

Multidrug-resistant *Klebsiella pneumoniae*: genetic diversity, mechanisms of resistance to polymyxins and clinical outcomes in a tertiary teaching hospital in Brazil

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ABSTRACT

Increased resistance to polymyxin in *Klebsiella pneumoniae* (ColRKP) has been observed. Molecular epidemiology, as well as the clinical impact of these difficult to treat pathogens need to be better characterized. We present the clinical outcomes of 28 patients infected by ColRKP in a tertiary hospital. Isolates with MIC >2 by Vitek 2 were confirmed by the microdilution broth test. Polymerase chain reaction (PCR) was performed for *bla*_{KPC}, *bla*_{NDM}, *bla*_{OXA-48} and *bla*_{mcr-1} genes in the isolates, and Whole Genome Sequencing (WGS) was performed in six isolates. Seventeen (61%) patients were female and the mean age was 50 years old. In-hospital and 30-day mortality were 64% (18/28) and 53% (15/28), respectively. Central line-associated bloodstream infection in addition to bacteremia episodes due to other sources were the most frequent (61%). Mean APACHE and Charlson comorbidity index were 16 and 5, respectively. Twenty patients (71%) received at least one active drug and ten (35%) received two drugs: tigecycline 46% (13/28); amikacin 21% (6/28) and fosfomicin 3% (1 case). Twenty-six out of 28 tested cases were positive for *bla*_{KPC}. Eight different clusters were identified. Four STs were detected (ST11, ST23, ST340, and ST437). Mutations on *pmrA*, *arnB*, *udg*, and *yciM* genes were present in all six isolates submitted to WGS; *lpxM* and *mgrB* mutations were also detected in all but one isolate. In conclusion, we observed resistance to polymyxin in severely ill patients mostly from intensive care units and/or immunosuppressed patients with high mortality rates in whom a diversity of ColRKP clusters was identified and might indicate selective pressure.

KEYWORDS: Colistin resistance. Hospital epidemiology. Antibiotic therapy. Genome sequencing. Polymyxins.

INTRODUCTION

As in other parts of the world, carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has established in Brazil as a major public health problem in the last decade¹⁻³. Infections caused by CRKP most frequently occur in severely ill patients and mortality rates are usually as high as 40% to 70%⁴. Polymyxins (colistin and polymyxin B) remain as one of the last options for severe infections caused by carbapenem-resistant (CR) *Enterobacteriaceae* and other CR Gram-negative bacteria. As a consequence, we have seen an increased use of polymyxins in our country. Data from the Health State Department in Sao Paulo, Brazil, revealed an increase in the use of these drugs (data not published) from 2008 to 2015. We have used colistin empirically in intensive care units in our hospital for the treatment of severe cases

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due to our marked high incidence rates of infections caused by CRKP. Concomitantly, we have experienced in the last years the emergence of colistin-resistant *K. pneumoniae* (ColRKP)^{5,6}. Resistance to polymyxins has been reported in many parts of the world and several mechanisms have been identified⁷. Resistance to other antimicrobial classes like quinolones, aminoglycosides and all beta-lactams usually accompany resistance to colistin. The breakpoints for resistance to colistin, as well as the best approach to test it in the clinical laboratory is still a matter of debate and due to the extremely limited options for the treatment of this agent, we face a very challenging task to diagnose and treat patients especially in the intensive care setting. Furthermore, the understanding of resistance mechanisms for ColRKP is currently in progress. Our aim was to describe 28 clinical cases of infection caused by ColRKP *K. pneumoniae* and their clinical outcomes, as well as the molecular characterization of the isolates regarding the resistance to colistin and the polymyxins-resistance genes harbored by the isolates.

METHODS

We describe a case series of 28 patients older than 18 years old identified as presenting a bloodstream and/or sterile site acquired infection caused by ColRKP at the Hospital das Clinicas, a 1,000 bed major teaching hospital affiliated to the University of Sao Paulo, Brazil, from December 2010 to October 2013. All cases were retrieved from the Hospital Infection Control Department database. Whenever there was a multiple culture diagnosis, we have only considered the first one. Concomitant infections, defined as those occurring seven days before or after the ColRKP diagnosis, were excluded. The primary outcome was 30-day mortality elapsed from the day of the ColRKP laboratory diagnosis. Associated demographic data and comorbidities were retrieved from medical charts, as well as information on antimicrobial therapy and in-hospital mortality. Therapy with active drugs was defined as receiving, after diagnosis, an antimicrobial with a minimal inhibitory concentration (MIC) within the susceptibility range, as described by the CLSI⁸. Patient's illness severity was scored by APACHE II and comorbidities by Charlson score.

Microbiology

The identification and the susceptibility test were first performed by routine Vitek 2 (bioMérieux®) automated method and further confirmed by the broth microdilution test. On a routine basis, every single isolate that presented with a colistin MIC > 2 µg/mL were re-tested by the

E-test and confirmed by the disk-diffusion method. We further compared Vitek 2 and the broth microdilution test. Susceptibility breakpoints were those of the Clinical Standards Laboratory Institutes (CLSI)⁸. The Food and Drug Administration (FDA) breakpoint for *Enterobacteriaceae* was used specifically to test tigecycline⁹. The Software Whonet® was used to distribute MIC50 and MIC90 based on broth microdilution test.

Molecular biology

Polymerase chain reaction (PCR) and pulsed field gel electrophoresis (PFGE)

Firstly, the routine laboratory performed real time PCR searching for the genes *bla*_{KPC}, *bla*_{NDM} and *bla*_{OXA-48} in all isolates using the BD Max platform (BD Diagnostics, Sparks, MD)⁹. After that, in the research laboratory, *mcr-1* and *bla*_{NDM} genes were searched by conventional PCR in 26 isolates, using the primers NDMup168 – 5'-GAATGTCTGGCAGCACACTT and NDMdw647 – 5'-TTGGCCTTGCTGTCCTTGAT-3' for *bla*_{NDM}; CLR5-F – 5'-CGGTCAGTCCGTTTGTTC-3' and CLR5-R – 5'-CTTGGTCGGTCTGTAGGG-3' for *mcr-1*¹⁰. In each reaction, strains previously identified in our hospital, harboring the genes (confirmed by Sanger's sequencing), were used as positive controls. Three negative controls were used: an initial and a final control during the PCR master mix preparation and sterilized water instead of the primers and another negative control using only sterilized water. The isolates were submitted to PFGE using the *Xba*I enzyme and restriction fragments were obtained by separation using a CHEF DRIII system (Bio-Rad, Hercules, California, USA). Patterns were interpreted according to the Bionumerics software, version 7.1 (Applied-Maths, Sint-Martens-Latem, Belgium).

Whole Genome Sequencing (WGS)

We performed WGS in six isolates from three different clusters, randomly selected corresponding to A, D and E (Figure 1) and all of them were recovered from blood. Total DNA extraction was performed with Illustra bacteria genomicPrep Mini Spin Kit (GE Healthcare Life Sciences) and DNA quality verified using the NanoDrop spectrophotometer (Thermo Scientific, Delaware, USA).

The DNA concentration was checked using the Qubit® fluorometer (Thermo Scientific, Delaware, USA) and integrity on 1.5% agarose gel. The whole genome of the isolates and the libraries were sequenced by MiSeq Illumina™ methodology and prepared with the

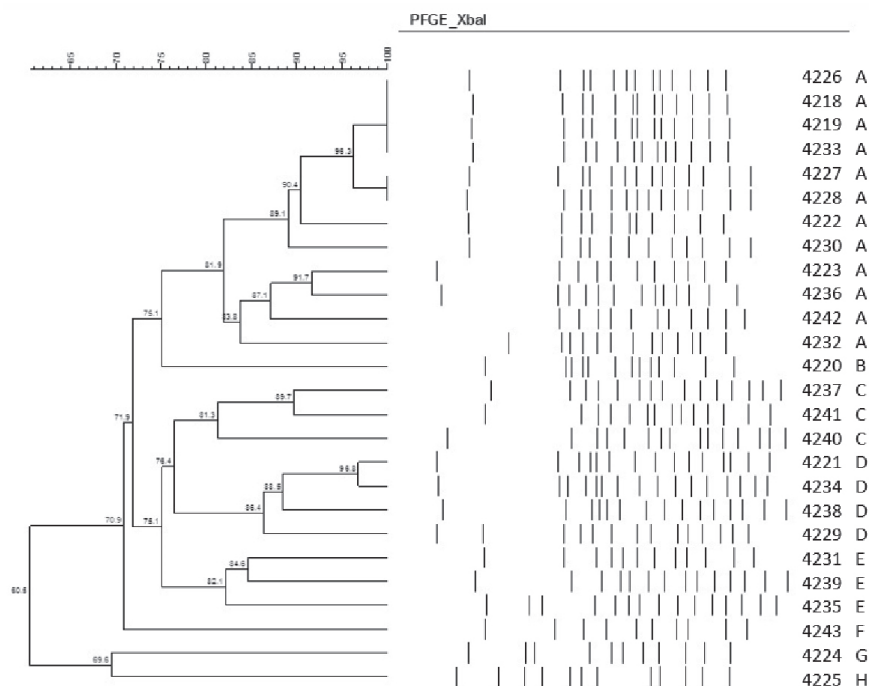


Figure 1 - Dendrogram of 28 strains of colistin-resistant *Klebsiella pneumoniae*. The four numbers refer to the identification of patients and letters A to H refer to the clusters.

commercial kit Nextera XT Illumina™ according to the manufacturer's instructions. The quality control of the library was evaluated in the Tape Station System (Agilent). Paired reading segments (paired end reads) with over 500 base pairs were processed on the Illumina™ MiSeq sequencing platform.

FastQC v programs. 0.11.3 and Trimmomatic v. 0:33 were used to evaluate the quality of the files generated by sequencing. The genome assembly was performed using the Velvet Optimiser v program. 2.2.5, and the contigs were ordered by Abacas v. 1.3.1 using the reference strain *K. pneumoniae* MGH78578 whose genome is available on the website of the National Center for Biotechnology Information¹¹. The genome was annotated with the Prokka v. 1:11. using a custom *Klebsiella* reference library¹². The sequence type (ST) was checked by the MLST finder tool (Multilocus Sequence Typing)¹³. Genes related to resistance were searched with the Artemis 16.0.0 program and by the ResFinder v2.1¹⁴. Single Nucleotide Polymorphisms (SNPs) were identified by mapping the sequencing reads of the isolates using the *K. pneumoniae* MGH78578 as the reference, using BWA, SAM tools and Genome Analysis Toolkit (GATK). All SNPs were manually checked.

Restriction fragments were obtained by separation using a CHEF DRIII system (Bio-Rad, Hercules, California, USA). Patterns were interpreted according to the Bionumerics version 7.1 (Applied-Maths, Sint-Martens-Latem, Belgium).

Statistics

The Chi-square test was used for categorical variables and the Fisher test when adequate. Mann-Whitney was used for continuous variables. Data were analyzed using the EPIINFO 7.0.

RESULTS

Twenty-eight patients were included, and their clinical characteristics are summarized in [Table 1](#). Seventeen (61%) were female and their mean age was 50 years old (range 20-73). Crude in-hospital and 30-day mortality were 64% (18/28) and 53% (15/28), respectively. Sixteen patients (57%) were in intensive care units (surgical, liver transplant recipients, hematopoietic stem cell transplant recipients and infectious diseases units) and twelve (43%) were in different wards, mostly surgical units. The main comorbidities were chronic liver disease (14%), hematopoietic stem cell transplantation (HSCT) (7%), solid tumors (32%), hematological malignancy other than HSCT (11%). Sources of infection were intra-abdominal (n=15, 53%), central line associated bloodstream infection (CLABSI) (n=11, 39%) and two others (3%) that were abdominal wall abscesses. Thirteen patients (43%) were exposed to polymyxins (twelve to colistin and one to polymyxin B) prior to diagnosis of infection caused by ColRKp. All bacteremia episodes (CLABSI + secondary

Table 1 - Clinical data of 28 patients with Colistin-resistant *Klebsiella pneumoniae* infection.

Pt	Sex	Age	Site of infection	Underlying disease at admission	Bacteremia	Outcome	Treatment	
							Active drugs	Non active drugs
1	F	49	IAB	Liver disease	Yes	Death	TG, AMK	
2	M	42	IAB	Liver disease	No	Death		
3	M	48	IAB	Bariatric surgery	No	Survival		COL
4	M	64	CLABSI	ALL HSCT	Yes	Survival	TG, AMK	MER
5	M	73	IAB	Colon cancer	No	Death	AMK	MER, TG
6	F	34	CLABSI	ALL HSCT	Yes	Death	AMK	
7	M	26	IAB	Kidney-pancreas transplantation	No	Death	TG	MER, COL
8	F	63	CLABSI	Diabetic ketoacidosis	Yes	Survival	TG, AMK	MER
9	M	67	other	Coronary heart disease	Yes	Death	TG	MER, COL
10	F	23	CLABSI	Liver disease	Yes	Survival		MER, COL
11	M	36	CLABSI	AML	Yes	Survival	TG	MER, COL
12	M	64	IAB	Cholangio-carcinoma	No	Death	TG, AMK	IMI, COL
13	F	20	IAB	Perforative peritonitis	No	Death		IMI, COL
14	F	68	CLABSI	Chagasic megacolon complications	Yes	Death	TG	MER, COL
15	M	51	CLABSI	Burns	Yes	Death	TG, GEN	COL
16	M	59	IAB	Liver disease	Yes	Death	TG, AMK	COL, CIP
17	F	37	IAB	Kidney-pancreas transplantation	No	Survival	TG	IMI
18	M	60	IAB	Gastric adeno-carcinoma	No	Death		TG, COL
19	F	50	IAB	Cholangio-carcinoma	No	Death		
20	F	51	CLABSI	AML	Yes	Death		MER, COL
21	F	58	CLABSI	Mesenteric ischemia	Yes	Death	TG, FOS	MER
22	M	49	IAB	Liver transplantation complications	Yes	Survival		IMI
23	F	52	CLABSI	T cells non- Hodgkin lymphoma	Yes	Survival		IMI
24	M	60	CLABSI	Liver transplantation complications	Yes	Survival	AMK	
25	M	60	IAB	Abdominal wall hernia repair complications	Yes	Survival		COL
26	M	28	other	Polytrauma	No	Death		
27	M	61	IAB	Liver transplantation complications	No	Death		COL
28	M	59	IAB	Liver transplantation complications	Yes	Death		SMX-TMP

Pt – patient; F – female; M – male; IAB – intra-abdominal infection; CLABSI – central line associated bloodstream infection; ALL – acute lymphoid leukemia; HSCT – hematopoietic stem cell transplantation; AML – acute myeloid leukemia; TG – tigecycline; AMK – amikacin; COL – colistin; MER – meropenem; IMI – imipenem; Fos – Fosfomycin; SMX-TMP – sulfamethoxazole-trimethoprim

to other sources) were 17 (61%). The mean time elapsed from admission to infection diagnosis was 35 days (range 1 – 129), from infection to death 28 days (range 1- 128) with a median of 15 days. The mean APACHE was 16 (5 – 36) and Charlson comorbidity index was 5 (2 – 12). Fifteen patients (53%) received active drugs to treat ColRkp

infections based on the broth microdilution method, seven of them received two active drugs and eight received one active drug. Among these fifteen patients, twelve patients received additional non-active drugs associated to the active regimen. Other ten patients (35%) received only non-active drugs in monotherapy or dual regimen. Three patients (10%)

did not receive any drug. Prescribed regimens are described in Table 1. The mean time elapsed from diagnosis (day when positive blood culture result was released) to the first dose of active drug was 2.8 days ranging from 0 to 7.

The distribution of the minimal inhibitory concentration of antimicrobials tested by the broth microdilution method for 26 isolates is presented in Table 2. Regarding carbapenemase-encoding genes, 26 isolates (93%) had *bla*_{KPC}, and one of them was also positive also to *bla*_{NDM-1}. The genes *bla*_{OXA-48} and *mcr-1* were not identified. The dendrogram of the 26 isolates tested by PFGE revealed the presence of eight different clusters (Figure 1).

WGS was performed for six randomly selected ColRKP isolates. Four STs were prevalent (ST11, ST23, ST340, and ST437), three isolates were from ST11.

The analysis of resistance genes detected the presence of *bla*_{KPC-2} in all 6 isolates, one of them co-harboring the *bla*_{NDM-1} gene. All the isolates sequences had *ompK35* and *ompK36* genes, except for the isolate 4219, which presented a disrupted *ompK35* gene due to a frameshift.

Plasmid mediated colistin-resistance related to the presence of *mcr-1* was not found. All 6 isolates presented chromosome non-synonymous mutations on genes related to colistin-resistance. Mutations on *pmrA*, *arnB*, *udg*, and *yciM* were present in all six isolates; *lpxM*, and *mgrB* mutations were also present in all but one isolate. All non-synonymous mutations found are described in Table 3. Other antimicrobial resistance-related genes were described in Table 4.

DISCUSSION

In December 2010, we identified the first isolate of *K. pneumoniae* presenting elevated inhibitory concentration (ColRKP) to colistin in blood. Since then, we have faced an increasing incidence of this agent causing healthcare-associated infection in our hospital, reaching 30% of all

K. pneumoniae carbapenem-resistant isolates (data not shown) in 2016. The emergence of polymyxin-resistant strains, especially to colistin (polymyxin E) has been identified in different countries¹⁵. In Italy, the emergence of colistin-resistant carbapenemase-producing *K. pneumoniae* has been reported since 2010¹⁶ and in an Italian nationwide cross-sectional survey in 2011, colistin resistance was found in 22.4% of CRKP isolates¹⁷.

We presented here 28 cases of bloodstream and/or sterile site infections that were well characterized in terms of molecular epidemiology and clonal relatedness. Severe comorbidities were associated to all cases. Fifteen patients received one or two active drugs, ten did not receive any active drug and three did not receive any antimicrobial drug for this infection. All of the treated patients received standard doses during treatment, unless an acute renal failure occurred.

Polymyxins are cationic peptides that exert their bactericidal effect by binding a peptide to the bacterial lipid A portion of lipopolysaccharide A (LPS A) that presents a negative charge in the outer membrane. Once it is bound, polymyxin displaces the cations Mg⁺⁺ and Ca⁺⁺, disrupting the membrane integrity, leading to cell lysis¹⁸. A strategy used by Gram-negatives pathogens to overcome these drugs is to alter their LPS. More often, LPS modification takes place substituting the phosphate groups by 4-amino-4-deoxy-L-arabinose (L-Ara4N) which decreases the negative charge of LPS A reducing its binding to polymyxins⁷. Another mechanism that reduces the binding to the drug by altering the negative charge is the addition of phosphoethanolamine (PEtN). Chromosome mutations on the two-component transcriptional regulatory systems PmrAB and PhoPQ, or on the MgrB, leads to upregulation of modified LPS being associated to polymyxin resistance¹⁹⁻²². Less frequently is the expression of an efflux pump and capsule formation⁷. Cassu-Corsi *et al.*²³ have recently reported two well characterized

Table 2 - Distribution of the minimal inhibitory concentration of antimicrobials tested by the broth microdilution method for the 26 isolates of *Klebsiella pneumoniae*.

Antimicrobial	breakpoint	N	%R	%I	%S	MIC50	MIC90	MIC variation
Imipenem	S≤1 R≥4	26	85.7	10.7	3.6	16	16	1 – 16
Meropenem	S≤1 R≥4	26	92.9	0	7.1	16	16	1 - 16
Amikacin	S≤16 R≥64	26	17.9	3.6	78.6	4	64	2 - 64
Gentamicin	S≤4 R≥16	26	78.6	0	21.4	16	16	1 – 16
Ciprofloxacin	S≤1 R≥4	26	96.4	0	3.6	4	4	1 – 4
Colistin	S≤2 R≥8	26	96.4	3.6	0	16	16	4 – 16
Tigecycline	S≤2 R≥8	26	10.7	7.1	82.1	2	8	0.5 - 8

MIC – minimal inhibitory concentration; R – resistant; I – intermediate; S - susceptible

Table 3 - Sequence typing, Minimal Inhibitory Concentration to Colistin, and Non-synonymous mutations in colistin-resistance genes found in six colistin-resistant *Klebsiella pneumoniae* isolates.

Isolates	Age	Unit	Source	<i>ompK35</i>	<i>pmrA</i>	<i>pmrC (eptA)</i>	<i>phoP</i>	<i>phoQ</i>	<i>mgrB</i>	<i>lpxM</i>	<i>armB</i>	<i>udg</i>	<i>yciM</i>
4219	48	ICU	IAB	115 frame shift	T245A; R255G	WT	WT	WT	M1V; Disrupted gene	S285G	D112A	I17V; Yins217; N353D; A375K; D376S	N212T
4223	61	ICU	IAB	+	T245A; P345L	C27F; V39L; Q319R	Disrupted	G150D	-	S285G; P321T	D112A	I17V; Yins217	WT
4224	60	ICU	Blood	+	T245A; R255G	WT	WT	WT	M1V; N25K; V26E; M27G; C28A; D29Y; stop codon 30	N6K	D112A	I17V; Yins217; N353D	N212T
4229	51	HSCT	Blood	+	T245A; R255G	WT	WT	WT	WT	WT	D112A	I17V; Yins217; N353D; A375V	N212T
4234	60	ICU	IAB	+	T245A; R255G	WT	WT	WT	M1V; I45R; P46L; W47F; A ins 48; F ins 49	S285G	D112A	Yins217; A273G; N353D; N386A	N212T
4235	52	ICU	Blood	+	T245A; R255G	WT	WT	- WT	M1V; I41D; N42L; K43D; F44P; I45P; P46S; Pns47; Nins48; Sins49; Fins50; Cins51; Iins52; Lins53; Sins54	S285G	D112A	I17V; Yins217; N353D	N212T

ICU – intensive care unit; IAB – intra-abdominal fluid; HSCT – hematopoietic stem cell transplant unit

Table 4 - Whole genome sequencing of six isolates of colistin-resistant *Klebsiella pneumoniae*.

Isolates	MLST	Aminoglycoside	Beta-lactam	Fluoroquinolone	Fosfomycin	Chloramphenicol	Sulphonamide	Trimethoprim
4219	11	<i>aadA2</i> ; <i>aacA4</i> ; <i>aph(3')-Ia</i> ; <i>aac(3)-IIa</i>	<i>blaKPC-2</i> ; <i>blaSHV-11</i> ; <i>blaTEM-1B</i> ; <i>blaOXA-2</i>	<i>oqxA</i> ; <i>oqxB</i> ; <i>aac(6')Ib-cr</i>	<i>fosA</i>	-	<i>sul1</i>	<i>dfrA12</i>
4223	23	<i>aac(3)-IIa</i> ; <i>aacA4</i>	<i>blaKPC-2</i> ; <i>blaOXA-2</i> ; <i>blaOXA-9</i> ; <i>blaCTX-M-2</i> ; <i>blaTEM-1A</i> ; <i>blaSHV-3</i>	<i>oqxA</i> ; <i>oqxB</i> ; <i>aac(6')Ib-cr</i>	<i>fosA</i>	-	<i>sul1</i>	-
4224	11	<i>aac(6')-Iq</i> ; <i>aacA4</i> ; <i>aph(3')-Ia</i> ; <i>aac(3)-IIa</i> ; <i>aadA1</i>	<i>blaKPC-2</i> ; <i>blaSHV-11</i> ; <i>blaOXA-2</i> ; <i>blaTEM-1B</i> ; <i>blaCTX-M-2</i>	<i>aac(6')Ib-cr</i> ; <i>oqxA</i> ; <i>oqxB</i>	<i>fosA</i>	<i>cmlA1</i> ; <i>catA1</i> ; <i>catA2</i>	<i>sul1</i> ; <i>sul2</i>	<i>dfrA15</i>
4229	11	<i>aadA2</i> ; <i>aacA4</i> ; <i>aac(3)-IIa</i>	<i>blaCTX-M-2</i> ; <i>blaSHV-11</i> ; <i>blaTEM-1B</i> ; <i>blaOXA-2</i>	<i>aac(6')Ib-cr</i>	<i>fosA</i>	<i>catA1</i>	<i>sul1</i>	<i>dfrA12</i>
4234	340	<i>aph(3')-Ia</i> ; <i>aph(3')-Via</i> ; <i>aadA2</i>	<i>blaSHV-11</i> ; <i>blaCTX-M-15</i> ; <i>blaKPC-2</i> ; <i>blaNDM</i>	<i>oqxA</i> ; <i>oqxB</i>	<i>fosA</i>	-	<i>sul1</i>	<i>dfrA12</i> ; <i>dfrA14</i>
4235	437	<i>aph(3')-Ia</i> ; <i>aadA2</i>	<i>blaKPC-2</i> ; <i>blaSHV-11</i> ;	<i>oqxA</i> ; <i>oqxB</i>	<i>fosA</i>	-	<i>sul1</i>	<i>dfrA30</i>

MLST = Multilocus sequence typing; (-) = absence of the gene; Aminoglycoside-resistance genes = chromosomal aminoglycoside phosphotransferase gene, *aph(3')-Ib*; gene *aac(6')*- Streptomycin 3'- adenylyltransferase; AAC = aminoglycoside acetyltransferase (3-2"); AAD = aminoglycoside adenylyltransferase; *aph* = aminoglycoside phosphotransferase; Quinolone-resistance genes = acetyltransferase AAC(6')-Ib-cr; *oqxA*, *oqxB* = efflux pump; *fosA* = plasmid-mediated phosphomycin-resistance gene; *cmlA1* = Chloramphenicol efflux protein; *catA1*, *catA2* = Chloramphenicol acetyltransferase; *Sul1* and *Sul2* = Sulfonamide-resistant dihydropteroate synthase; *dfrA12*, *dfrA14*, *dfrA15*, *dfrA30* = Dihydrofolate reductase.

K. pneumoniae strains expressing loss of porins *ompk35* and *ompk36* associated with other mechanisms.

We identified 26 strains harboring *bla_{kpc}* gene. The two remaining isolates tested negative for *bla_{kpc}*, *bla_{OXA-48}* and *bla_{NDM}* polymerase chain reaction. In six isolates, WGS detected several mutations in important genes related to LPS modification, such as *pmrA*, *mgrB*, *lpxM*, *arnB*, *udg* and *yciM*. The *pmrAT245A* and R255G; *arnB* D112A; and *udg* I17V and Yins217 mutations were present in all six isolates; the *yciMN212T* and *lpxMS285G* mutations were also present in 5 and 4 isolates, respectively. The role of these mutations is not yet clear, but their simultaneous presence in all those genes that are already known to be involved with colistin-resistance in the colRKP isolates suggest that they may also lead to polymyxin-resistance²⁰⁻²⁴. Four different MLST were identified, and although there were three isolates from MLST 11, they did not share the same mutations, which suggest that they were not from a clonal lineage, corroborating the clustering analysis results. These results in addition to the

fact that we did not find the plasmid *mcr-1* indicate that colistin-resistance is more likely due to selective pressure by the use of colistin, rather than a plasmid or clonal spread amongst our isolates.

We did not find the presence of gene *mcr-1* among these six isolates submitted to WGS, although we had identified in our hospital two isolates in 2016 (data not shown). Gene *mcr-1* has been described as an important mechanism of colistin-resistance. Liu *et al.*²⁵ reported a plasmidial *mcr-1* gene in *E. coli* identified in raw meat, animals and humans conferring elevated MICs (8 µg/ml) to colistin. Plasmid-mediated resistance has epidemiological implications due to a facilitated way of spread, including *K. pneumoniae*, as seen previously with ESBL and KPC²⁶.

Moreover, isolates of *E. coli* and *S. enterica* with elevated MIC to colistin were found in pigs, in Brazil²⁷. Although interspecies transmission is not completely understood, the use of polymyxins by veterinarians is a colistin-resistance growth promoter and their consequences to human health are of great concern.

We have seen in our institution, the emergence of carbapenem non-susceptible *Enterobacteriaceae*, increasing from 0.01 to 1.6/1000 patient-day in 2007 and 2016, respectively. Most of them (70%) harbor carbapenemase-producing *K. pneumoniae* (data not shown). As a result of this scenario, colistin has been used as a key drug to treat patients affected by this pathogen, often associated with other drugs especially, aminoglycosides, tigecycline, fosfomicin (study protocol)²⁸ and meropenem, regardless of susceptibility³. Thus, the extensive use of polymyxins due to increasing carbapenem-resistant pathogens may have exerted a selective pressure leading to the emergence of resistant strains. Twelve patients and one patient were exposed to colistin or polymyxin B, respectively, prior to the occurrence of ColRKP infection in this cohort. The rate of polymyxins use has been on average 27.7 DDD/1000 patients-day in our hospital in the last ten years (2007-2016) which is positioned in percentile 75 among 321 intensive care units in Sao Paulo State (Southeastern Brazil), the region in which we are located²⁹. In our series, the fact that 28 isolates were distributed in eight clusters reinforced the possibility of selective pressure. Although efforts to prevent cross transmission have been employed, the judicious use of polymyxins is a primary and urgent need. Moreover, the epidemiology of naturally polymyxin-resistant pathogens, such as *Proteus*, *Providencia*, *Morganella* and *Serratia* might change in the near future due the increasing use of these drugs³⁰.

Our study is limited by the small number of strains, especially of those studied by WGS. Although we identified several mutations in genes previously related to colistin-resistance we cannot assure that they were directly involved in these cases. Further transcriptomics studies are needed to verify if these mutations led to LPS alterations.

Colistin-resistance in carbapenem resistant *K. pneumoniae* is of great concern as few therapeutic options if any, are left. Strict control measures that prevent dissemination, as well as the judicious use of polymyxins are important measures until new drugs or alternative therapeutic approaches are available.

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