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CLINICAL MICROBIOLOGY - SHORT COMMUNICATION

High-risk clones of carbapenem resistant *Klebsiella pneumoniae* recovered from pediatric patients in Southern Brazil

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Abstract

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) exhibit high mortality rates in pediatric patients and usually belong to international high-risk clones. This study aimed to investigate the molecular epidemiology and carbapenem resistance mechanisms of *K. pneumoniae* isolates recovered from pediatric patients, and correlate them with phenotypical data. Twenty-five CRKP isolates were identified, and antimicrobial susceptibility was assessed using broth microdilution. Carbapenemase production and β-lactamase genes were detected by phenotypic and genotypic tests. Multilocus sequence typing was performed to differentiate the strains and whole-genome sequencing was assessed to characterize a new sequence type. Admission to the intensive care unit and the use of catheters were significantly positive correlates of CRKP infection, and the mortality rate was 36%. Almost all isolates showed multidrug-resistant phenotype, and most frequent resistant gene was bla_{KPC}. We observed the dissemination of ST307 and clones belonging to CG258, which are considered high risk. In pediatric patients, these clones present with high genomic plasticity, favoring adaptation of the KPC and NDM enzymes to healthcare environments.

Keywords *Klebsiella pneumoniae* · Pediatric · KPC · NDM · MLST · ST6407

Introduction

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) is the main cause of healthcare-associated infections (HAIs), including bacteremia, meningitis, pneumonia, and urinary tract infections, responsible for high mortality rates. *K. pneumoniae* is a ubiquitous bacteria present in the human gut microbiota and usually causes outbreaks associated with high-risk clones which are associated with the convergence of antimicrobial resistance and virulence genes [1–3]. CRKP is considered a pathogen of public health concern and included in the World Health Organization (WHO) priority pathogens' list; thus, it deserves attention regarding the development of new drugs [4].

Resistance to β-lactams has increased the use of carbapenems for the treatment of HAIs, and these antibiotics can exert selective pressure, favoring resistant strains. Resistance to carbapenems may be attributed to acquisition of carbapenemase genes by mobile genetic elements (MGEs); porin-mediated resistance to reduce uptake of carbapenems and efflux pumps, which pump the carbapenem outside the cells [5].

High-risk clones are usually associated with multidrug-resistant (MDR) phenotype that presents resistance to at least one drug in three classes of antibiotics [6]. For *K. pneumoniae,* these clones mainly belong to the internationally disseminated CG258, which is associated with clinical outbreaks and the occurrence of important carbapenemases enzymes (KPC, NDM, VIM, and OXA-48-like) [7, 8]. In Brazil, the endemic STs are ST11, ST258, and ST437 belonging to CG258 [2, 9].

Surveillance studies in pediatric patients are relevant for defining empirical treatments and evaluating the

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dissemination of MDR isolates to adopt infection control measures. In this context, this study aimed to evaluate the molecular epidemiology and carbapenem resistance mechanisms of CRKP recovered from pediatric patients and correlate them with phenotypical data.

Methods

Ethics

The Institutional Review Board approved this study (approval no. #2.096.359/2016). Investigations were performed by ensuring the anonymity of each patient, consent terms were waived.

Study design

This retrospective cross-sectional study using a quantitative experimental approach was conducted at a 372-bed pediatric tertiary care teaching hospital in southern Brazil.

Characterization of bacterial isolates, and antimicrobial resistance

We analyzed 25 non-redundant CRKP isolates (one per patient) obtained from sterile body fluid samples (blood, cerebrospinal fluid [CSF], bone fragment, peritoneal fluid, and pleural fluid) from pediatric patients (age, 0–16 years) collected between August 2016 and October 2021. All isolates were resistant to at least one carbapenem (ertapenem, imipenem, or meropenem) according to the clinical breakpoints of the current guidelines. All samples were stored in Brain Heart Infusion (BHI) broth with 20% glycerol (v/v) and frozen at -80 °C until further analysis.

Bacterial identification was performed by MALDI-TOF MS using a Microflex LT Biotyper 3.0 system (Bruker Daltonics, Bremen, Germany) and antimicrobial susceptibility testing using broth microdilution and interpreted as recommended by EUCAST/BRCAST [11, 12]. The modified carbapenem (mCIM) and EDTA-carbapenem (eCIM) inactivation methods were performed to phenotypically detect carbapenemase producers [13, 14].

Demographic and clinical data were collected from the medical records. For these analyses, the definition of mortality was based on 30-days after positive culture [10].

Antibiotic resistance characterization and molecular typing

Genomic DNA was extracted using the PureLink™ Genomic DNA Mini Kit (Invitrogen, Waltham, Massachusetts, USA) according to the manufacturer's recommendations. To identify β-lactamase ($bla_{\text{CTX-M-[M-1, M-2, M-8, and M-9 groups]}}$; bla_{TEM}, bla_{SHV}, bla_{PER}, and bla_{GES}) and carbapenemase genes (bla_{KPC}, $bla_{\text{OXA-48-like}}$, bla_{NDM}, bla_{IMP}, and bla_{VIM}), PCR was performed using primers and amplification conditions previously described [15, 16].

Multilocus sequence typing (MLST) was performed for seven housekeeping genes following the protocol 1 available on the Pasteur MLST website (https://bigsdb.pasteur.fr/klebsiella/) [17]. All the amplicons were sequenced using an ABI 3500 Genetic Analyzer (Applied Biosystems, Waltham, Massachusetts, USA). Nucleotide sequences were analyzed using BioEdit version 7.2. β-lactamase genes were compared with those available in GenBank using the BLAST nucleotide algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

A maximum likelihood tree based on the MLST alignment was constructed using IQ-TREE version 2 [18]. The phylogeny was tested against 1000 bootstrap replicates and the resulting tree was edited using iTOL version 5.6.1 [19]. *K. pneumoniae* strain FF1043 from GenBank was used as an out-group because it presented a better rooted result. Even though it belongs to the same species, it was distantly related to the isolates.

Genome sequencing and assembly

A new allele combination for Kpn10 was observed and thus, it was selected for genomic sequencing. DNA was quantified using NanoDrop One (Thermo Fisher Scientific Inc., Waltham, MA, USA). Illumina sequencing libraries with an average insert size of 600 bp fragments were generated using an Illumina Nextera XT DNA library kit (Illumina Inc., San Diego, CA, USA). Libraries were quantified and their quality was verified using a Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). Whole-genome sequencing of paired-end libraries (2 × 250 bp) was performed using the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA). Raw read quality was checked using FastQC version 0.11.9 [20] and quality-based trimming and filtering were performed using Trimmomatic version 0.39 [21]. SPAdes version 3.14.1 was used to produce a de novo assembly of genomic DNA [22]. The completeness and taxonomic classification were verified using the DFAST tool [23]. Resistome was predicted using the Comprehensive Antibiotic Resistance Database (CARD) version 3.2.7 and ResFinder database version 4.1 [24, 25].

Statistical analysis

Clinical data were evaluated via statistical analysis of frequencies using Pearson's chi-squared tests. Monte Carlo simulations were used to increase robustness, and p-values ≤ 0.05 were considered statistically significant [26–28]. All tests were carried out using R (version 4.2.2) [29].

Results

Clinical data

A total of 25 cases (60% male patients) of invasive infections caused by *K. pneumoniae* were identified during the study period; 15 patients (60%) were aged ≤ 1 year (Table 1). Herein, intensive care unit (ICU) admission ($p = 0.04687$) or catheter use ($p = 0.0000042$), known risk factors of infection, were observed in almost all patients. The main underlying disease was neurological in 40% (10/25) of the cases, and the mortality rate was 36% (9/25). Detailed results are presented in Table 1 and Fig. 1.

Antimicrobial resistance

All isolates (100%, 25/25) were resistant to ceftazidime, cefotaxime, and ertapenem. 96% (24/25), 92% (23/25), and 68% (17/25) of isolates were resistant to cefepime, imipenem, and meropenem, respectively (Table 1). Regarding therapeutic options, 36% of isolates were resistant to polymyxin B, and 32% to colistin. Almost all isolates 96% (24/25) showed MDR profile. The most frequent carbapenemase was bla_{KPC} (84%, 21/25), followed by bla_{NDM} (8%, 2/25). Two isolates were carbapenem-resistant, but no carbapenemase genes tested were detected by PCR (Fig. 1). All the amplified resistance genes were sequenced, however, the β-lactamase variants were not defined.

Molecular typing

MLST analysis revealed a polyclonal spread of nine different STs (ST11, ST111, ST6407, ST76, ST258, ST307, ST437, ST443, and ST133). A novel ST6407 (Kpn10) was identified. Several antimicrobial resistance genes were detected in this isolate ($bla_{CTX\text{-}M\text{-}8}$, $bla_{TEM\text{-}1A}$, $bla_{OXA\text{-}9}$, bla_{SHV}, *qnrE1*, *aadA1*, *aac(6')-Ib*, *aac(6')-Ib-cr*), and mutations in *oqxA*, *oqxB*, *ompK36* and *ompK37* were compared with CARD and ResFinder database. Seventeen isolates belonged to CG258 (ST11, ST258, and ST437); all these isolates had bla_{KPC}, and some carried other β-lactamases. Herein, ST11 was the most prevalent ST among the isolates (40%, 10/25). Kpn111 and Kpn112 are members of ST307, and both carried bla_{KPC}, and the first one also carrying $bla_{CTX\text{-}M\text{-}1}$. ST76 (Kpn11) carried bla_{KPC} and bla_{TEM}; ST133 (Kpn150) presented only bla_{KPC} and was resistant to polymyxin B. Kpn6 and Kpn13 isolates belonged to the same ST111 and carried bla_{NDM}. Detailed results are presented in Table 1 and Fig. 1.

Discussion

We investigated the molecular epidemiology and carbapenem resistance mechanisms of *K. pneumoniae* in pediatric patients using genetic and clinical data. Worldwide, there has been an increase in CRKP infections in pediatric patients over the years; however, studies on this population are still scarce. Herein, most infections occurred in male patients and most patients were aged ≤ 1 year, this group deserves more attention because they have an immature immune system, which makes them more susceptible to MDR infections [30]. This group of patients also has high mortality rates.

Some risk factors for infection in this population are like those for adults, especially underlying diseases and admission to the ICU. However, some risk factors for infection or colonization are specifically observed in pediatric patients, such as previous use of broad-spectrum antibiotics, invasive medical devices (e.g., mechanical ventilation, central line catheters), long hospital stays, and prior surgery [31]. Our patients presented at least one of these risk factors, namely principal ICU admission and the use of catheters. The mortality rate were similar to those observed in other pediatric studies [31, 32].

The high rates of resistance to β-lactams observed can be associated with the presence of one or more β-lactamase genes. The most commonly detected enzymes were KPC (96%), CTX-M-1 (32%), CTX-M-9 (12%), and NDM (8%). The carbapenem-resistance isolates with no carbapenemase genes can be explained by the presence of extended spectrum β-lactamases (ESBL) combined with other mechanisms, such as efflux pumps, porin loss or mutations (*ompK*) [5]. The isolate Kpn10, which was resistant to ertapenem and meropenem, did not amplify for any carbapenem resistance gene via PCR, but several mutations in *ompK36* and *ompK37* were identified from whole genome sequencing that could explain this resistance.

The main cause of carbapenem resistance in our study was the presence of bla_{KPC}, which is considered endemic in Brazil [33]. During the COVID-19 pandemic, in addition to the presence of KPC producers, an increase in the number of NDM-producing isolates was observed as well as carbapenemase co-producing isolates [33]. In our study, which took place during the pandemic, we did not observe a significant increase in NDM or carbapenemase-coproducing. Only two isolates (Kpn6 and Kpn13) were recovered in 2017, which both belonged to ST111 and were NDM producers.

A worrying number of isolates were defined as MDR, showing resistance to at least one drug in three classes of antibiotics (broad spectrum cephalosporins, carbapenems,

Table 1 Clinical data, antibiotic susceptibility, molecular typing, and phenotypic features of 25 K. *pneumoniae* isolates

Isolates	Clinical Data				Minimal Inhibitory Concentration (mg/L)[d]											MLST	Phenotypic test	
	Risk-factors[a]	Age[b]	Outcome	Clinic[c]	CTX	CAZ	CPM	ERT	IMI	MER	CIP	AMI	GEN	COL	POL		mCIM	eCIM
Kpn1	S	16 y	Recovery	Neurology	32	> 64	16	64	32	16	> 16	16	4	2	1	11	+	-
Kpn6	C, PN, S	5 m	Death	SICU	64	> 64	16	8	2	8	2	8	1	1	1	111	+	+
Kpn10	C	8 m	Death	GICU	> 128	64	> 128	128	4	16	> 16	64	32	1	2	6407	-	-
Kpn11	C	10 m	Death	MC42	> 128	> 64	> 128	> 128	> 128	128	> 16	32	16	> 32	> 32	76	+	+
Kpn13	C	11 m	Recovery	MC51	64	> 64	4	4	8	2	0.25	2	0.5	2	2	111	+	+
Kpn22	C, S	6 m	Recovery	Neurology	> 128	> 64	> 128	> 128	> 128	128	> 16	> 128	> 128	> 32	> 32	258	+	-
Kpn61	C, S	1 y	Recovery	GICU	32	> 64	32	> 128	64	> 128	> 16	16	> 128	1	1	11	+	+
Kpn64	C, MV, S	6 y	Recovery	GICU	32	32	32	> 128	128	> 128	> 16	32	128	1	1	11	+	+
Kpn89	C, MV, S	1 y	Recovery	Neurology	> 128	32	> 128	16	64	64	> 16	> 128	> 128	32	32	258	+	+
Kpn90	C	6 y	Death	HO	> 128	> 64	64	> 128	16	8	> 16	4	128	2	1	11	+	-
Kpn101	C, MV, S	9 y	Recovery	SICU	> 128	64	> 128	> 128	64	16	> 16	> 128	> 128	> 32	16	258	+	-
Kpn104	C, MV, NP, S	2 m	Recovery	SICU	> 128	64	> 128	> 128	64	8	> 16	> 128	> 128	> 32	32	258	+	+
Kpn108	C	10 y	Death	MC42	> 128	> 64	64	16	16	4	> 16	32	> 128	4	1	11	+	-
Kpn111	C	8 m	Recovery	HO	> 128	64	> 128	> 128	32	16	> 16	8	2	1	2	307	+	-
Kpn112	C, MV, S	5 m	Recovery	GICU	> 128	64	> 128	16	16	4	> 16	4	0.5	2	2	307	+	-
Kpn117	C, MV, S	10 y	Recovery	SICU	> 128	> 64	> 128	> 128	> 128	> 128	16	> 128	> 128	> 32	> 32	437	+	-
Kpn122	C, MV, PN, S	4 m	Recovery	NICU	> 128	64	128	64	64	128	16	> 128	> 128	> 32	> 32	437	+	-
Kpn123	C	14 y	Death	MC42	> 128	> 64	64	32	16	2	> 16	2	0.5	1	1	11	+	-
Kpn127	C, S	3 y	Recovery	MC42	> 128	> 64	128	32	16	8	> 16	8	128	1	1	11	+	-
Kpn130	C, MV, PN, S	4 m	Recovery	NICU	> 128	64	> 128	64	16	16	2	> 128	> 128	1	1	443	-	-
Kpn134	C, MV, S	1 y	Recovery	SICU	> 128	> 64	> 134	> 128	> 128	64	> 16	> 128	> 128	> 32	> 32	437	+	-
Kpn138	C	7 y	Death	HO	> 128	64	64	32	64	16	> 16	16	> 128	1	1	11	+	-
Kpn143	C, S	5 m	Death	NICU	64	16	128	128	32	16	> 16	8	> 128	1	2	11	+	-
Kpn147	C, MV	9 y	Recovery	MC52	> 128	> 64	> 128	128	32	16	> 16	4	64	1	1	11	+	-
Kpn150	C, PN, S	4 m	Death	ID	> 128	> 64	> 128	> 128	> 128	> 128	1	128	1	1	4	133	+	-

[a]Risk-factors abbreviations: *C* catheter, *MV* mechanical ventilation, *PN* parenteral nutrition, *S* surgery

[b]Age abbreviations: *y* years (s), *m* months

[c]Inpatient unit abbreviations: *SICU* surgical intensive care unit, *GICU* general intensive care unit, *MC42* medical clinic 42, *MC51* medical clinic 51, *MC52* medical clinic 52, *HO* hematology-oncology, *NICU* neonatal intensive care unit, *ID* infectious disease

[d]Antibiotic abbreviations: *CTX* cefotaxime, *CAZ* ceftazidime, *CPM* cefepime, *ERT* ertapenem, *IMI* imipenem, *MER* meropenem, *CIP* ciprofloxacin, *AMI* amikacin, *GEN* gentamicin, *COL* colistin, *POL* polymyxin B

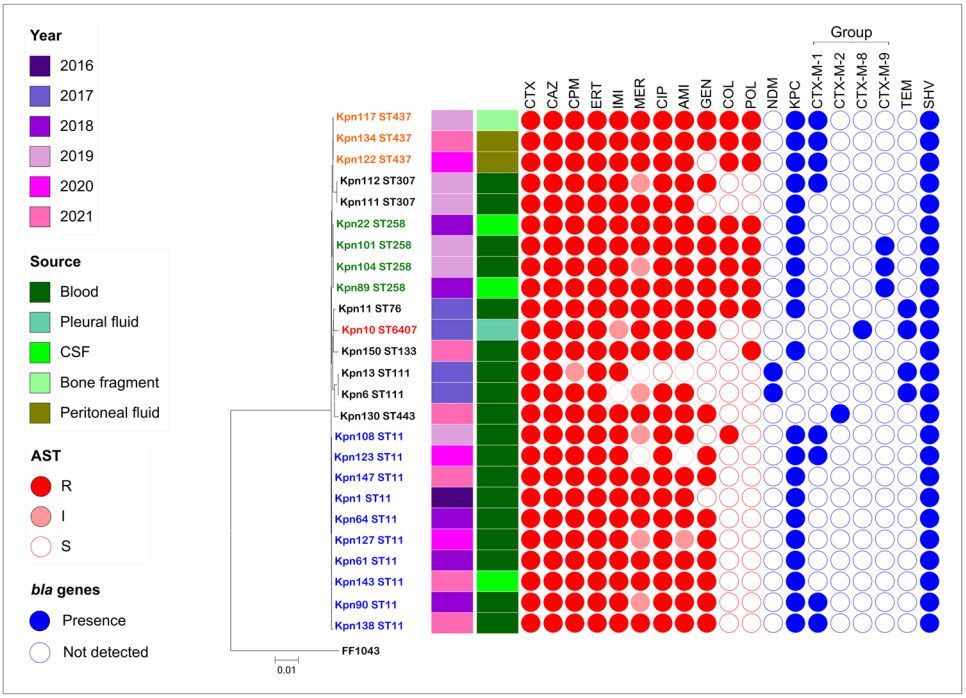

Fig. 1 Multilocus sequence typing (MLST) phylogenetic relationships with resistance phenotypic profile and genes of 25 *K. pneumoniae* isolated from pediatric patients. Legend: CSF, cerebrospinal fluid. R: Resistant; I: Susceptible, with increased exposure; S: Susceptible. ST6407 in bold type is the new ST. Antimicrobial susceptibility test (AST) for the following antimicrobials were performed: cefotaxime (CTX); ceftazidime (CAZ); cefepime (CPM); ertapenem (ERT); imipenem (IMI); meropenem (MER); ciprofloxacin (CIP); amikacin (AMI); gentamicin (GEN); colistin (COL); and polymyxin B (POL)

and aminoglycosides and/or fluoroquinolones) [6]. This may be associated with high mortality rates and dwindling therapeutic options as described in previous studies [31, 34]. In this scenario, polymyxins are one of the few therapeutic options for treating CRKP isolates. Another possible treatment option could be ceftazidime-avibactam, a novel β-lactam/β-lactamase inhibitor effective against strains harboring bla_{KPC}, however, it is not active against NDM producers and its clinical use is limited by its high cost [35].

MLST analysis revealed a polyclonal spread of nine different STs. Multiple clones may indicate that they evolved independently, possibly because of the selective pressure of antimicrobials. ST6407 (Kpn10) was considered a novel ST due to a new allele combination. Multiple antimicrobial resistance genes were observed in Kpn10, which may have contributed to the patient's death.

Seventeen isolates belonged to CG258 harbored bla_{KPC}, and some carried other β-lactamases. This CG is endemic in Brazil, and its members are considered international high-risk clones due to the presence of several antimicrobial resistance genes [2, 9, 36]. The most prevalent ST among our isolates was ST11, which was also reported in a pediatric study in China [30].

The isolates Kpn111 and Kpn112 belonged to ST307 and harbored $bla_{KPC,}$ and Kpn112 also carried the $bla_{CTX\text{-}M\text{-}1}$ gene. ST307 has already been observed across all continents from various samples (humans, animals, and environmental), and is an emergent high-risk clone that usually harbors $bla_{CTX\text{-}M\text{-}15}$. This ST is spreading rapidly and is considered an important carbapenemase carrier [37].

ST76, ST111, and ST133 were detected in our study, and have not been previously described in Brazil. The isolate Kpn11 belongs to ST76, which is prevalent in China, carries the β-lactamase genes bla_{KPC} and bla_{TEM} [38]. Kpn6 and Kpn13 isolates which were recovered from different patients and a clinical unit belonged to the same ST111. Additionally, these isolates carried bla_{NDM}, an important carbapenemase that is increasing in prevalence in Brazil, which has mainly been reported in ST11 and ST15 [33, 40]. Other studies in the USA and South India have identified ST111 in clinical and/or environmental samples [41].

Kpn150 belonged to ST133, which is considered a highly virulent clone in Europe and Japan [39]. This isolate possessed the KPC enzyme combined with other resistance mechanisms, including resistance to polymyxin B. This may have contributed to the rapid death of the patient.

In this context, the presence of different STs and the identification of new ST in the analyzed period did not provide evidence of horizontal transmission, even though some of them were found in the same unit. Although these clones have already been described in adults, studies on pediatric patients are scarce. This is an alarming report demonstrating the dissemination of high-risk clones, CG258 and ST307, in *K. pneumoniae*, which present high genomic plasticity, favoring adaptation to healthcare environments [42].

A limitation of our study is the lack of characterization of the resistance mechanisms of other antimicrobials. Further

studies are required to assess the infections by MDR in this population.

Conclusions

In summary, our study reports the presence of MDR *K. pneumoniae* in patients admitted to a large pediatric hospital in Brazil. Knowledge of circulating clones in pediatric patients showed that they are like those present in adult patients. Considering that there are few studies on CRKP infections in pediatric patients, our results may contribute to defining better empirical treatments and monitoring epidemiological data.

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Authors' contributions Conceptualization: L.S.R., F.A.M., L.M. D-C.; Methodology: D.K., L.S.R., A.C.S., É. M.S., T.M.V., R.N.S.; Formal analysis and investigation: D.K., L.S.R, A.C.S., D.M., R.C., M.C.R., F.A.M., D.C.; Funding acquisition: L.M.D-C. Writing—original draft preparation: D.K., D.C.; Writing—review and editing: D.K, L.S.R., D.M., D.C., L.M.D-C; Supervision: L.S.R., D.C., L.M.D-C.

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Data Availability The draft genome of Kpn10 was submitted to GenBank under the accession number JASEKY000000000.2.

Declarations

Competing interests The authors declare that they have no competing interests.

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