International Journal of Antimicrobial Agents 54 (2019) 579–586

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Colistin resistance emerges in pandrug-resistant *Klebsiella pneumoniae* epidemic clones in Rio de Janeiro, Brazil

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ARTICLE INFO

Article history:
Received 12 February 2019
Accepted 24 August 2019

Editor: Jean-Marc Rolain

Keywords:
Klebsiella pneumoniae
Colistin resistance
Pandrug resistance
Multi-drug resistance
Epidemic clones

ABSTRACT

Klebsiella pneumoniae is an important human pathogen, able to accumulate and disseminate a variety of antimicrobial resistance genes. Resistance to colistin, one of the last therapeutic options for multi-drug-resistant bacteria, has been reported increasingly. Colistin-resistant *K. pneumoniae* (ColRKp) emerged in two hospitals in Rio de Janeiro state, Brazil in 2016. The aim of this study was to investigate if these ColRKp isolates were clonally related when compared between hospitals, to identify the molecular mechanisms of colistin resistance, and to describe other antimicrobial resistance genes carried by isolates. Twenty-three isolates were successively recovered, and the whole-genome sequence was analysed for 10, each of a different pulsed-field gel electrophoresis (PFGE) type. Although some PFGE clusters were found, none of them included isolates from both hospitals. Half of the isolates were assigned to CC258, three to ST152 and two to ST15. One isolate was pandrug resistant, one was extensively drug resistant, and the others were multi-drug resistant. Colistin resistance was related to mutations in *mgrB, pmrB, phoQ* and *crrB*. Eleven new mutations were found in these genes, including two nucleotide deletions in *mgrB*. All isolates were carbapenem resistant, and seven were associated with carbapenemase carriage (bla_{KPC-2} in six isolates and $bla_{OXA-370}$ in one isolate). All isolates had a bla_{CTX-M}, and two had a 16S ribosomal RNA methyltransferase encoding gene (*armA* and *rmtB*). ColRKp were composed of epidemic clones, but cross-dissemination between hospitals was not detected. Colistin resistance emerged with several novel mutations amid highly resistant strains, further restricting the number of drugs available and leading to pandrug resistance.

1. Introduction

Klebsiella pneumoniae is among the most frequently reported pathogens causing infections worldwide. Genetic plasticity, high plasmid burden and a wide variability of G + C content are among the traits that enable *K. pneumoniae* to accumulate and disseminate antimicrobial resistance genes and to occupy various niches [1]. Resistance has a significant impact on clinical outcome, with high mortality related to the virulence of strains and lack of appropriate antimicrobial therapy [2]. Use of colistin has re-emerged as one of the last therapeutic options for multi-drug-resistant (MDR) *K. pneumoniae*; however, colistin-resistant *K. pneumoniae* (ColRKp) recovered from clinical isolates has been reported increasingly [3,4], with a frequency as high as 27% in Brazil [5].

Colistin resistance in *K. pneumoniae* is mediated by several mechanisms. It frequently involves the addition of 4-amino-deoxy-arabinose (L-Ara4N) and phosphoethanolamine (PEtN) to the lipid A moiety of lipopolysaccharide (LPS), which decreases the electrostatic interaction between colistin and LPS [4]. Colistin resistance emerges with mutations in genes encoding two-component regulatory systems such as PhoPQ and PmrAB [3]. These systems regulate the expression of *pmrC* and *pmrHFIJKLM* operon responsible for the addition of PEtN and L-Ara4N to lipid A [6]. Additional mutations involved in colistin resistance are found in *mgrB* gene, a negative

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https://doi.org/10.1016/j.ijantimicag.2019.08.017
0924-8579/

regulator of the PhoPQ regulatory system, and *crrB* gene, a regulator of *pmrC* and *pmrHFIJKLM* operon [3,6].

Colistin resistance mechanisms have been rarely described in consecutive clinical isolates, providing a sense of frequency of mutations [7]. Starting in November 2016, ColRKp emerged in two hospitals in Rio de Janeiro state, Brazil. To understand the nature of the emerging pathogen, the aim of this study was to investigate if ColRKp isolates were clonally related when compared between hospitals, as well as to describe the molecular mechanisms of colistin resistance and other antimicrobial genes carried by isolates.

2. Materials and methods

2.1. Study setting, bacterial isolates and antimicrobial susceptibility tests

Twenty-three *K. pneumoniae* isolates (10 from Hospital A and 13 from Hospital B) were recovered consecutively from rectal and oropharynx screening swabs, urine and blood specimens of 16 patients between November 2016 and June 2017. Ten of the 16 patients had prior exposure to polymyxin B (30,000 UI/kg/day) for a median of 12 days (range 4–32 days), as seen in Table 2. The hospital laboratories reported that all 23 isolates had polymyxin minimum inhibitory concentration (MIC) ≥4 μg/mL by E-test. Isolates were named as follows: Cr (colistin resistant); A or B (name of the hospital); arabic numeral referring to each patient; and roman numeral in italic to distinguish different isolates when more than one were obtained from the same patient. Hospital A is a 182-bed university hospital and Hospital B is a 188-bed referral hospital for cancer treatment, both with 17 beds in the medical-surgical intensive care units and several clinical and surgical wards.

Bacterial identification was assessed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Biotyper 3.1, Bruker Daltonics, Bastrop, TX, USA). Colistin resistance was assessed by MIC by broth microdilution in triplicate (Sigma, St Louis, MO, USA) [8]. Susceptibility to meropenem and tigecycline was determined by broth microdilution method (Sigma), and susceptibility to amikacin, amoxicillin-clavulanate, aztreonam, cefepime, cefotaxime, cefoxitin, ceftazidime, chloramphenicol, ciprofloxacin, ertapenem, fosfomycin, gentamicin, meropenem, tetracycline and trimethoprim-sulfamethoxazole was determined by disk diffusion (Cefar, São Paulo, Brazil) [8]. Susceptibility test results were interpreted according to the Clinical and Laboratory Standards Institute [8] for all antimicrobial agents with the exception of tigecycline, interpreted according to the criteria of the European Committee on Antimicrobial Susceptibility Testing [9]. Intermediate results were considered as resistant.

2.2. Strain typing

Isolates were typed by pulsed-field gel electrophoresis (PFGE) [10] and band profiles were analysed with BioNumerics v. 7.6 (Applied Maths, Sint-Martens-Latem, Belgium). The similarity matrix was calculated by Dice coefficient, and a dendrogram was generated using the unweighted pair-group method with arithmetic averages with 1.0% tolerance. Types were defined with ≥90% similarity.

2.3. Bacterial whole-genome sequencing, and genome assembly and annotation

Genomic DNA was extracted from overnight cultures of selected isolates using QIAGEN DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol, and sent for whole-genome sequencing (WGS) at MicrobesNG (Birmingham, UK). Briefly, DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, Los Angeles, CA, USA) following the manufacturer's instructions. DNA quantification and library preparation were performed on a Hamilton Microlab STAR automated liquid handling system. Pooled libraries were quantified using Kapa Biosystems Library Quantification Kit for Illumina on a Roche light cycler 96 qPCR machine. Libraries were sequenced on the Illumina HiSeq using a 250bp paired-end protocol. Adapters were removed and reads trimmed using Timmomatic 0.30 with a sliding window cut-off of Q15 [11].

Reads were de-novo assembled using SPADES in PATRIC genome assembly, and annotated with PATRIC tools (www.patricbrc.org). Assemblies were mapped with reference genome *K. pneumoniae* subsp. *pneumoniae* HS11286 (accession number NC_016845.1) on Geneious R10 (Biomatters Ltd, Auckland, New Zealand).

2.4. General genomic features, acquired resistance genes, plasmid identification and CRISPR detection

General genomic features were defined by PATRIC automatic annotation tools. Sequence types (STs) were determined with MLST 2.0 (https://cge.cbs.dtu.dk/services/MLST/). Acquired resistance genes were determined by ResFinder 3.0 (https://cge.cbs.dtu.dk/services/ResFinder/), and plasmids were identified by PlasmidFinder 2.0 (https://cge.cbs.dtu.dk/services/PlasmidFinder/) with a minimum threshold of 90% identity, both hosted by the Center for Genomic Epidemiology. CRISPR presence was also detected with CRISPRCasFinder (https://crisprcas.i2bc.paris-saclay.fr/CrisprCasFinder/Index).

2.5. Mutations in genes related to colistin resistance

Mutations in *mgrB, pmrA, pmrB, phoP, phoQ* and *crrB* were inspected by alignment with reference genome *K. pneumoniae* subsp. *pneumoniae* MGH78578 (# NC_009648.1). PROVEAN tool (Protein Variation Effect Analyzer) v.1.1.5 (http://provean.jcvi.org/index.php) was applied to predict the effect of amino acid substitutions on protein function [12]. PROVEAN score ≤-2.5 was deleterious for protein function, and a score >-2.5 was taken as a neutral effect on protein function.

2.6. Comparative genomic and phylogenetic analysis

PubMed was searched for all publicly available *K. pneumoniae* genomes of Brazilian isolates using the following keywords: '*Klebsiella pneumoniae*', 'whole-genome' and 'Brazil'. All of these genomes and the reference genome *K. pneumoniae* subsp. *pneumoniae* HS11286 were downloaded. The *K. quasipneumoniae* subsp. *similipneumoniae* strain KPC142 was used as an outgroup reference. The downloaded genomes are listed in Table 1.

All *K. pneumoniae* genomes, including those from this study, were annotated with Prokka v.1.13 [13]. Genomes were aligned and the core genome was inferred with Roary v.3.11.2 [14]. Single nucleotide polymorphisms (SNPs) were detected and extracted with SNP-sites v.2.4.1 [15]. A maximum-likehood phylogenetic tree was inferred by PhyML v.3.1 [16] using the GTR evolutionary model with 100 bootstraps. The phylogenetic tree was visualized in MEGAx [17].

2.7. GenBank accession numbers

The *K. pneumoniae* genomes were deposited in GenBank under accession numbers SAMN10904698 (CrA1i), SAMN10904699 (CrB8i), SAMN10904700 (CrB9i), SAMN10904701 (CrA11ii), SAMN10904702 (CrA10i), SAMN10904703 (CrA11iii), SAMN10904704 (CrB6i), SAMN10904705 (CrB13i), SAMN10904706 (CrB15i) and SAMN10904707 (CrB5ii).

Table 1
Genomes downloaded for phylogenetic analysis.

Strain	Accession number
K. pneumoniae subsp. *pneumoniae* HS11286	NC_016845.1
K. pneumoniae strain B03[a]	NTHT01000000
K. pneumoniae strain B04[a]	NTGK01000000
K. pneumoniae strain KPC05[a]	NTGJ01000000
K. pneumoniae strain B11[a]	NTHU02000000
K. pneumoniae strain B16[a]	NTCW01000000
K. pneumoniae strain B17[a]	NTHV01000000
K. pneumoniae strain B29[a]	NTHW02000000
K. pneumoniae strain B30[a]	NTHX01000000
K. pneumoniae strain B35[a]	NTHY02000000
K. pneumoniae strain KPC40[a]	NIRG01000000
K. pneumoniae strain SJRP/Kp10[b]	MUDD01000000
K. pneumoniae strain KPHU468[c]	NBYZ01000000
K. pneumoniae strain CCBH6984[d]	MCNT01000000
K. pneumoniae strain 606B[e]	LYMZ01000000
K. pneumoniae subsp. *pneumoniae* Kp13[f]	CP003999
K. quasipneumoniae strain KPC142[g]	CP023478

K. pneumoniae, Klebsiella pneumoniae.

[a] Araújo BF, Ferreira ML, Campos PA, Royer S, Gonçalves IR, da Fonseca Batistão DW, et al. Hypervirulence and biofilm production in KPC-2-producing Klebsiella pneumoniae CG258 isolated in Brazil. J Med Microbiol 2018;67:523-528.

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[g] Nicolás MF, Ramos PIP, Marques de Carvalho F, Camargo DRA, de Fátima Morais Alves C, Loss de Morais G, et al. Comparative genomic analysis of a clinical isolate of Klebsiella quasipneumoniae subsp. similipneumoniae, a KPC-2 and OKP-B-6 beta-lactamases producer harboring two drug-resistance plasmids from Southeast Brazil. Front. Microbiol 2018;9:220.

3. Results

3.1. General characteristics of bacteria

The 23 isolates, all confirmed as ColRKp, with colistin MIC ranging from 4 to ≥256 μg/mL (mode 32 μg/mL), are shown in Table 2. PFGE defined 10 types, each with one to four isolates. One isolate from each of the 10 PFGE types was selected for WGS.

STs and antimicrobial resistance profiles are shown in Table 3. Five of the isolates belonged to CC258 (three ST258 and two ST437), three belonged to ST152, and two belonged to ST15. One isolate, belonging to ST437, was resistant to all drugs tested; one isolate, of ST15, was resistant to all but one drug tested, and all other isolates were resistant to several drugs. Tigecycline MIC ranged from ≤0.5 to 4 μg/mL (mode 1 μg/mL).

3.2. General genomic features

A summary of genomic features of the 10 sequenced *K. pneumoniae* genomes is presented in Table 4. Whole-genome sizes ranged from 5,450,540 to 6,151,245 bp with all isolates carrying various numbers of plasmids (three to eight). All isolates showed a similar G+C content (56.58–57.41%). CRISPR arrays were only found in the two ST15 isolates, each with two CRISPR arrays.

3.3. Antimicrobial resistance gene content

A wide variety of antimicrobial resistance genes was found in the ColRKp genomes, presented in Table 5. All isolates had $bla_{CTX\text{-}M}$ extended-spectrum beta-lactamase (ESBL) encoding genes, seven with a $bla_{CTX\text{-}M\text{-}15}$ type. Seven isolates had carbapenemase genes, six of which were $bla_{KPC\text{-}2}$ and one was $bla_{OXA\text{-}370}$. All isolates had *fosA* and *sul1* genes, which confer resistance to fosfomycin and sulphonamides, respectively. All isolates, but one, had *aac(6')-Ib-cr*, which confers resistance to both aminoglycoside and fluoroquinolones. All five CC258 isolates carried the $bla_{SHV\text{-}182}$ gene, which may be an additional ESBL. In addition to antimicrobial resistance encoding genes, two multi-drug-efflux systems were found in all isolates: MexAB-OprM and AcrAB-TolC.

3.4. Mutations in genes related to colistin resistance

Mutations in *mgrB* were found in seven isolates, four of these by means of IS insertions. The other three isolates, of ST152, had deletion of nt17 and nt18. Two isolates assigned to ST258 presented novel alterations in the PhoQ encoding gene: the deletion of amino acids L26 and V27, and deleterious amino acid substitutions T84K and D90E. All isolates had wild-type PhoP and PmrA. None of the isolates had any *mcr* plasmid-mediated colistin resistance gene. Mutations in genes related to colistin resistance are summarized in Table 6.

3.5. Plasmids

Several plasmids were identified in *K. pneumoniae* genomes, listed in Table 7. IncF plasmids were detected in all isolates. IncN plasmids were present in all isolates assigned to CC258, with one exception. Col-like plasmids were detected in all isolates except for one assigned to ST258. IncA/C2 plasmids were detected in all ST258 isolates.

3.6. Phylogenetic analysis

Multiple alignments of the core genome of 26 *K. pneumoniae* strains in this study and others from Brazil (excluding the outgroup strain) showed that CC258 formed a large cluster with 19 (73%) isolates (Fig. 1). Only two study isolates were clustered with other genomes from Brazil (both ST437). The other eight isolates formed three clusters, each including exclusive isolates from the present study, of ST15, ST152 and ST258.

4. Discussion

ColRKp emerged in two hospitals in neighbouring cities in Rio de Janeiro state within 20 days of each other. It was not possible to demonstrate a correlation between exposure to polymyxin before isolation of ColRKp and level of resistance. Moreover, few isolates were obtained from the same patient, so it was not possible to determine if resistance could evolve in an individual patient. Although Patient 11 had two clinical isolates (one of pulsotype 6 and the other of pulsotype 5) preceded by a surveillance culture (pulsotype 5), the pair of isolates from surveillance and infection of the same pulsotype (5) had the same MIC (4 μg/mL).

Isolates belonging to the same ST (ST258 and ST15), but with different PFGE types, were detected in the two hospitals. These institutions are located 21 km apart, and if episodes of ColRKp

Table 2
Colistin-resistant *Klebsiella pneumoniae* study isolates.

Isolate	Date of isolation	Clinical specimen	Days of polymyxin use before isolate detection	Colistin MIC (μg/mL)	PFGE type
CrA1*i*	12/11/2016	Blood	13	32	7
CrA2*i*	21/11/2016	Rectal swab	11[a]	≥256	7
CrA2*ii*	21/11/2016	Rectal swab	11[a]	≥256	7
CrA2*iii*	25/11/2016	Blood	11[a]	≥256	9
CrA3*i*	21/11/2016	Blood	27	128	7
CrA4*i*	22/11/2016	Rectal swab	0	64	7
CrB5*i*	14/12/2016	Blood	10[b]	128	2
CrB5*ii*	03/06/2017	Blood	32[b]	128	1
CrB5*iii*	03/06/2017	Urine	32[b]	128	1
CrB6*i*	14/12/2016	Urine	14	32	2
CrB7*i*	18/12/2016	Blood	4[c]	64	10
CrB7*ii*	28/12/2016	Blood	4[c]	32	2
CrB8*i*	20/12/2016	Urine	22	128	3
CrB9*i*	04/01/2017	Blood	19	64	8
CrA10*i*	11/01/2017	Urine	0	≥256	9
CrA11i	07/02/2017	Oropharynx swab	0	4	5
CrA11*ii*	13/02/2017	Urine	0	32	6
CrA11*iii*	15/02/2017	Blood	0	4	5
CrB12*i*	02/03/2017	Urine	11	≥256	2
CrB13*i*	14/03/2017	Blood	15	64	4
CrB14*i*	30/03/2017	Blood	0	64	1
CrB15*i*	13/04/2017	Urine	0	32	10
CrB16*i*	13/04/2017	Urine	0	8	10

MIC, minimum inhibitory concentration; PFGE, pulsed-field gel electrophoresis.
[a,b,c]Isolates belonged to the same patient.
Isolates selected for whole-genome sequencing are marked in bold.

Table 3
Sequence types, antimicrobial minimum inhibitory concentrations (MIC) and resistance profiles of *Klebsiella pneumoniae* isolates.

Isolate	ST	CC	MIC (μg/mL)		Antimicrobial resistance profile
			MEM	TGC	MIC (μg/mL)
CrA1*i*	258	258	64	1	AMC, CPM, CTX, FOX, CAZ ATM, ETP, AMI, CIP, SXT, CHL
CrB8*i*	258	258	128	2	AMC, CPM, CTX, FOX, CAZ, ATM, ETP, GEN, CIP, SXT, CHL
CrB9*i*	258	258	≥256	2	AMC, CPM, CTX, FOX, CAZ, ATM, ETP, CIP, SXT, CHL
CrA11*ii*	437	258	≥256	4	AMC, CPM, CTX, FOX, CAZ, ATM, ETP, GEN, AMI, TET, CIP, SXT, CHL, FOS
CrA10*i*	437	258	64	1	AMC, CPM, CTX, FOX, CAZ, ATM, ETP, GEN, AMI, TET, CIP, SXT, CHL
CrA11*iii*	15	SN	32	4	AMC, CPM, CTX, FOX, CAZ, ATM, ETP, TET, CIP, SXT, CHL
CrB6*i*	15	SN	64	1	AMC, CPM, CTX, FOX, CAZ, ATM, ETP, CIP, SXT
CrB13*i*	152	SN	32	≤0.5	AMC, CPM, CTX, FOX, CAZ, ATM, ETP, GEN, AMI, CIP, SXT, CHL
CrB15*i*	152	SN	4	1	AMC, CPM, CTX, FOX, CAZ, ATM, ETP, GEN, AMI, CIP, SXT, CHL
CrB5*ii*	152	SN	32	≤0.5	AMC, CPM, CTX, CAZ, ATM, GEN, AMI, CIP, SXT, CHL

ST, sequence type; CC, clonal complex; SN, singleton; TGC, tigecycline; AMC, amoxicillin-clavulanate; CPM, cefepime; CTX, cefotaxime; FOX, cefoxitin; CAZ, ceftazidime; ATM, aztreonam; ETP, ertapenem; MEM, meropenem; GEN, gentamicin; AMI, amikacin; TET, tetracycline; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; CHL, chloramphenicol; FOS, Fosfomycin.

Table 4
General features of colistin-resistant *Klebsiella pneumoniae* genomes.

Isolate	Genome features										
	Genome length (bp)	Contigs	No. CDS	G+C content (%)	Proteins with functional assignment	Hypothetical proteins	rRNA	tRNA	Crispr array	CRISPR repeats	CRISPR spacers
CrA1*i*	5,682,444	509	5634	57.12	4772	862	25	88	0	0	0
CrB8*i*	6,151,245	276	6397	56.58	5205	1,192	12	82	0	0	0
CrB9*i*	5,776,334	110	5844	57.05	4896	948	12	80	0	0	0
CrA11*ii*	5,595,697	117	5579	57.18	4759	820	12	81	0	0	0
CrA10*i*	5,513,361	151	5508	57.01	4674	834	12	80	0	0	0
CrA11*iii*	5,707,759	147	5690	57.01	4865	825	27	86	2	22	20
CrB6*i*	5,649,448	124	5635	56.98	4842	793	10	83	2	22	20
CrB13*i*	5,502,620	116	5426	57.40	4727	699	6	78	0	0	0
CrB15*i*	5,483,391	154	5464	57.40	4760	704	4	78	0	0	0
CrB5*ii*	5,450,540	149	5452	57.41	4764	688	13	83	0	0	0

Table 5
Antimicrobial resistance encoding genes present in the genome of *Klebsiella pneumoniae* isolates.

Isolate	Resistance genes in antimicrobial group									
	Aminoglycoside	Beta-lactam	Fluoroquinolone	FOS	MLS	PHEN	RIF	SULPHO	TET	TMP
CrA1*i*	*aac(3')-Ia, aac(3')-IId, aph(6')-Id, aac(6')-Ib3, aph(3')-IIb, aph(3')-Ib, aac(6')-II, rmtB, aadA2*	$bla_{OXA-370}$, bla_{TEM-1B}, $bla_{CTX-M15}$, $bla_{CTX-M-14}$, $bla_{SHV-182}$, bla_{PAO}, $bla_{OXA-395,}$ bla_{KPC-2}	*aac(6')-Ib-cr, oqxA, oqxB, crpP*	*fosA*	*mph(A), erm(42)*	*catB7, catB3, catA1*	ARR-4	*sul1, sul2*	*tet(G)*	*dfrA12*
CrB8*i*	*aac(6')-Ib-cr, aac(3')-IId, aph(3')-Ia, aadA2, aph(3')-Ib, aph(6')-Id*	bla_{TEM-1B}, $bla_{SHV-182}$, bla_{KPC-2}, $bla_{CTX-M-14}$, bla_{OXA-1}	*oqxA, oqxB, aac(6')-Ib-cr*	*fosA*	*mph(A), erm(42)*	*catA1, catB3*	-	*sul1, sul2*	*tet(A), tet(D)*	*dfrA12*
CrB9*i*	*aph(3')-Ia, aadA2, aph(3')-Ib, aph(6')-Id*	$bla_{CTX-M-14}$, $bla_{SHV-182}$, bla_{KPC-2}, bla_{TEM-1B}	*oqxA, oqxB*	*fosA*	*mph(A), erm(42)*	*catA1*	-	*sul1, sul2*	-	*dfrA12*
CrA11*ii*	*aac(3')-IId, aac(6')-Ib-cr, aph(3')-Ia*	bla_{TEM-1B}, bla_{KPC-2}, $bla_{SHV-182}$, $bla_{CTX-M-15}$, bla_{OXA-1}	*aac(6')-Ib-cr, oqxA, oqxB*	*fosA*	*mph(A)*	*catB3*	-	*sul1*	*tet(A), tet(D)*	*dfrA30*
CrA10*i*	*aac(6')-Ib-cr, armA*	bla_{OXA-1}, $bla_{SHV-182}$, bla_{TEM-1A}, $bla_{CTX-M-9}$	*aac(6')-Ib-cr, qnrA1, oqxA, oqxB*	*fosA*	*msr(E), mph(E)*	*catA1, catB3*	ARR-3	*sul1*	-	*dfrA30*
CrA11*iii*	*aph(3')-Ib, aac(6')-Ib-cr, aph(6')-Id, aph(3')-Ia, aadA2*	bla_{TEM-1A}, bla_{KPC-2}, bla_{SHV-28}, $bla_{CTX-M-15}$, bla_{OXA1}	*oqxA, oqxB, aac(6')-Ib-cr*	*fosA*	*mph(A), erm(B)*	*catB3*	-	*sul1*	*tet(A)*	*dfrA12*
CrB6*i*	*aph(3')-Ia, aadA2, aph(3')-Ib, aph(6')-Id*	bla_{SHV-28}, bla_{TEM-1A}, bla_{OXA-1}, $bla_{CTX-M-15}$, bla_{KPC-2}	*oqxA, oqxB, aac(6')-Ib-cr*	*fosA*	*mph(A), erm(B)*	*catB3*	-	*sul1*	-	*dfrA12*
CrB13*i*	*aadA16, aac(3')-IIa, aph(3')-Ib, aph(6')-Id, aac(6')-Ib-cr*	$bla_{CTX-M-15}$, bla_{KPC-2}, $bla_{SHV-187}$, bla_{OXA-1}, bla_{TEM-1B}	*aac(6')-Ib-cr, qnrB6*	*fosA*	*mph(A)*	*catA1, catB3*	ARR-3	*sul1, sul2*	-	*dfrA27*
CrB15*i*	*aac(6')-Ib-cr, aadA16, aac(3')-IIa, aph(3')-Ib, aph(6')-Id*	$bla_{SHV-187}$, bla_{TEM-1B}, $bla_{CTX-M-15}$, bla_{OXA-1}	*aac(6')-Ib-cr, qnrB6, oqxA, oqxB*	*fosA*	*mph(A)*	*catA1, catB3*	ARR-3	*sul1, sul2*	-	*dfrA27*
CrB5*ii*	*aadA16, aac(6')-Ib-cr, aac(3')-IIa, aph(3')-Ib, aph(6')-Id*	$bla_{CTX-M-15}$, $bla_{SHV-187}$, bla_{TEM-1B}, bla_{OXA-1}	*aac(6')-Ib-cr, qnrB6*	*fosA*	*mph(A)*	*catA1, catB3*	ARR-3	*sul1, sul2*	-	*dfrA27*

FOS, fosfomycin; MLS, macrolide, lincosamide, streptogramin B; PHEN, phenicol; RIF, rifampicin; SULPHO, sulphonamide; TET, tetracycline; TMP, trimethoprim.

Table 6
Mutations in genes related to colistin resistance in *Klebsiella pneumoniae* isolates.

Isolate	Gene/protein			
	mgrB/MgrB	*pmrB*/PmrB	*phoQ*/PhoQ	*crrB*/CrrB
CrA1*i*	None	R256G[a]T246A[b]	None	C68S[b,c]S195N[b,c]Q296L[b,c]
CrB8*i*	None	R256G[a]T246A[b]	L26del[a,c] V27del[a,c]D90E[a,c]T84K[b,c]	C68S[b,c]Q296L[b,c]
CrB9*i*	None	R256G[a]T246A[b]	L26del[a,c]V27del[a,c]D90E[a,c]T84K[b,c]	C68S[b,c]Q296L[b,c]
CrA11*ii*	Insertional inactivation, IS*Kpn*25 element	R256G[a]T246A[b]	None	ND
CrA10*i*	Insertional inactivation, IS903 element	R256G[a]T246A[b]	None	ND
CrA11*iii*	Insertional inactivation, IS5-like element	None	None	ND
CrB6*i*	Insertional inactivation, IS5-like element	P95L[a,c]	L37P[a,c]	ND
CrB13*i*	deletion of nt17 and 18[c,d]	T246A[b]	H410Y[b,c]	ND
CrB15*i*	deletion of nt17 and 18[c,d]	T246A[b]	H410Y[b,c]	ND
CrB5*ii*	deletion of nt17 and 18[c,d]	T246A[b]	H410Y[b, c]	ND

ND, gene not detected; nt, nucleotides.
[a] Deleterious mutation.
[b] Neutral mutation.
[c] Present work.
[d] Deletion of nucleotides resulting in a truncated protein.

Table 7
Plasmid incompatibility groups found in *Klebsiella pneumoniae* isolates.

Isolate	Plasmid incompatibility group
CrA1*i*	IncA/C2, IncFIB(K), IncFII(K), IncX5, IncN
CrB8*i*	IncA/C2, IncFIB(K), IncFIB (pQil), IncFII(K), IncN, IncFIB(pKPHS1), Col440I, Col440II
CrB9*i*	IncA/C2, IncFIB(K), IncFII(K), IncN
CrA11*ii*	IncFIB(K), IncFIB (pQil), IncFII(K), IncN, IncFIB(pKPHS1), Col440I, Col440II
CrA10*i*	IncFIB (pQil), IncHI1B, IncFIB(Mar), ColpVC, Col440II
CrA11*iii*	ColRNAI, IncFIB(pKPHS1), IncFIB(K), IncFII(K), Col(MGD2)
CrB6*i*	ColRNAI, IncFIB(K), IncFII(K), Col(MGD2)
CrB13*i*	IncFIB(K), IncFII(K), ColRNAI
CrB15*i*	IncFIB(K), IncFII(K), ColRNAI, Col440I
CrB5*ii*	IncFIB(K), IncFII(K), ColRNAI

emergency are related, bacterial strains may be carried by health-care workers who work in both places or patients who may have been admitted to both hospitals. As PFGE is more discriminative than multi-locus sequence typing, and ST258 and ST15 have been detected previously in Brazil [18–20], both possibilities must be considered: ColRKp emerged independently in each hospital, or a strain was transmitted from one hospital to the other, but isolates with indistinguishable PFGE band profiles obtained from both hospitals were missing in the study collection. Unfortunately, it is not possible to make any assumptions. Another reason for possibly having missed ColRKp in the hospital was that the E-test method was used to detect isolates. The clinical laboratory at the hospital was unable to perform broth microdilution, which is the method of choice to detect colistin resistance.

Genomic features of isolates are consistent with data described previously for other sequenced *K. pneumoniae* genomes [1]. Half of the isolates were assigned to CC258, the predominant clone in Brazil and many parts of the world. Three isolates were ST152, already detected elsewhere in the world, and two isolates were ST15, a virulent and epidemic clone [21] detected in both hospitals. This indicates a scenario filled with high-risk clones. To the authors' knowledge, this is the first paper to describe genomes of ST258, ST15 and ST152 isolates from Brazil.

As expected for colistin-resistant isolates, all were also resistant to other clinically useful antimicrobial agents. One isolate was pandrug resistant, one was extensively drug resistant, and the other eight were MDR [22]. Meropenem was not a choice for monotherapy as the isolates' MICs (with one exception) were all

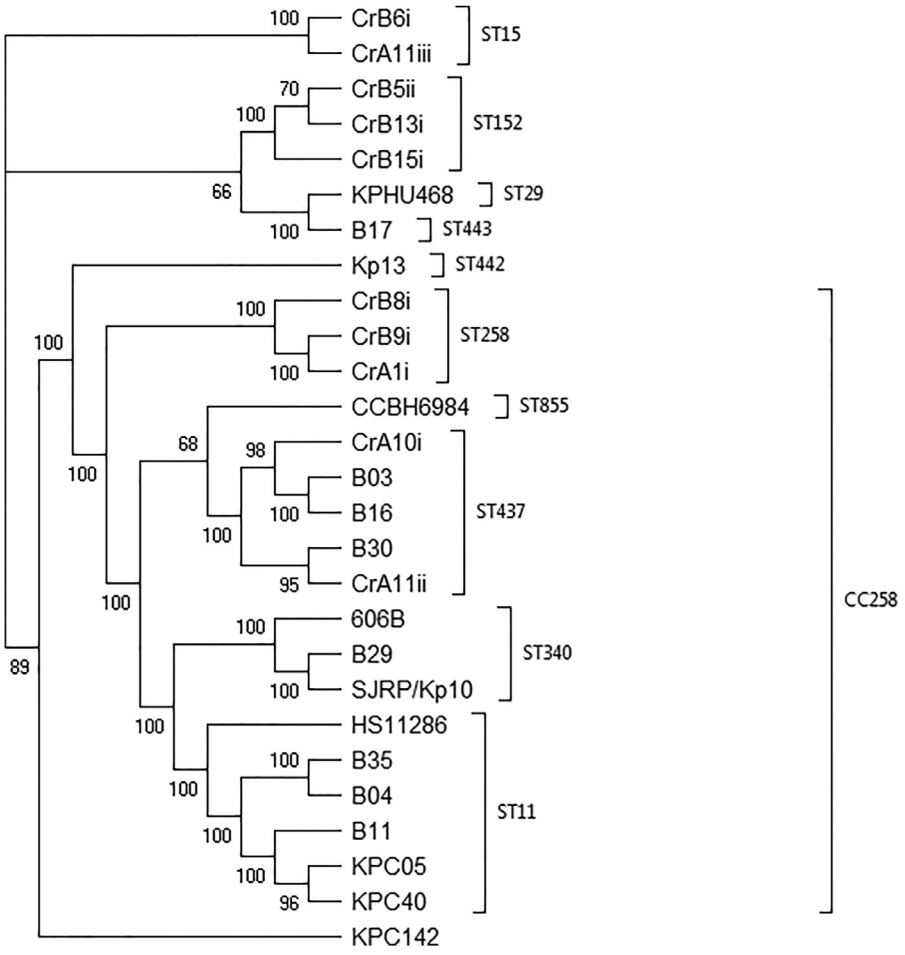

Fig. 1. Phylogenetic tree (maximum likehood) of the sequenced *Klebsiella pneumoniae* isolates, the reference strain HS11286 (NC_016845.1), and the publicly available *K. pneumoniae* genomes from Brazilian isolates: B03 (NTHT01000000), B04 (NTGK01000000), KPC05 (NTGJ01000000), B11 (NTHU02000000), B16 (NTCW01000000), B17 (NTHV01000000), B30 (NTHX01000000), B35 (NTHY02000000), KPC40 (NIRG01000000), SJRP/Kp10 (MUDD01000000), KPHU468 (NBYZ01000000), CCBH6984 (MCNT01000000), 606B (LYMZ01000000), Kp13 (CP003999), and the *K. quasipneumoniae* subsp. *similipneumoniae* strain KPC142 (CP023478).

≥32 μg/mL. To address clinical infections, patient-specific characteristics were taken into consideration. Many ColRKp remained susceptible to a few antimicrobial agents such as fosfomycin, amikacin and tigecycline. For urinary tract infections (usually less severe events), patients were treated with fosfomycin or amikacin as monotherapy; however, for bloodstream infections, combination therapy of carbapenem plus amikacin, carbapenem plus tigecycline, or carbapenem plus tigecycline plus amikacin was preferred; finally, one patient was treated with dual carbapenem therapy of meropenem plus ertapenem.

The study isolates presented a diversity of mutations in colistin resistance genes. Seven isolates had new mutations, illustrating the dependence on whole-genome analysis to elucidate the molecular mechanisms of such resistance. Seven isolates had mutations in *mgrB*, the most frequently reported molecular mechanism of colistin resistance [6,23,24]. Some of these isolates had additional mutations in *pmrB* and *phoQ*. The other three isolates had mutations in *pmrB, phoQ* and *crrB* alone. Among the isolates with mutations in *mgrB*, two assigned to ST15 had a truncated gene due to the insertion of IS5-like elements between nt74 and nt75 [6]. Another ST15 isolate had the novel deleterious mutations P95L in PmrB, and L37P in PhoQ.

All isolates assigned to CC258 had amino acid substitutions R256G and T246A, described previously in other Brazilian isolates [7]. Additional findings in ST437 were insertion disruptions in *mgrB* with IS*Kpn*25 between nt141 and nt142 in one isolate, and IS903 at nt75 in another isolate, as described in other isolates from Brazil [25]. Concerning ST258 isolates, all presented C68S and Q296L substitutions in CrrB, and two isolates had three novel deleterious mutations in PhoQ: ΔL26 and ΔV27, and D90E substitutions; and a neutral mutation T84K.

The three ST152 isolates had deletions of nt17 and nt18 leading to a premature stop codon at amino acid 24 of MgrB; deletions of nt17 and nt18 are reported here for the first time. Deletion of nt19 of *mgrB* has been reported recently [26]. Other mutations were found in *pmrB* and *phoQ*. Thus, in a series of 23 consecutively obtained isolates, 10 genomes were studied and 11 novel mutations related to colistin resistance were identified. Although some of these mutations had a neutral effect on protein function, these findings illustrate the local micro-evolution of these genes.

Resistance phenotypes observed in the isolates, in addition to colistin resistance, correlated with a diversity of resistance encoding genes. Two multi-drug efflux pumps, MexAB-OprM and AcrB-TolC, were found in all genomes. The substrate profile of MexAB-OprM includes macrolides, lincosamides, ketolides, fluoroquinolones, tetracyclines, glycylcyclines, beta-lactams and aminoglycosides [27]; and the substrate profile of AcrAB-TolC includes chloramphenicol, fluoroquinolone, tetracycline, novobiocin, rifampin, fusidic acid, nalidixic acid and beta-lactam antibiotics [28].

ColRKp isolates are usually carbapenem resistant. Although all study isolates showed phenotypic resistance to meropenem and/or ertapenem, carbapenemase encoding genes were found in only seven isolates, six of them with bla_{KPC-2}, the predominant carbapenemase encoding gene in the world [29] and in Brazil [30]. An ST258 isolate had $bla_{OXA-370}$, a carbapenemase described in Rio de Janeiro in *K. pneumoniae* ST16 and ST1041, and *Enterobacter cloacae* and *Enterobacter aerogenes* [31]; this is the first description of such carbapenemase in *K. pneumoniae* ST258, suggesting an increased trend to dissemination. Carbapenem resistance in the three other isolates with no carbapenemase encoding gene may be mediated by MexAB-OprM multi-drug-efflux pump [32]. Other beta-lactamase encoding genes of note are CTX-M type ESBL, found in all isolates. These genes are disseminated worldwide, particularly in the developing world [19]. Therefore, the universal presence of bla_{CTX-M} in study isolates was a predictable picture. Other beta-lactamase genes were $bla_{SHV-187}$, present in the three ST152 isolates, which encodes an ESBL (GenBank accession number LN515533.1), and $bla_{SHV-182}$, present in the five CC258 isolates, but with no information on ESBL status (GenBank accession number KP050489.1). Still of note, with respect to wide-spectrum aminoglycoside resistance, is the presence of 16S ribosomal RNA methyltransferase encoding genes *armA* and *rmtB*, the most frequently reported worldwide, in two isolates belonging to CC258 [33,34].

CRISPR arrays were found in only two ST15 isolates and belonged to type I-E. This is congruent with the literature, which shows that this system is not widely distributed in *K. pneumoniae* genomes and mainly belongs to this same type [35]. CRISPR/Cas systems are involved in preventing the entry of exogenous genetic elements into bacterial cells, and perform an important role in biofilm formation, colonization and virulence regulation in multiple pathogenic bacteria [36]. Information about CRISPR systems in *K. pneumoniae* are scarce [35,37]. In a series of 176 *K. pneumoniae* clinical isolates from Taiwan, CRISPR systems were found in 54 (31%), nine of them of ST15 [37], which suggests that this is an ST15 clone feature. None of 16 ST258 isolates from Taiwan had CRISPR systems, as observed in the present study. Another study, with undefined STs and antimicrobial susceptibility profiles of isolates with eight complete genomes and 44 draft genomes publicly available, found CRISPR systems in only four of the draft genomes and two of the complete genomes, confirming that CRISPR systems are uncommon in *K. pneumoniae* [35].

An important mechanism of mobilization of antibiotic resistance and virulence traits is plasmids. The study isolates had a diversity of plasmids, including IncF, IncN, IncA/C, IncX and Col-like families. All isolates had IncF family plasmid, reported in Enterobacteriaceae species frequently associated with antimicrobial resistance and virulence genes [38]. IncF, IncN and IncA/C are known because of their tendency to acquire resistance genes and disseminate quickly among Enterobacteriaceae [39]. Col-like plasmids, detected in all of the study isolates with one exception, have been reported increasingly as carrying antimicrobial resistance genes in Enterobacteriaceae strains, and considered as versatile gene capture platforms [40].

5. Conclusions

ColRKp emerged in two hospitals in Rio de Janeiro state, Brazil, but cross-dissemination between hospitals was not detected with polyclonal strains. Nevertheless, the independent emergence of virulent and epidemic clone ST15 in both hospitals, apparently simultaneously, is of note. Colistin resistance was related to mutations in PhoQ or PmrB in all isolates, with one exception, and with additional findings in CcrB in three isolates; 11 new mutations were found in seven isolates, including the deletion of two nucleotides in *mgrB*. One isolate was pandrug resistant, and the other isolates were resistant to nearly all drugs tested. ColRKp isolates presented carbapenemases, ESBL and several other resistance genes in strains. Colistin resistance emerged with several novel mutations amid highly resistant strains, restricting further antimicrobial drugs available and leading to pandrug resistance.

Funding: This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Process Number: 88881.068043/2014-01, Instituto Nacional de Pesquisa em Resistência Antimicrobiana–Brazil, CNPq 465718/2014-0, and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro.

Competing interests: None declared.

Ethical approval: The Ethics Committee of Hospital Universitário Antonio Pedro of Universidade Federal Fluminense approved this study [32570 (CAAE 02759912.9.0000.5243)].

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