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

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Short report

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Abstract

Carbapenem-resistant Enterobacteriaceae are a worldwide health problem and isolates carrying both blaKPC-2 and blaNDM-1 are unusual. Here we describe the microbiological and clinical characteristics of five cases of bloodstream infections (BSI) caused by carbapenem-resistant Klebsiella pneumoniae and Serratia marcescens having both blaKPC-2 and blaNDM-1. Of the five blood samples, three 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 neighbourhoods and had no travel history. Despite antibiotic treatment, four of five patients died. The phenotypic susceptibility assays showed that meropenem added either EDTA, phenylboronic acid (PBA) or both, increased the zone of inhibition in comparison to meropenem alone. Molecular tests showed the presence of blaKPC-2 and blaNDM-1 genes. K. pneumoniae isolates were assigned to ST258 or ST340 by whole genome sequencing. This case-series showed a high mortality among patients with BSI caused by Enterobacteriaceae harbouring both carbapenemases. The detection of carbapenemase-producing isolates carrying both blaKPC-2 and blaNDM-1 remains a challenge when using only phenotypic assays. Microbiology laboratories must be alert for K. pneumoniae isolates producing both KPC-2 and NDM-1.

Introduction

In the last decade, several studies have been reported the emergence of Gram-negative bacteria carrying multiple carbapenemases, isolated from patients with distinct pathologies [1,2,3,4,5,6]. However, clinical isolates harbouring simultaneously KPC-2 and NDM-1 carbapenemases are less common [7,8,9,10,11,12]. Previous reports describing the production of both carbapenemases includes clinical isolates of Klebsiella pneumonia [7,8,9,10], Enterobacter cloacae [11,12] and Enterobacter hormaechei [13], collected from rectal swabs, blood samples, urinary tract infections and wound infections as well. Regarding Serratia marcescens clinical isolates harbouring simultaneously KPC-2 and IMP-10 [14] or KPC-2 and SRT-2 [15] were described.

The production of carbapenemases, such as Klebsiella pneumoniae carbapenemase (KPC) and the New Delhi metallo-β-lactamase (NDM) constitutes one of the most important mechanisms of resistance to β-lactam antibiotics [16]. As the emergence of carbapenem-resistant clinical isolates has turned a serious clinical challenge due to the limited treatment options, the presence of multiple carbapenemases by the same strain further aggravates this issue.

Concerning the Brazilian data, NDM producers were originally detected in the southern regions of Brazil and have since moved into other states [17]. Bacterial pathogens producing KPC alone are the main cause of bloodstream infections (BSI) in intensive care unit patients (21.2%) in the state of São Paulo, according to the surveillance data [18]. Reports from Brazil also indicate that NDM and KPC producers carry resistance genes other than KPC-2 and NDM-1 conferring resistance to other antimicrobials classes rather than β-lactam [12,13,19]. The present study describes the clinical and microbiological features of five, namely Klebsiella pneumoniae (n=4) and Serratia marcescens (n=1), co-harboring KPC-2 and NDM-1, collected from bloodstream infections (BSI).

Materials And Methods

Four carbapenem-resistant K. pneumoniae and one carbapenem-resistant S. marcescens were obtained from blood cultures during the years 2012 and 2016. The patients were hospitalized at Hospital das Clínicas, Faculdade de Medicina da Universidade de São Paulo, a tertiary teaching hospital in São Paulo, Brazil. These isolates belong to the Microbiology Laboratory Biobank.

Vitek II (bioMerieux, Marcy-l’Étoile, France) was used to identify the isolates KP1411, KP4301, KP158 and SM1581 species. The isolate KP4990 was identified using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS; Bruker, Billerica, Massachusetts, USA).

To confirm the susceptibility pattern, the minimum inhibitory concentrations (MICs) were determined using Sensititre Gram Negative GNX3F AST Plate (TREK Diagnostic Systems, Cleveland, OH, USA). The antimicrobials tested were aztreonam, meropenem, imipenem, ceftazidime, colistin, amikacin, tigecycline and ciprofloxacin [20]. The susceptibility test results were interpreted according to the criteria recommended by Clinical Laboratory Standards Institute (CLSI) M100 [21]. Isolates were considered susceptible if the MIC of carbapenem were ≤1mg/mL, intermediate if MIC=2mg/mL and resistant with MICs ≥4mg/mL [22]. Escherichia coli ATCC25922 was used as quality control strain and Klebsiella pneumoniae ATCC700603 as carbapenem-resistant Enterobacteriaceae control strain.

To precisely detect the genes coding for the carbapenemases KPC and NDM, we performed PCR using previously described primers for blaKPC-2 and blaNDM-1 [22,23,24]. The amplicons were submitted to Sanger Sequencing using MegaBACE 1000 (ABI 3730 DNA Analyser; Applied Biosystems, Alameda, CA) to confirm the gene identity.

To determine the genotypic profile including resistance and virulence genes, whole genome sequencing (WGS) was performed by Illumina MiSeq. For WGS, total DNA was extracted with Illustra bacteria genomicPrep Mini Spin Kit (GE Healthcare Life Sciences, Marlborough, USA). DNA quality was verified using the NanoDrop spectrophotometer (Thermo Scientific, Delaware, USA). The quality of the files generated in the sequencing was evaluated by FastQC v.0.11.3 and Trimmomatic v.0.33. Genome assembly was performed using Velvet Optimiser v.2.2.5 and annotated with Prokka v.1.11 [25,26,27]. The sequence type (ST) of the isolates was determined with MLSTfinder tool [28] and confirmed in the database PubMLST (https://pubmlst.org/kpneumoniae/info/primers.shtml). The gene blaKPC-2 was manually investigated using Artemis v.16.0.0.

Additional phenotypic analysis was done to determine the resistance to carbapenem using the disk diffusion (DD) method described by Migliavacca et al. [29], and the CLSI breakpoints for carbapenems. Meropenem (MPM) commercial disks containing 10 μg (Sensidisc DME, Araçatuba, BRA) were added with 0.05 M of ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, St. Louis, USA) and/or 20 μg/mL of phenylboronic acid (PBA) (Sigma-Aldrich, St. Louis, USA). According to Migliavacca et al. [29], a difference of ≥4mm in the zone of inhibition diameter was used as criteria to determine whether the isolate produce a serine carbapenemase, a metallo-β-lactamase or both, in presence of EDTA and/or PBA, respectively, when compared to the MPM disk alone. E. coli ATCC25922 was used as negative quality control.
Results

The five clinical isolates analysed in the present study were collected from BSI of five patients submitted to hematopoietic stem cell transplant (n=3), renal transplant (n=1) and soft tissue surgery (n=1). All patients lived in low-income neighbourhoods in São Paulo, have less than eight years of schooling and have no history of international travel.

The patients in this study were individuals with severe conditions and prolonged hospitalization, varying between 16 up to 51 days at the diagnostic of BSI by multi-drug resistant (MDR) agents. Three of the four patients infected by K. pneumoniae died within the course of the treatment, two of them deceased two days after the diagnosis, and one died 40 days after BSI culture turned positive. The patient infected by S. marcescens died 11 days after the diagnosis. Patients infected by K. pneumoniae (n=4) had been treated with meropenem or ceftazime for previous infections. Afterwards the blood culture results Tigecycline and colistina were added to the treatment; the patient infected with S. marcescens was receiving ciprofloxacin and clindamycin, increased to tigecycline and fosfomycin after the blood culture result. Detailed clinical data is shown in the Table 1.

Microdilution susceptibility testing demonstrated that all isolates were resistant to at least two carbapenems. All the isolates were susceptible to ceftazidime plus avibactam and tigecycline. However, ceftazidime plus avibactam was not available in Brazil for clinical use during the study. The isolates KP1411, KP4301 and KP4990 were susceptible to colistin, been the KP1411 also susceptible to amikacin (Table 1). Phenotypic tests with specific β-lactamase inhibitors revealed the presence of serine carbapenemases and metallo-β-lactamases as well. Meropenem added with either EDTA and/or PBA showed an increased zone of inhibition in comparison to meropenem disk alone.

The isolates KP1411, KP4301 and KP4990 had full growth around the meropenem disk and, when either EDTA and/or PBA was added to meropenem disk, an inhibition zone higher than 9mm was obtained. However, the isolates KP1411 and KP4301 presented a double ring reaction surrounding the disk with MPM and EDTA, two clear different zones of growth of the same bacteria. The experiment was repeated three times and the same pattern was sustained. The isolate KP4301 presented a phantom zone around the disk with MPM, EDTA and PBA. The remaining two isolates, S. marcescens SM1581 and KP1581 did not show differences higher than 4mm in the zone of inhibition in any if the combinations (Table1).

The WGS analysis led to the identification of resistance and virulence genes, such as fosA in the isolate KP4301 which never had been exposure to fosfomycin. However, the WGS data did not show blaNDM-1 in none of the samples. Besides the resistance genes, the WGS analysis showed virulence genes related with adhesion, efflux pumps, iron acquisition, regulation and secretion systems were present in the four K. pneumoniae isolates. Each of the genes were analyzed separately (Table 1). The WGS data were used for further MLST analysis of the isolates representing the two common STs for K. pneumoniae, ST258 and ST340. Isolates KP1411, KP4301 and KP1581 were assigned to ST340 and isolate KP4990 to ST258.

Due to the phenotypic profile of the samples, PCR analysis was performed and detected blaKPC-2 and blaNDM-1 genes in all five strains. The PCR result was confirmed by Sanger sequencing.

Discussion

To the best of our knowledge, this is the first report describing K. pneumonia and S. marcescens clinical isolates harbouring simultaneously blaKPC-2 and blaNDM-1. The presence of these resistance genes was not previously described in K. pneumoniae ST258 and ST340 worldwide.

The ST258 emerged during the early 2000s as a hybrid clone created by recombination between ST11, ST442 and ST340. Isolates KP1411, KP4301 and KP1581 were assigned to ST340 and isolate KP4990 to ST258. The presence of these resistance genes was not previously described in K. pneumoniae ST258 and ST340 worldwide.

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 [19]. Subsequently, Rozales et al, 2014 [17] published a study analyzing 1134 isolates of Enterobacteriaceae, among which 11 isolates (0.97%) harbored blaNDM-1. Noteworthy, none of these cases had a history of travel outside Brazil, which suggest local acquisition. The isolates analysed in the present study were collected from patients with risk factors contributing to infection by multidrug resistant pathogens, e.g. low socioeconomic class, low educational background, severe clinical conditions and poor functional status [8,31,32].

Outside the hospital setting, another aggravating factor is the lack of basic sanitation in the country. Ecological surveillance studies found KPC-2 producing K. pneumoniae isolates (ST340) in urban rivers in the city of Sao Paulo, Brazil [33]. Such finding may confirm the improper treatment of hospital sewage being discarded on the rivers and rising a hypothesis regarding the correlation among environmental and hospital isolates.

Our isolates displayed high-level of resistance to β-lactams, aminoglycosides, and fluoroquinolones. Carbapenem-resistant enterobacteria isolates carrying blaNDM-1 are more likely to be resistant to several antibiotics. They are often accompanied by other resistance genes, associated with resistance to β-lactams, fluoroquinolones, and aminoglycosides. As described by Kumarasamy et al, 2010, who analysed 111 isolates of K. pneumoniae producing NDM-1 from India and United Kingdom. Their isolates displayed resistant to polymyxins and aminoglycosides [7].

Although the isolate KP1581 is colistin-resistant, the WGS did not show plasmid mediated resistance genes or previously described chromosomal mutations that lead to colistin resistance. Therefore, we speculate that the resistance derived from virulence genes encoded at lipopolysaccharide (LPS) rfb locus. The genes wzm and wzt codify a transmembrane ATP-binding cassette transporter (ABC). It plays an important role in the synthesis of cell surface LPS [34]. 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 isolate KP1581 carries just the wzm gene. Thus, our hypothesis is that it has wzt trapped by 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 [35].
The main limitations of this study are the small number of samples and the fact they belong to different genera. Even though, the presence of \( \text{bla}_{\text{KPC-2}} \) and \( \text{bla}_{\text{NDM-1}} \) might have contributed to an unfavourable clinical outcome, considering that four of the patients died from the same conditions without blood culture clearance.

In conclusion, the detection of carbapenemase-producing isolates carrying both \( \text{bla}_{\text{KPC-2}} \) and \( \text{bla}_{\text{NDM-1}} \) remains a challenge. Additional effort is required to identify isolates when using only phenotypic assays. Thus, routine microbiology laboratories must be on alert for isolates possessing both \( \beta \)-lactamases.

**Declarations**

**Ethics approval and consent to participate**

The isolates belong to the Microbiology Laboratory biobank 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 (approval reference numbers 1.310.231, 2.158.859 and 2.452.282).

**Consent for publication**

As the previous item.

**Availability of data and materials**

These isolates WGS are placed at GeneBank under the numbers:

- KP1411 - *Klebsiella pneumoniae* - QOIJ00000000
- KP1581 - *Klebsiella pneumoniae* - QOII00000000
- KP4301 - *Klebsiella pneumoniae* - JABBZC00000000
- SM1756 - *Serratia marcescens* - QJPQ00000000
- KP4990 - *Klebsiella pneumoniae* - QOTZ00000000

These isolates Sanger sequence are placed at GeneBank under the numbers: (Gene \( \text{blaKPC} \) e \( \text{blaNDM} \))

- \( \text{blaNDM}_{1411} \) - MT721962
- \( \text{blaNDM}_{4990} \) - MT721963
- \( \text{blaNDM}_{1756} \) - MT721964
- \( \text{blaNDM}_{4301} \) - MT721965
- \( \text{blaNDM}_{1581} \) - MT721966
- \( \text{blaKPC}_{4990} \) - MT721967
- \( \text{blaKPC}_{4301} \) - MT721968
- \( \text{blaKPC}_{1581} \) - MT721969
- \( \text{blaKPC}_{1411} \) - MT721970
- \( \text{blaKPC}_{1756} \) - MT665969

**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**
TB wrote the main manuscript and the genotypic and phenotypic analyses. DN worked phenotypic and genotypic analyzes, as well as with the whole genome sequencing analyzes. AM worked phenotypic and genotypic analyzes. HH, GP and TG were the infectious diseases attendants who manage the patient clinically. LP, AL and SC 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.
| Isolate | Underlying diseases/Infection | Time (d)  | Phenotype Test 1 | Phenotype Test 2 | Confirmation | Resistance (MIC) | Phage Typing | MLST | Strain Identification |
|---------|-------------------------------|----------|-----------------|-----------------|--------------|-----------------|--------------|------|----------------------|
| M. pneumoniae (MIC) | 10/10/2014 | MPM+PBA: 12mm | MPM+EDTA: 10mm | Positive | ≥ 32 [R] | ≥ 16 [R] | ≥ 16 [R] | ≥ 2 [S] | ≥ 16 [R] |
| M. pneumoniae (MIC) | 10/10/2014 | MPM+PBA: 12mm | MPM+EDTA: 10mm | Positive | ≥ 32 [R] | ≥ 16 [R] | ≥ 16 [R] | ≥ 2 [S] | ≥ 16 [R] |
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| M. pneumoniae (MIC) | 10/10/2014 | MPM+PBA: 12mm | MPM+EDTA: 10mm | Positive | ≥ 32 [R] | ≥ 16 [R] | ≥ 16 [R] | ≥ 2 [S] | ≥ 16 [R] |
| M. pneumoniae (MIC) | 10/10/2014 | MPM+PBA: 12mm | MPM+EDTA: 10mm | Positive | ≥ 32 [R] | ≥ 16 [R] | ≥ 16 [R] | ≥ 2 [S] | ≥ 16 [R] |
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| M. pneumoniae (MIC) | 10/10/2014 | MPM+PBA: 12mm | MPM+EDTA: 10mm | Positive | ≥ 32 [R] | ≥ 16 [R] | ≥ 16 [R] | ≥ 2 [S] | ≥ 16 [R] |
| M. pneumoniae (MIC) | 10/10/2014 | MPM+PBA: 12mm | MPM+EDTA: 10mm | Positive | ≥ 32 [R] | ≥ 16 [R] | ≥ 16 [R] | ≥ 2 [S] | ≥ 16 [R] |
| M. pneumoniae (MIC) | 10/10/2014 | MPM+PBA: 12mm | MPM+EDTA: 10mm | Positive | ≥ 32 [R] | ≥ 16 [R] | ≥ 16 [R] | ≥ 2 [S] | ≥ 16 [R] |
| M. pneumoniae (MIC) | 10/10/2014 | MPM+PBA: 12mm | MPM+EDTA: 10mm | Positive | ≥ 32 [R] | ≥ 16 [R] | ≥ 16 [R] | ≥ 2 [S] | ≥ 16 [R] |
| M. pneumoniae (MIC) | 10/10/2014 | MPM+PBA: 12mm | MPM+EDTA: 10mm | Positive | ≥ 32 [R] | ≥ 16 [R] | ≥ 16 [R] | ≥ 2 [S] | ≥ 16 [R] |
| M. pneumoniae (MIC) | 10/10/2014 | MPM+PBA: 12mm | MPM+EDTA: 10mm | Positive | ≥ 32 [R] | ≥ 16 [R] | ≥ 16 [R] | ≥ 2 [S] | ≥ 16 [R] |
| M. pneumoniae (MIC) | 10/10/2014 | MPM+PBA: 12mm | MPM+EDTA: 10mm | Positive | ≥ 32 [R] | ≥ 16 [R] | ≥ 16 [R] | ≥ 2 [S] | ≥ 16 [R] |

MM: Meropenem; IMP: Imipenem; ERT: Ertapenem; PBA: Phenylboronic Acid; DD: Diffusion Disk.

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.