-beta-lactamase or both in presence of EDTA, PBA or either, respectively, when compared to the MPM disk alone. *E. coli* ATCC 25922 was used as negative quality control.

To detect carbapenemases genes, we performed PCR using previously described primers for *bla*_{KPC-2} and *bla*_{NDM-1} genes(20). The amplicons were submitted to Sanger Sequencing using MegaBACE 1000 (ABI 3730 DNA Analyser, Applied Biosystems, Alameda, CA) to confirm the gene identity.

Whole Genome Sequencing (WGS) was done by Illumina MiSeq. For the WGS, total DNA was extracted with Illustra bacteria genomicPrep Mini Spin Kit (GE Healthcare Life Sciences). DNA quality was verified using the NanoDrop spectrophotometer (Thermo Scientific, Delaware, USA). The whole genome was sequenced by MiSeq IlluminaTM. Libraries were prepared with the commercial kit Nextera XT IlluminaTM according to manufacturer's instructions. The quality of the files generated in the sequencing was evaluated by FastQC v.0.11.3 and Trimmomatic v.0.33. The genome assembly was performed using Velvet Optimiser v.2.2.5. The genome was annotated with Prokka v.1.11. The ST of the isolates was checked by MLSTfinder tool (Larsen et al, 2012) and confirmed in the database PubMLST (<http://pubmlst.org>). The gene *bla*_{KPC-2} was manually investigated using Artemis v.16.0.0.

Declarations

Ethics approval and consent to participate

The five isolates were from the Microbiology Laboratory samples bank and were involved in previous studies, when informed consent term was obtained. This is a retrospective study and all methods were performed in accordance with the guidelines and regulations, approved by the Ethics Committee of Hospital das Clínicas of University of São Paulo, Brazil.

Consent for publication

As the previous item.

Availability of data and materials

The data used to support the findings of this study are all included within the article and references.

Competing interests

The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest in the subject matter or materials discussed in this manuscript.

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Authors' contributions

Taniela Bes wrote the main manuscript and the genotypic and phenotypic analyses;

Debora Nagano worked phenotypic and genotypic analyzes, as well as with the whole genome sequencing analyzes;

Roberta Martins worked with the whole genome sequencing analyzes;

Ana Paula Marchi worked phenotypic and genotypic analyzes;

Hermes Higashino, Gladys Prado and Thais Guimaraes were the infectious diseases attendants who manage the patient clinically;

Lauro Perdigão Neto, Anna Levin and Sílvia Costa were the main reviewers;

All authors reviewed the manuscript

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Table

Table 1: Clinical characteristics, outcome, phenotypic analyze, virulence and resistance-associated genes identified by whole genome sequence.

M: Meropenem ME: Meropenem + EDTA MA: Meropenem + Phenylboronic Acid (PBA) MEA: Meropenem + EDTA + PBA

Isolate MLST	Underlying diseases/Approach	Treatment and outcome	Phenotypic Tests - serina-β- lactamases (DD mm)	Phenotypic Tests - MBLs (DD mm)	Carbapenembac	beta- Lactamics resist genes	Aztreonam (MIC)	Me (MI
<i>K. pneumoniae</i> (1411) 10/19/2012 ST340	Myelodysplastic syndrome Leukocytes: 100 Neutrophils: 0 Allogeneic BMT (10/11/2012)	Meropenem + Colistin Death 2 days after blood culture- positive	IMP: 0 ERT: 0 MPM: 0 MPM+PBA: 14mm MPM+EDTA+PBA: 17mm	IMP: 0 ERT: 0 MPM: 0 MPM+EDTA: 0 (phantom zone 14) MPM+EDTA+PBA: 17mm	Positive	blaCTX-M- 15 blaKPC-2 blaSHV- 11 blaTEM- 1B	≥ 32 [R]	≥ 1
<i>K. pneumoniae</i> (1581) 12/14/2012 ST340	Severe aplastic anemia Leukocytes: 100 Neutrophils: 0 Not related allogeneic TCTH (12/09/2012)	Meropenem + Colistin Death 2 days after blood culture- positive	IMP: 23mm ERT: 16mm MPM: 20mm MPM+PBA: 23mm MPM+EDTA+PBA: 23mm	IMP: 23mm ERT: 16mm MPM: 20mm MPM+EDTA: 21mm MPM+EDTA+PBA: 23mm	Positive	blaKPC-2 blaSHV- 11	≥ 32 [R]	4 [F]
<i>K. pneumoniae</i> (4301) 10/16/2014 ST340	Non-Hodgkin lymphoma Peripheral T-cell lymphoma Leukocytes: 3,33 Neutrophils: 2,43 Autologous BMT 9/17/2014	Meropenem + Colistin Discharge from hospital 23 days after blood culture- positive, for a total of 64 days hospitalization	IMP: 0 ERT: 0 MPM: 0 MPM+PBA: 13mm MPM+EDTA+PBA: 18mm	IMP: 0 ERT: 0 MPM: 0 MPM+EDTA: 10 (phantom zone 14) MPM+EDTA+PBA: 18mm	Positive	blaKPC-2 blaSHV-5 blaSRT-2	≥ 32 [R]	≥ 1
<i>S. marcescens</i> (1756) 6/14/2013	Necrotizing fasciitis Amputation of the right arm (05/31/13) Complication: Ischemic cecum perforation (6/07/2013)	Ciprofloxacin + Clindamycin Tigecycline + Fosfomycin Death 11 days after blood culture- positive	IMP: 13mm ERT: 17mm MPM: 23mm MPM+PBA: 25mm MPM+EDTA+PBA: 26mm	IMP: 13mm ERT: 17mm MPM: 23mm MPM+EDTA: 19mm MPM+EDTA+PBA: 26mm	Positive	blaKPC-2 blaSHV-5 blaSRT-2	≥ 32 [R]	4 [F]
<i>K. pneumoniae</i> (4990) 10/21/16 ST258	Kidney transplant due Chronic Kidney Disease (10/13/16) Urinary tract infection (10/20/16) Graft Nephrectomy (11/23/16)	Ceftazidime + Avibactam Death 40 days after blood culture- positive	IMP: 6mm ERT: 0 MPM: 0 (phantom zone 7) MPM+PBA: 14mm MPM+EDTA+PBA: 16mm (phantom zone 20)	IMP: 6mm ERT: 0 MPM: 0 (phantom zone 7) MPM+EDTA: 9mm (phantom zone 20) MPM+EDTA+PBA: 16mm (phantom zone 20)	Positive	blaCTX-M- 14 blaKPC-2 blaSHV- 11 blaTEM- 1B	≥ 32 [R]	≥ 6

Figures

Figure 1

Phylogenies of *K. pneumoniae* showing the same clone ST340 in the BMT unit and the globally disseminated, ST258 in Renal Transplant Unit.