



Molecular and epidemiological surveillance of polymyxin-resistant *Klebsiella pneumoniae* strains isolated from Brazil with multiple *mgrB* gene mutations

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ABSTRACT

The prevalence of polymyxin-resistant *Enterobacteriaceae* is increasing worldwide. Their emergence is worrisome and limits therapeutic options for severely ill patients. We aimed to investigate the molecular and epidemiological characteristics of polymyxin-resistant *Klebsiella pneumoniae* circulating in Brazilian hospitals. Polymyxin-resistant *K. pneumoniae* isolates from two Brazilian healthcare facilities were characterized phenotypically and subjected to whole genome sequencing (WGS). Using the WGS data we determined their sequence type, resistance gene content (resistome), their composition of virulence genes and plasmids. ST11 was the most common (80 %) sequence type among the isolates followed by ST345, ST15 and ST258. A resistome analysis revealed the common presence of *bla*_{KPC-2} and less frequently *bla*_{SHV-11}, *bla*_{TEM-1}, *bla*_{CTX-M-15}, and *bla*_{OXA-9}. Genes conferring resistance to aminoglycosides, fluoroquinolones, phenicols, sulphonamides, tetracyclines, trimethoprim and macrolide-lincosamide-streptogramin were also detected. We observed a clonal spread of polymyxin-resistant *K. pneumoniae* isolates, with polymyxin-resistance associated with various alterations in the *mgrB* gene including inactivation by an insertion sequence and nonsense point mutations. We additionally identified a novel 78-bp repeat sequence, encoding a MgrB protein with 26 amino acids duplicated in six isolates. This is the first observation of this type of alteration being associated with polymyxin resistance. Our findings demonstrate that *mgrB* alterations were the most common source of polymyxin-resistance in Brazilian clinical settings. Interestingly, distinct genetic events were identified among clonally related isolates, including a new amino acid alteration. The clinical implications and investigation of the resistance mechanisms is of great importance to patient safety and control of these infections, particularly in long-term care facilities.

1. Introduction

Infections caused by multi-drug resistant (MDR) Gram-negative bacteria have become a major problem in Intensive Care Units (ICUs) worldwide (Brusselaers et al., 2011) and are often associated with a high rate of morbidity and mortality (Codjoe and Donkor, 2017; Friedman et al., 2016). *Klebsiella pneumoniae* is a Gram-negative bacteria that can carry a variable compendium of virulence genes and cause a range of human infections (Ergonul et al., 2016; Lee et al., 2017). Notably, *K. pneumoniae* has the capacity to rapidly acquire novel antimicrobial

resistance mechanisms and carbapenem-resistance *K. pneumoniae* has been recognized as key agent of hospital-acquired infections. The spread of carbapenem-resistance *K. pneumoniae* is being facilitated by mobile genetic elements that carry genes conferring resistance to beta-lactams, such as OXA-48-like, NDM-type and particularly KPC-type enzymes (Abodakpi et al., 2017; Guducuoglu et al., 2017; Sampaio and Gales, 2016).

Colistin/polymyxin regimes represent some of the last options to treat infections caused by KPC-producing *K. pneumoniae* and other MDR Gram-negative (Rojas et al., 2017; Zheng et al., 2017). Although these

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antimicrobials are effective for the treatment of a variety of infections, the emergence and dissemination of polymyxin-resistance *K. pneumoniae* pose a significant threat to the treatment management of nosocomial infections and increase the potential for pan-resistant bacteria (Guducuoglu et al., 2017; Mansour et al., 2017; Sampaio and Gales, 2016). Resistance to polymyxins can arise through adaptive or mutational mechanisms that alter the outer membrane via modification of lipopolysaccharide (LPS) via cationic substitutions (Olaitan et al., 2014). Previous studies have demonstrated that modifications in the PmrA/PmrB and PhoP/PhoQ two-component systems and the inactivation of the *mgrB* gene (a regulator of the PhoP/PhoQ system) lead to polymyxin resistance by LPS modification (Aires et al., 2016). More recently, the plasmid-mediated transferable polymyxin resistance *mcr-1* gene, which causes resistance by modification of lipid A, was found in *Escherichia coli* and *K. pneumoniae* in China (Liu et al., 2016).

Polymyxin is commonly used in Brazilian hospitals to treat infections caused by MDR Gram-negative pathogens. This use has been associated with an increase in the prevalence of polymyxin-resistant *K. pneumoniae*, which increased from 1.8 % in 2009 to 15 % in 2013 and 35.5 % in 2015 (Bartolletti et al., 2016; Pereira et al., 2013; Sampaio and Gales, 2016). However, despite their commonality, little has been done to identify circulating clones or define the associated resistance mechanisms, which may provide some welcome insight into their prevention and treatment and also potentially reduce their transmission in Brazilian healthcare facilities. Here we aimed to investigate the molecular and epidemiological characteristics of polymyxin-resistant *Klebsiella pneumoniae* circulating in Brazilian hospitals. Specifically, we exploited whole-genome sequencing (WGS) to explore the molecular mechanisms associated with polymyxin resistance in *K. pneumoniae* found in ICUs in Brazil.

2. Material and methods

2.1. Study design

Polymyxin-resistant *K. pneumoniae* were recovered from patients hospitalized in two public tertiary care hospitals located in distinct cities in Brazil: Tertiary Hospital, Dourados (Hospital A) and Regional Hospital, Campo Grande (Hospital B), between September 2015 and October 2016. These locations are 187 and 352 bed facilities respectively, which are distributed among infirmaries, maternal and infant areas, ICUs adult, pediatric, neonatal and intermediate care units (IU). Clinical records from all patients diagnosed with polymyxin-resistant strains hospitalized in Hospital A and B were reviewed and the following data were recorded: demographics; medical history and comorbid conditions; location prior to admission; hospital course (duration and ward location); invasive procedures (devices use and surgery) receiving of mechanical ventilation; treatment with immunosuppressant drugs; antimicrobial exposure history; source of infection and outcome (recovery/death).

Strict CDC definitions were used to determine whether an isolated organism was associated with colonization or infection. Clinical infection was defined by medical diagnosis according to clinical criteria (sepsis, fever, changes in frequency or color of secretions, or new radiological findings) associated with the decision to initiate antimicrobial therapy, as well as, isolation of one polymyxin-resistant *K. pneumoniae* organism (Horan et al., 2008). Colonizers were defined as bacteria permanently or temporarily present in the skin or mucous membranes of the patient, dissociated from signs or symptoms of infectious disease. The endemic level of colonization and infection by polymyxin-resistance per 1000 patient-days was calculated using a previously described method (Arantes et al., 2003).

2.2. Bacterial identification and antimicrobial susceptibility testing

Bacterial species identification and antimicrobial susceptibility testing were performed using the Vitek®2 (bioMérieux, Hazelwood,

MO) according to the manufacturer's instructions. After isolation, the susceptibility profile was confirmed with the evaluation of the minimal inhibitory concentrations (MICs) against various antimicrobials by broth microdilution following the recommendations of the Clinical and Laboratory Standards Institute (CLSI) guidelines (Institute, 2017). Susceptibility results were interpreted according to the most recent CLSI guidelines (Institute, 2017).

2.3. Outer membrane protein analysis

To investigate carbapenem resistance the outer membrane protein (OMP) composition of *K. pneumoniae* were analyzed by SDS-PAGE by taking membrane extracts from bacteria grown overnight in nutrient broth, and gels stained with Coomassie blue (Carvalhoes et al., 2010). Alterations of OmpK35- and OmpK36-encoding genes were also investigated by sequencing (Correa et al., 2013).

2.4. Whole-genome sequencing and phylogenetic analysis

Genomic DNA was extracted from fresh cultures of polymyxin-resistant organisms using QIAamp® DNA Mini Kit (Qiagen, Germany). The concentration and purity of DNA were determined using a Qubit® 2.0 fluorometer using the dsDNA BR Assay Kit (Life Technologies, Carlsbad, CA). Sequencing libraries were prepared using the Nextera library kit (Illumina). The prepared libraries were sequenced with 150 bp paired-end reads via IlluminaMiSeq Platform (Illumina, San Diego, USA), as previously described (Dung et al., 2017). FastQC version 0.11.2 was used to preprocess the reads (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>) and QC stats were exported to graph and manually assessed.

Species identification was confirmed with Kraken (Wood and Salzberg, 2014). Each read set was assembled using SPAdes version 3.6.1, (Bankevich et al., 2012) with k-mer sizes 21, 33, 55, 77, 99 and 127 with mismatch correction. These sequences were annotated using Prokka (Seemann, 2014). The “core” *K. pneumoniae* genome was defined as concatenation of coding sequences presenting one copy in all of the final assemblies, with a sequence identity of 95 % and 100 % of coverage (Page et al., 2015). Reads were mapped to the reference genome *K. pneumoniae* HS11286/ST11 (accession number CP003200) using Bowtie2 (Langmead and Salzberg, 2012), then high quality Single nucleotide polymorphisms (SNPs) were extracted from the alignments using SAMtools (Li et al., 2009). Maximum likelihood (ML) phylogenetic trees were constructed using RAxML (v 8.1.23) (Stamatakis, 2014). Analyses were performed with 100 bootstrap replicates per run, with a generalized time-reversible model and a gamma distribution to model site specific recombination (GTR+ Γ substitution model; GTRGAMMA in RAxML) and was visualized using FigTree version 1.3.1 (<http://tree.bio.ed.ac.uk/software/figtree/>). We selected the single tree with the highest ML as the best tree and annotation was performed in R with the *ape* package. SRST2 (Inouye et al., 2014) was used to mapping known alleles (Diancourt et al., 2005) and identify MLSTs directly from reads according to the *K. pneumoniae* MLST allele definition database (<https://bigdb.pasteur.fr/klebsiella/klebsiella.html>).

2.5. Gene content analysis

Read sets were screened for known alleles of genes using a read mapping approach with SRST2. For acquired resistance genes we used the ARG-ANNOT database (Gupta et al., 2014). Plasmid replicons were identified with ARIBA using the PlasmidFinder database (Carattoli et al., 2014). Virulence genes were identified by comparison to the gene databases for virulence and wzi alleles (*K. pneumoniae* BIGSdb) hosted by the Institut Pasteur MLST database (<http://bigdb.web.pasteur.fr>). PROVEAN software tool was used to predict whether sequence variants at the nucleotide level resulted in amino acid substitutions with an impact on the biological function of proteins (Choi and Chan, 2015).

Insertion Sequence (IS) elements were identified using ISFinder (Siguier et al., 2006). Whole genome sequences generated for this work have been deposited in DDBJ/ENA/GenBank; Accession numbers: ERR2743730 to ERR2743758.

3. Results

3.1. Polymyxin-resistant *Klebsiella pneumoniae*

During the period of investigation, we isolated a total of 30 polymyxin-resistant *K. pneumoniae*; 25 from hospital A and 5 from hospital B. The distribution of polymyxin-resistant *K. pneumoniae* isolates among the different hospital wards was as follows: intensive care unit (ICU) (n = 18); intensive care unit neonatal (ICU neo) (n = 10) and intensive care unit pediatric (ICU ped) (n = 2). Organisms were recovered from 30 individual patients on a median of 10 days following admission (range 2–40 days); 27 patients had a history of previous hospitalization. The clinical characteristics of 30 patients infected or colonized by polymyxin-resistant *K. pneumoniae* are shown in Table 1. The patients in the adult ICU ranged from 38 to 86 years; 12 (40 %) of the patients included in the study were neonates hospitalized in the neonatal ICU and with a median gestational age of 31 weeks.

Prior to isolation of polymyxin-resistant *K. pneumoniae*, all patients had received antimicrobial regimens, which included penicillins, third- or fourth-generation cephalosporins, quinolones, aminoglycosides, carbapenems, and polymyxins. However, empiric antimicrobial therapy did not provide appropriate antibacterial coverage based on the antimicrobial susceptibility testing. The analysis of data on patient outcomes revealed that polymyxin-resistant *K. pneumoniae* patients had a high mortality rate (60 %). On the basis of epidemiological data an outbreak was identified in hospital A. We constructed a timeline representing the isolation of polymyxin-resistant *K. pneumoniae* on each ward (Fig. 1). This timeline showed that the ICU was involved continuously between September 2015 and November 2015, whereas the ICU neo had two different distinct periods separated by 180 days respectively (September to November 2015 and June to October 2016) (Fig. 2).

Regarding the endemicity of polymyxin-resistant organisms, rates of occurrence of infection and/or colonization per 1000 patient-days in September 2015 and November 2015 exceeded the control limit established, above the average incidence of colonization/infection (Fig. 2). The acquisition rate of infection and/or colonization was 0.004 per 1000 patient-days in August 2015, increasing to 0.01 per 1000 patient-days in October 2015. Although it has not exceeded the control limit, from July 2016 to September 2016 other cases subsequently occurred and the acquisition rate of polymyxin-resistant *K. pneumoniae* strains was above the average prevalence of colonization or infection, reaching the alert limit. Furthermore, 67 % (n = 20) of the identified strains were recovered from blood culture, 10 % (n = 3) from tracheal aspirates, 10 % (n = 3) from catheter, 7 % (n = 2) from rectal swab, 3 % (n = 1) from surgical wound and 3 % (n = 1) from cerebrospinal fluid.

3.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed at the time of isolation. Polymyxin-resistant strains showed resistance to all the antimicrobials tested by broth microdilution as follows: ceftazidime (MIC₅₀, >256 mg/L), cefotaxime (MIC₅₀, >256 mg/L), ceftriaxone (MIC₅₀, >256 mg/L), cefepime (MIC₅₀, >256 mg/L), aztreonam (MIC₅₀, >32 mg/L), imipenem (MIC₅₀, >16 mg/L), meropenem (MIC₅₀, >16 mg/L), ertapenem (MIC₅₀, >32 mg/L), amikacin (MIC₅₀, 64 mg/L), gentamicin (MIC₅₀, 32 mg/L), ciprofloxacin (MIC₅₀, 16 mg/L), levofloxacin (MIC₅₀, 16 mg/L) and polymyxin B (MICs ranging from MIC₅₀, 4 mg/L MIC₅₀, 32 mg/L).

3.3. Outer membrane protein analysis

Alterations in the OmpK35 and OmpK36 proteins were found in four strains. According to the SDS-PAGE results, three *K. pneumoniae* strains presented two bands, probably corresponding to OmpA and one of the main porins (either OmpK35 or OmpK36), suggesting that they have lost at least one of the main porins. PCR analysis of OMP-encoding genes showed altered amplicons of at least one main OMP-encoding gene, including a lack of amplification (four isolates). Sequencing results showed OmpK35 was not detected in four isolates (KP32, KP34, KP37 and KP42) and OmpK36 in one isolate (KP32).

3.4. Phylogenetic analysis of whole genome sequences

Core genome analysis of the 30 *K. pneumoniae* whole genome sequences revealed 3792 unique genes that were present in 99.0 % of isolates. MLST analysis of the genome sequences identified four different sequence types; ST11 was the most common (n = 24) and both non-KPC2-producing ST11 and KPC-2-producing ST11 isolates were genotypically closely related. Other STs were also represented including ST345 (n = 3), ST15 (n = 2) and ST258 (n = 1). A maximum likelihood tree of the 30 polymyxin-resistant *K. pneumoniae* sequences was constructed (Fig. 3). All 6 isolates carrying the *mgrB* repeated sequence at nt 89 were closely related and grouped together in the tree, these strains have from 2 to 29 SNPs of difference. SNP analysis also demonstrated that KP32 the only isolate harboring the *mgrB* repeated sequence detected in the neonatal ICU had a median difference of 630 (62–1373) SNPs between KP032 and isolates that were found in the second period in the neonatal ICU.

3.5. Antimicrobial resistance and virulence genes

All organisms harbored several antimicrobial-resistant genes encoding resistance against beta-lactams, aminoglycosides, fluoroquinolones and tetracyclines (Fig. 3). The genomic data showed that the *bla*_{KPC-2} gene, conferring resistance to carbapenems, was present in 29 organisms and all carried >1 ESBL gene, including *bla*_{CTX-M-15} (100 %), *bla*_{TEM-1} (77 %) and *bla*_{SHV-11} (40 %). In addition, genes conferring resistance to streptomycin, rifampin, chloramphenicol, trimethoprim, macrolides and sulphonamides were also identified. The plasmid-mediated colistin resistance gene *mcr-1* was not detected. The identified virulence genes included *clbA* (colibactin), *fyuA*, *irp1*, *irp2*, *ybtA*, *ybtE*, *ybtQ*, *ybtS*, *ybtT*, *ybtU*, *ybtX* (yersiniabactin), *iutA* (aerobactin), *kfuA*, *kfuB*, *kfuC* (iron uptake), *kvgA*, *kvgS* (iron regulation) *mrkA*, *mrkB*, *mrkC*, *mrkD*, *mrkF*, *mrkH*, *mrkI* and *mrkJ* (fimbrial adherence determinants) (Fig. 3).

The diversity of loci encoding capsule serotypes were identified in the 30 study isolates. In brief, four capsule loci and 5 *wzi* alleles were identified (Table 2). We found correlation between capsular polysaccharide serotypes and clonal type. Most isolates of the same ST did possess the same K-locus type and same *wzi* allele. For instance, *K. pneumoniae* ST11 isolates possessed two *wzi* alleles (*wzi*75 and *wzi*50). In contrast, all ST345 isolates showed *wzi*245 associated with capsule type KL15. ST15 isolates contained *wzi*93 associated with KL112 and the single ST258 isolate possessed *wzi*154 and belonging to KL107 capsule type.

Plasmid replicon sequences were identified using PlasmidFinder. The isolates were found to harbor between one and six plasmids and a total of 16 plasmid replicon types were identified. Seventy-three percent of KPC-2-producing strains harbored IncR plasmid in addition to IncFIB or IncL. The non-KPC-2-producing strain had IncFIB, IncL and ColRNAI incompatibility groups (Fig. 3).

Table 1Clinical characteristic of 30 patients infected/colonized with polymyxin-resistant *K. pneumoniae* strains.

Patient	Age/ Sex	Clinical specimen	Strain	Hospital admission from	Hospital unit	Length of stay (days)	Device	Outcome	Exposure to Antimicrobials	Treatment/ Dosage/Days of therapy
1	73/M	Blood	I	Another hospital	ICU	84	CVC/ MV/UC	Death	Aminoglycosides/ Carbapenems/Glycopeptide/ Polymyxin	AMK(500 mg)/ CST-PMB (500 mg)/13
2	69/M	Tracheal aspirates	I	Another hospital	ICU	56	CVC/ MV/UC	Discharge	Aminoglycosides/ Carbapenems/	TGC (50 mg)/ CST-PMB (500 mg)/17
3	70/M	Tracheal aspirates	I	Another hospital	ICU	44	CVC/ MV/UC	Discharge	Aminoglycosides/ Carbapenems/Glycopeptide	TGC (50 mg)/ CST-PMB (500 mg)/14
4	8days/ F	Blood	I	Home	ICUneo	75	ETT/ PVA/ CVA/ NGT	Death	Aminoglycosides/ Cephalosporins/ Penicillins	AMK(50 mg)/ CST-PMB (500 mg)/14
5	46/F	Blood	I	Home	ICU	92	CVC/ MV/UC	Death	Aminoglycosides/ Carbapenems/Cephalosporins/ Polymyxin	AMK(250 mg)/ CST-PMB (500 mg)/17
6	67/M	Blood	I	Another hospital	ICU	89	CVC/ MV/UC	Death	Aminoglycosides/ Carbapenems/Fluoroquinolone	AMK(250 mg)/ CST-PMB (500 mg)/14
7	45 days/F	Rectal swab	C	Home	UI	42	PVA/NGT	Discharge	Aminoglycosides/ Carbapenems/Cephalosporins/	TGC(150 mg)/ CST-PMB (500 mg)/14
8	30 days/M	Rectal swab	C	Another hospital	UI	52	PVA/NGT	Discharge	Cephalosporins/Penicillins	TGC(50 mg)/ CST-PMB (500 mg)/14
9	80/F	Blood	I	Another hospital	ICU	34	CVC/ MV/UC	Death	Aminoglycosides/ Carbapenems/Glycopeptide	AMK(500 mg)/ CST-PMB (500 mg)/13
10	56/M	Blood	I	Home	ICU	40	MV/UC	Death	Aminoglycosides/ Carbapenems/Glycopeptide	TGC(150 mg)/ CST-PMB (500 mg)/18
11	41/M	Blood	I	Another hospital	ICU	32	CVC/ MV/UC	Death	Aminoglycosides/ Carbapenems/Glycycycline/ Polymyxin	AMK(500 mg)/ CST-PMB (500 mg)/20
12	58/F	Blood	I	Another hospital	ICU	38	CVC/ MV/UC	Death	Aminoglycosides/ Carbapenems/Glycopeptide/ Polymyxin	AMK(500 mg)/ CST-PMB (500 mg)/12
13	72/F	Blood	I	Home	ICU	28	CVC/ MV/UC	Death	Carbapenems/Penicillins	TGC(50 mg)/ CST-PMB (500 mg)/18
14	56/M	Blood	I	Another hospital	ICU	35	CVC/ MV/UC	Death	Aminoglycosides/ Carbapenems/Cephalosporins /Polymyxin	AMK(500 mg)/ CST-PMB (500 mg)/10
15	34/F	Blood	I	Another hospital	IUC	85	MV	Death	Carbapenems /Polymyxin	AMK(250 mg)/ CST-PMB (500 mg)/14
16	61/F	Blood	I	Another hospital	ICU	35	MV/UC	Death	Aminoglycosides/ Carbapenems/ Fluoroquinolone	AMK(500 mg)/ CST-PMB (500 mg)/18
17	45 days/F	Surgical wond	C	Home	ICUneo	90	ETT/ CVA/NEC	Discharge	Aminoglycosides/Penicillins	TGC(50 mg)/ CST-PMB (500 mg)/20
18	29 days/M	Blood	I	Home	ICUneo	38	ETT/ CVA/ NGT	Death	Aminoglycosides/ Carbapenems/Penicillins	TGC(50 mg)/ CST-PMB (500 mg)/17
19	15 days/M	Blood	I	Home	ICUneo	28	ETT/ CVA/ PVA/NGT	Death	Aminoglycosides/ Cephalosporins/Penicillins	AMK(50 mg)/ CST- PMB (500 mg)/17
20	8days/ F	Catheter	C	Home	ICUneo	35	AVP/ CVA/ NGT	Discharge	Aminoglycosides/ Cephalosporins/Penicillins	AMK(250 mg)/ CST- PMB (500 mg)/15
21	13 days/M	Blood	I	Home	ICUneo	59	ETT/ CVA/ PVA/NGT	Death	Aminoglycosides/Penicillins	AMK(50 mg)/ CST- PMB (500 mg)/17
22	2/M	Cerebrospinal fluid	I	Another hospital	ICUped	90	CVA/ NGT	Discharge	Carbapenems/Cephalosporins	TGC(50 mg)/ CST- PMB (500 mg)/21
23	1/F	Blood	I	Home	ICUped	27	–	Discharge	Aminoglycosides/Penicillins	TGC(50 mg)/ CST- PMB (500 mg)/18
24	30 days/F	Catheter	I	Home	UI	30	PVA/NGT	Discharge	Aminoglycosides/ Cephalosporins/Penicillins	

(continued on next page)

Table 1 (continued)

Patient	Age/ Sex	Clinical specimen	Strain	Hospital admission from	Hospital unit	Length of stay (days)	Device	Outcome	Exposure to Antimicrobials	Treatment/ Dosage/Days of therapy
25	30 days/F	Blood	I	Home	UI	28	PVA/ CVA/ NGT	Discharge	Aminoglycosides/Penicillins	AMK(50 mg)/ CST- PMB (500 mg)/17 AMK(50 mg)/ CST- PMB (500 mg)/10
26	76/M	Blood	I	Home	ICU	15	–	Death	Carbapenems/Cephalosporins	TGC(50 mg)/ CST- PMB (500 mg)/14 AMK(500 mg)/ CST- PMB (500 mg)/14
27	79/F	Blood	I	Home	ICU	25	NGT	Death	Aminoglycosides/ Carbapenems/Glycycycline/ Glycopeptide/Polymyxin	AMK(500 mg)/ CST- PMB (500 mg)/14
28	81/F	Blood	I	Another hospital	ICU	34	PVA/NGT	Discharge	Aminoglycosides/ Carbapenems/Glycycycline	AMK(500 mg)/ CST- PMB (500 mg)/18
29	46/M	Tracheal aspirates	I	Home	ICU	92	NGT	Death	Aminoglycosides/ Carbapenems/Cephalosporins/ Polymyxin	AMK(500 mg)/ CST- PMB (500 mg)/21
30	85/F	Catheter	C	Another hospital	ICU	26	CVA	Death	Carbapenems/ Fluoroquinolone	TGC (50 mg)/ CST- PMB (500 mg)/18

Abbreviations: M- Male; F- Female; I- Infection; C- Colonizers; ICU- Intensive care unit; ICU neo- Intensive care unit neonatal; ICU ped- Intensive care unit pediatric; CVC- central venous catheter; MV- mechanical ventilation; UC- urinary catheter; ETT- endotracheal tube; CVA- central venous access; PVA-peripheral venous access; NGT- nasogastric tube; NEC- nasoenteral catheter; AMK- amikacin; CST- colistin; PMB-polymyxin B; TGC- Tigecycline.

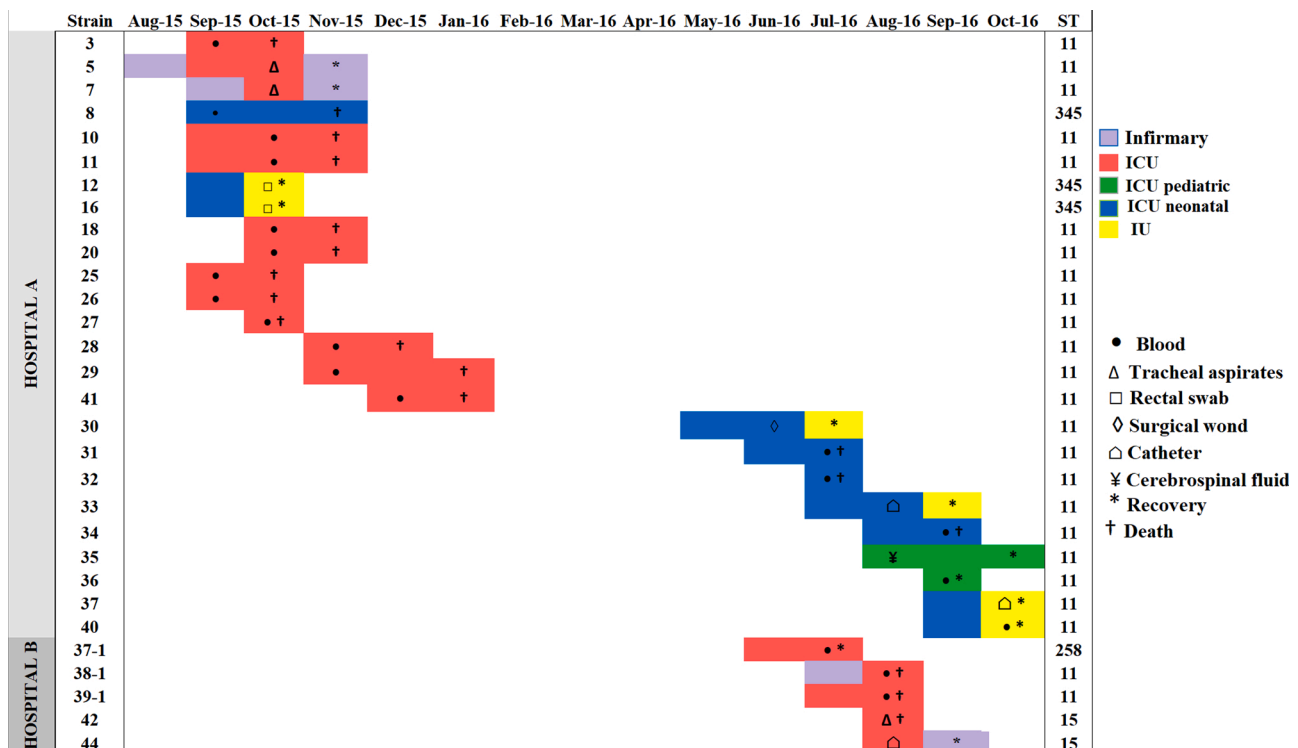
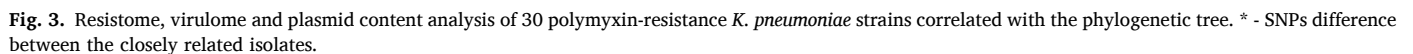
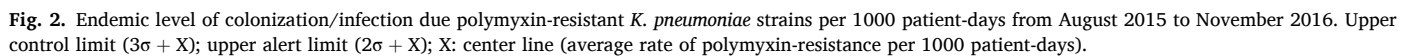


Fig. 1. Duration and location of hospital stays of patients infected and/or colonized with polymyxin-resistant *K. pneumoniae* strains.

3.6. mgrB alterations

To investigate potential polymyxin-resistance mechanisms the nucleotide sequences of *pmrA*, *pmrB*, *phoP*, *phoQ*, *csrB* and *mgrB* genes were analyzed and compared to sequences from a polymyxin-susceptible *K. pneumoniae* organism (NTUH-K2044). None of the 30 isolates had mutations in the PmrAB/PhoP-related genes, however all organisms had a point mutation in the *phoQ* gene leading to amino acid substitution (Asp150Gly), which was predicted to have a neutral impact on the

protein function. Out of 30 organisms, 22 exhibited IS mediated disruptions in the *mgrB* coding sequence (Table 2). The ISs were of five different types, IS903 (n = 7), IS5-like (n = 7), ISKpn13 (n = 4), ISEcp1 (n = 3) and ISKpn18 (n = 1) and were found to be inserted at six different positions and in various orientations (Table 2/Fig. S1). One isolate had a point mutation (A7T) in the *mgrB* coding sequence creating a stop codon. Six strains harbored a 78-bp repeat sequence at nucleotide position 89, encoding an amino acid sequence with 26 amino acids duplicated which altered function of the protein and predicted to be



3.7. Infection control measures

patients with previous hospitalization. Sharing of patient equipment was limited as much as possible; hand hygiene promotion on ICU; isolation of patients colonized/infected by carbapenem-resistant strains; general environmental cleaning and disinfection of reusable medical equipment were properly executed. Cleaning of all surfaces including walls, floors, ceilings, windows, furniture, and medical equipment were intensified; exchange of antiseptic solutions; daily summary of all relevant microorganisms on ICU and communication between the departments of medical microbiology, infection prevention and other healthcare

Table 2Features and *mgrB* gene status of the 30 polymyxin-resistant strains.

Strains	Year	Hospital	Source	ST	K locus (wzi)	Carbapenemase	Polymyxin MIC (µg/L)	<i>mgrB</i> status
KP03	2015	A	Blood	11	KL105 (wzi75)	KPC-2	32	<i>mgrB</i> repeated sequence at nt 89
KP05	2015	A	Tracheal aspirates	11	KL105 (wzi75)	KPC-2	16	Insertional inactivation, <i>ISEcp1at</i> nt 124 (FW)
KP07	2015	A	Tracheal aspirates	11	KL105 (wzi75)	KPC-2	16	Insertional inactivation, <i>ISEcp1at</i> nt 124 (FW)
KP08	2015	A	Blood	345	KL15 (wzi245)	KPC-2	16	Insertional inactivation, <i>ISKpn13</i> at nt 125 (FW)
KP10	2015	A	Blood	11	KL105 (wzi75)	KPC-2	32	<i>mgrB</i> repeated sequence at nt 89
KP11	2015	A	Blood	11	KL105 (wzi75)	KPC-2	32	<i>mgrB</i> repeated sequence at nt 89
KP12	2015	A	Rectal swab	345	KL15 (wzi245)	KPC-2	16	Insertional inactivation, <i>ISKpn13</i> at nt 75 (RW)
KP16	2015	A	Rectal swab	345	KL15 (wzi245)	KPC-2	16	Insertional inactivation, <i>ISKpn13</i> at nt 125 (FW)
KP18	2015	A	Blood	11	KL105 (wzi75)	KPC-2	32	<i>mgrB</i> repeated sequence at nt 89
KP20	2015	A	Blood	11	KL15 (wzi50)	KPC-2	32	Insertional inactivation, <i>ISKpn18</i> at nt 122
KP25	2015	A	Blood	11	KL105 (wzi75)	KPC-2	16	Insertional inactivation, <i>IS903at</i> nt 89 (FW)
KP26	2015	A	Blood	11	KL105 (wzi75)	KPC-2	16	Insertional inactivation, <i>IS5</i> -like element at nt 89 (FW)
KP27	2015	A	Blood	11	KL105 (wzi75)	KPC-2	16	Insertional inactivation, <i>IS903at</i> nt 89 (FW)
KP28	2015	A	Blood	11	KL105 (wzi75)	KPC-2	8	Insertional inactivation, <i>IS903at</i> nt 89 (FW)
KP29	2015	A	Blood	11	KL105 (wzi75)	KPC-2	16	Insertional inactivation, <i>IS5</i> -like element at nt 89 (RW)
KP30	2016	A	Surgical wound	11	KL105 (wzi75)	KPC-2	8	Insertional inactivation, <i>IS5</i> -like element at nt 89 (RW)
KP31	2016	A	Blood	11	KL105 (wzi75)	KPC-2	8	Insertional inactivation, <i>ISEcp1</i> at nt 124 (FW)
KP32	2016	A	Blood	11	KL105 (wzi75)	None	32	<i>mgrB</i> repeated sequence at nt 89
KP33	2016	A	Catheter	11	KL105 (wzi75)	KPC-2	16	Insertional inactivation, <i>IS903at</i> nt 89 (FW)
KP34	2016	A	Blood	11	KL105 (wzi75)	KPC-2	8	Insertional inactivation, <i>IS903at</i> nt 89 (FW)
KP35	2016	A	Cerebrospinal fluid	11	KL105 (wzi75)	KPC-2	16	Insertional inactivation, <i>IS5</i> -like element at nt 89 (FW)
KP36	2016	A	Blood	11	KL105 (wzi75)	KPC-2	16	Insertional inactivation, <i>IS903at</i> nt 88 (FW)
KP37	2016	A	Catheter	11	KL105 (wzi75)	KPC-2	8	Insertional inactivation, <i>ISKpn13</i> at nt 89 (FW)
KP40	2016	A	Blood	11	KL105 (wzi75)	KPC-2	16	Insertional inactivation, <i>IS5</i> -like element at nt 89 (RW)
KP41	2016	A	Catheter	11	KL105 (wzi75)	KPC-2	32	<i>mgrB</i> repeated sequence at nt 89
KP37-1	2016	B	Blood	258	KL107 (wzi154)	KPC-2	8	Insertional inactivation, <i>IS5</i> -like nt 75 (FW)
KP38-1	2016	B	Blood	11	KL105 (wzi75)	KPC-2	8	Insertional inactivation, <i>IS5</i> -like element at nt 89 (RW)
KP39-1	2016	B	Blood	11	KL105 (wzi75)	KPC-2	16	Insertional inactivation, <i>IS903at</i> nt 89 (FW)
KP42	2016	B	Tracheal aspirates	15	KL112 (wzi93)	KPC-2	8	Substitution at nt 7 (a > t)
KP44	2016	B	Catheter	15	KL112 (wzi93)	KPC-2	8	WT

Abbreviations: KPC – *Klebsiella pneumoniae* carbapenemase; MIC – Minimal inhibitory concentration; nt – nucleotide; FW – Forward; RW – Reverse; WT– Wild type.

personnel was intensified through regular instruction meetings performed at the hospital wards. There was no intervention on antimicrobial administration and patients with infections were treated according to standard antimicrobial treatment policy (amikacin/polymyxin or tigeciclyne/polymyxin combined therapy). After implementing these measures, a considerably reduction in the incidence of carbapenem and polymyxin-resistant isolates was observed (Fig. 2).

4. Discussion

Polymyxin-resistance is rapidly emerging worldwide in *Enterobacteriaceae*, in particular in *K. pneumoniae*. Here we aimed to further understand the genetic basis for polymyxin-resistance in *K. pneumoniae* circulating in two Brazilian hospitals, including the report of an outbreak of polymyxin-resistant and KPC-2-producing *K. pneumoniae*. Given the epidemiological timeline and location, as well as, the genetic relatedness, transmission routes could represent direct transmissions. It was observed that the index case of the outbreak shared time and space with other patients in the ICU during almost the whole outbreak period and could be the source of polymyxin-resistant strain. There were no direct contacts between the first outbreak and the second period of isolation (four months among the last case of the first outbreak and the initial case of the second clonal spread) in spite of persuasive genetic relatedness, likely due to transmission through health care staff, colonization of another patient in the unit, or a contaminated environmental reservoir, which was not identified in this investigation.

As the increase of polymyxin-resistant *K. pneumoniae* was observed

and to prevent further spread, stringent infection control measures were introduced at ICU and neonatal ICU of hospital A. The outbreak was declared under control in November/2015 and extended infection control measures ended in December/2015. However, our data show that the infection control measures may have had a short-term effect, since gradually lost efficacy in the following months and the number of new acquisitions of MDR organisms dramatically rose in ICUs. Therefore, education about CRE, strict adherence to proper hand hygiene and compliance with contact precautions were resumed decreased drastically.

In the present study, ST11 was found to be the most common clone, similar to previous report, belonged to clonal complex 258 (CC258), the most important CC associated with KPC production (Pereira et al., 2013). ST11 and ST258 types are globally disseminated high-risk clones (Bowers et al., 2015; Dong et al., 2018; Guducuoglu et al., 2017). ST15 type was isolated only in Hospital B and had previously been identified sporadically in South America (Lee et al., 2016). In contrast, to our knowledge this is the first report of ST 345 in Brazil. In general, the epidemiological trend of polymyxin-resistance strains in hospital A can be divided into three stages. In the early stage of the outbreak only three ST345 strains were isolated restricted to ICU neonatal. Beginning in September/2015, ST11 strains began to be isolated in ICU adult remaining until December/2015. However, after four months, ST11 strains were recovered from ICU neonatal and pediatric. Based on this finding, we hypothesize that the polymyxin-resistance ST11 clone was a successful clone that established itself in our hospital.

Polymyxin-resistant *K. pneumoniae* strains frequently exhibit MDR

clinical characteristics of some patients from whom the polymyxin-resistant isolates were obtained, and these strains were excluded. Thus, the small number of isolates included may have impacted the significance of our findings. Additionally, our screening method to inclusion of polymyxin-resistance isolates was the semi-automatized system Vitek2, which could lead to false-susceptible results and underestimation of polymyxin-resistance rates. Besides the limitations, our data reveal the occurrence of clonal dissemination of polymyxin-resistant strains in our hospitals, which highlights the need to pay more attention to the emergence of these strains in similar settings.

5. Conclusions

In conclusion, we report a high rate of polymyxin resistance caused by different mechanisms affecting *mgrB* gene, including a novel protein alteration resulting in 26-amino-acid duplication which could lead to polymyxin resistance. Clonal spread of polymyxin-resistant isolates was late detected in the outbreak, highlighting the challenges of laboratory detection. The patients involved had complex and extended inpatient stays which made understanding the origin of polymyxin-resistant isolates challenging. This is cause for serious concern for public health, leading to high morbidity and mortality rates of hospitalized patients, as polymyxin is among the few remaining treatment options for infections by multidrug-resistant Gram-negative pathogens. Furthermore, infection control measures and use of stricter antimicrobial policies are required to control the spread of these organisms.

Ethical approval

This study was conducted with the approval of the Research Ethics Committee from the Universidade Federal da Grande Dourados (number 877.292/2014).

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reference

Holt et al. (2015)

Declaration of Competing Interest

None

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.ijmm.2020.151448>.

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