ORIGINAL RESEARCH

Genetic and Phenotypic Characteristics of Carbapenem-Resistant *Klebsiella pneumoniae* Isolates from a Tertiary Hospital in Beijing

Qin Ni[1,2,*], Xingwei Yao[3,*], Jinhui Li[2,*], Jinghan Ma[3,*], Kaiying Wang[2], Xiong Liu[2], Peihan Li[2], Lang Yang[2], Peng Li[2], Shenlong Li[1,2]

[1]Epidemiology, Zhengzhou University, Zhengzhou, People's Republic of China; [2]Biosafety, Chinese PLA Center for Disease Control and Prevention, Beijing, People's Republic of China; [3]Medical Clinical Laboratory, Dong Zhimen Hospital Beijing University of Chinese Medicine, Beijing, People's Republic of China

*These authors contributed equally to this work

Correspondence: Peng Li; Shenlong Li, Email jiekenlee@126.com; lishenlong@sohu.com

Objective: *Klebsiella pneumoniae* is a common multidrug-resistant pathogen that jeopardizes the health of hospitalized patients. We aimed to study the phenotypic and genotypic characteristics of carbapenem-resistant *K. pneumoniae* (CRKP) isolates from a hospital in Beijing.

Methods: Twenty-four CRKP clinical isolates were collected within a half-year to investigate antimicrobial resistance and genomic characteristics. Illumina and Nanopore sequencing were performed to assemble and annotate genomes.

Results: All strains were multi-drug resistant. Twenty-two strains carried the bla_{KPC-2} gene and two harbored bla_{NDM-5}. Multilocus sequence type(MLST) analysis identified five sequence types; most isolates belonged to ST11. Three strains were isolated from the same patient; each carried a different plasmid replicon, either IncFII $_{(pHN7A8)}$, IncX, or IncFIB $_{(K)}$.

Conclusion: This study furthers the understanding of CRKP antimicrobial resistance genotypes, and may facilitate the control of nosocomial infections caused by antimicrobial-resistant pathogens.

Keywords: *Klebsiella pneumoniae*, drug resistance, carbapenemase, ST11, bla_{KPC-2}

Introduction

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) comprise a set of Gram-negative bacteria that cause serious nosocomial infections, including pneumonia, meningitis, liver abscess, wound and urinary tract infections, especially in immunodeficient patients.[1,2] The rising prevalence and increasing multidrug resistance of CRKP are of critical concern.[3] A World Health Organization report on antimicrobial resistance disclosed that CRKP has a worldwide geographic distribution, and that the prevalence of CRKP among *K. pneumoniae* isolates exceeds 50% in some patient groups.[4] For example, carbapenem resistance among *K. pneumoniae* isolates in Greece increased from 0% in 2003 to 38.3% in 2010.[5] Surveillance reports from CHINET show that the prevalence of meropenem resistance among *K. pneumoniae* isolates in China has increased annually, from 2.9% in 2005 to 26.3% in 2018.[6]

Carbapenems are used to treat severe infections caused by multidrug-resistant *Enterobacteriaceae* (including *K. pneumoniae*). The emergence of carbapenemases and extended-spectrum beta-lactamases (ESBL) in *K. pneumoniae* has reduced bacterial sensitivity to almost all beta-lactams,[7] which confounds treatment and jeopardizes clinical outcomes.

bla_{KPC-2} is the major carbapenemase gene in *Enterobacteriaceae* isolated from Chinese adults and children.[6,8] bla_{NDM}-positive*K. pneumoniae* isolates often produce metallo-β-lactamase[9] and demonstrate high-level resistance to all carbapenems.[10] Understanding the types and distribution of *K. pneumoniae* antibiotic resistance genes (ARGs) facilitates hospital infection control and deserves attention.[11]

We employed Illumina and Nanopore sequencing to analyze the genomic epidemiology of CPKP clinical isolates. Our findings characterized the genomic and resistance features of *K. pneumoniae* and revealed an evolving genotype in isolates from a single patient, and may guide prevention and treatment.

Methods
Bacterial Classification and Antimicrobial Susceptibility Testing

Samples were collected in a tertiary hospital in Beijing, China, from June to December 2021. Twenty-four CRKP isolates were obtained from sputum (11/24), urine (7/24), blood (3/24), catheters (1/24), secretions (1/24) and throat swabs (1/24). The samples were collected from 15 (62.5%) men and 9(37.5%) women between 50 and 94 years old. A fully automated analysis system (PHOENIX-100 [BD, USA]) was used for species identification and antimicrobial susceptibility testing. Susceptibilities to 18 commonly used antibacterial agents were determined; these included ampicillin, amoxicillin/clavulanate, ampicillin/sulbactam, piperacillin/tazobactam, cefazolin, cefepime, cefotaxime, ceftazidime, meropenem, imipenem, gentamicin, amikacin, ciprofloxacin, moxifloxacin, levofloxacin, trimethoprim/sulfamethoxazole, tetracycline, and aztreonam. Results were interpreted according to the breakpoints and reporting method of the Clinical Laboratory Standards Institute (2021 version, https://clsi.org).

Whole-Genome Sequencing Using Illumina and Nanopore

Genomic DNA was extracted from cultured bacteria. The Illumina sequencing library was prepared using NEBNext Ultra II FS DNA Library Prep Kit for Illumina (New England BioLabs, CA, USA) according to the manufacturer's instructions. Sequencing was performed on the Illumina NovaSeq 6000 platform at Novogene company with a strategy of pair-end 150bp. Nanopore sequencing libraries were prepared using the Ligation Sequencing Kit SQK-LSK109 (Oxford Nanopore Technologies, Cambridge, UK). The prepared libraries were sequenced on a MinION MK1B sequencer (Oxford Nanopore Technologies, Cambridge, UK) with an R9.4 flow cell.

Dry Lab: Bioinformatics Analysis

The term dry lab denotes the bioinformatic portion of the technology.[12] Illumina and Nanopore sequencing data were filtered by Fastp[13] (version 0.19.7) and NanoFilt (v2.8.0). Hybrid assembly of clean data was conducted with Unicycler (v0.4.8).[14] The ARGs of CRKP were identified using the ABRicate (1.0.1) and Resfinder[15] databases (https://cge.food.dtu.dk/services/ResFinder/). Kleborate (v2.0.1)[16] was used for multilocus sequence typing (MLST). Plasmids were identified using the PlasmidFinder[17] database. Genomes were annotated using Prokka (1.14.6).[18] Core genome alignments were created by Roary,[19] and maximum likelihood phylogenetic trees were generated using RAxML-NG[20] (v1.0.3), visualized using FigTree (v1.4.4) and the R ggplot2 package. Plasmid genome circle map visualization using proksee (https://proksee.ca).

Results
CRKP Isolation and Antimicrobial Susceptibility Testing

From June to December 2021, 24 CRKP isolates were obtained from sputum (11/24), urine (7/24), blood (3/24), catheters (1/24), secretions (1/24), and throat swabs (1/24). Specimens were primarily from the Intensive Care Unit (ICU) (17/24), followed by neurology (3/24), neonatology (2/24) and nephrology (2/24) wards. All strains were ESBL-positive. Three strains (BJ10-0010, BJ10-0013 and BJ10-0015) were isolated from urine, blood, and sputum of the same ICU patient in June, September, and October 2021, respectively. The samples were collected from 15 (62.5%) men and 9(37.5%) women between 50 and 94 years old. Most strains were resistant to multiple agents, including meropenem and imipenem (Table 1). All 24 strains (100%) were resistant to ampicillin, cefazolin, amoxicillin/clavulanate, ampicillin/sulbactam, meropenem, piperacillin/tazobactam, cefepime, cefotaxime and ceftazidime, but most (66.67%) were sensitive to amikacin. Interestingly, the three strains from the same patient showed slightly different levels of imipenem resistance. The MIC values of the 22 strains are provided in Supplementary Table S1.

Table I Antimicrobial Susceptibility of *K. pneumoniae* Isolates

Drug Classes	Antimicrobials	Resistant n (%)	Intermediate n (%)	Susceptible n (%)
β-lactams	Ampicillin	24 (100.00)	0 (0.00)	0 (0.00)
	Amoxicillin/Clavulanate	24 (100.00)	0 (0.00)	0 (0.00)
	Ampicillin/Sulbactam	24 (100.00)	0 (0.00)	0 (0.00)
	Piperacillin/Tazobactam	24 (100.00)	0 (0.00)	0 (0.00)
	Cefazolin	24 (100.00)	0 (0.00)	0 (0.00)
	Cefepime	24 (100.00)	0 (0.00)	0 (0.00)
	Cefotaxime	24 (100.00)	0 (0.00)	0 (0.00)
	Ceftazidime	24 (100.00)	0 (0.00)	0 (0.00)
Carbapenems	Meropenem	24 (100.00)	0 (0.00)	0 (0.00)
	Imipenem	22 (91.67.00)	2 (8.33)	0 (0.00)
Aminoglycosides	Gentamicin	20 (83.33)	I (4.17)	3 (12.50)
	Amikacin	8 (33.33)	0 (0.00)	16 (66.67)
Fluroquinolones	Ciprofloxacin	22 (91.67)	I (4.17)	I (4.16)
	Moxifloxacin	22 (91.67)	I (4.17)	I (4.16)
	Levofloxacin	22 (91.67)	2 (8.33)	0 (0.00)
Sulfonamides	Trimethoprim/Sulfamethoxazole	17 (70.83)	0 (0.00)	7 (29.17)
Tetracyclines	Tetracycline	18 (75.00)	0 (0.00)	6 (25.00)
Monocyclic β- lactams	Aztreonam	22 (91.67)	2 (8.33)	0 (0.00)

Genomic Characterization of CRKP

The genomes of 24 CRKP strains were obtained by hybrid assembly using Illumina and nanopore sequencing, and have been deposited in NCBI under the BioProject accession number PRJNA874878. Whole genome MLST identified five sequence types. Most strains belonged to ST11 (19/24), and others belonged to ST15 (2/24), ST37 (1/24), ST403 (1/24) and ST1530 (1/24). Genomic annotation identified diverse carbapenemase- and β-lactamase-encoding ARGs. The bla_{LAP-2} (13/24), $bla_{CTX-M-65}$ (7/24) and bla_{SHV-12} (7/24) genes were chromosomal, while bla_{KPC-2} (13/24), bla_{TEM-1B} (12/24), bla_{OXA-1} (8/24), bla_{NDM-5} (2/24) bla_{SHV-12} (2/24) and $bla_{CTX-M-3}$ (1/24) $bla_{CTX-M-65}$ (1/24) were plasmid-encoded. The distribution of ARGs of strains of different STs is shown in Figure 1.
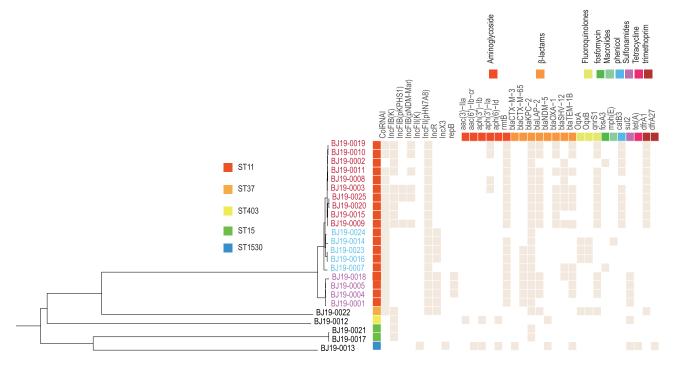

Figure I Distribution of antibiotic resistance genes, plasmids and STs of all strains. Colors represent different clades. Red represents subclade I, blue represents subclade 2, and purple represents subclade 3. The antique white squares represent corresponding ARGs or replicons.
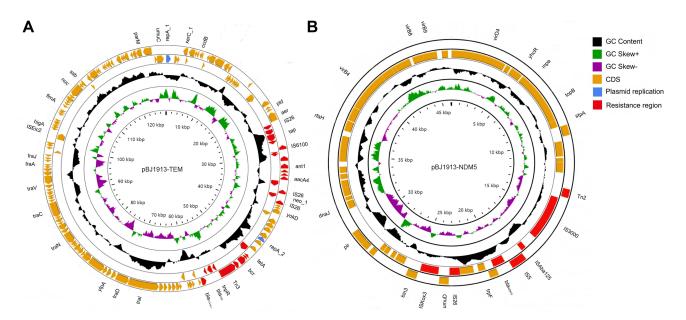
Figure 2 The genome circle map of plasmids pBJ1913-TEM (**A**) and pBJ1913-NDM5 (**B**) carried by strain BJ19-0013. The blue color in the outer circle represents the plasmid replicon protein, the red color represents the drug resistance gene region, and the Orange color represents the protein coding region. The inner-circle indicates the positive and negative GC skew of the plasmid in green and purple, respectively.

Distribution of Antibiotic Resistance Genes Among Plasmid Replicon Types

Plasmid incompatibility groups were determined according to replication sequences. The strains contained multiple plasmid replicon types, most of which distributed in ST11 strains. All ST11 strains containing plasmid replicons IncFII $_{(pHN7A8)}$. IncFIB $_{(K)}$, IncFIB $_{(pKPHS1)}$ or IncFIB $_{(pNDM-Mar)}$ were distributed exclusively in subclade1, while IncR was carried only in subclades 2 and 3. Twenty-five plasmids contained single replicons, and 14 carried multiple replicons. On average, each single-replicon plasmid carried 1.52 ARGs, and each multi-replicon plasmid encoded 2.71 ARGs. Plasmids containing multiple-replicons IncFIB$_{(K)}$/IncFII$_{(pHN7A8)}$ and IncFIB$_{(K)}$/IncFIB$_{(pKPHS1)}$ carried bla_{KPC-2}, bla_{TEM-1B}, and $rmtB$. The plasmids of BJ10-0013 included the IncFII $_{(K)}$ replicon and encoded eight ARGs, the most of all plasmids.

Comparison of BJ19-0010, BJ19-0013 and BJ19-0015 from a Single Patient

These three strains were isolated from different samples from the same patient. Interestingly, BJ19-0010 and BJ19-0015 belonged to ST11 and were resistant to ciprofloxacin and levofloxacin, while strain BJ19-0013 belonged to ST1503 and was fluoroquinolone-sensitive. In the phylogenetic tree, BJ19-0010 and BJ19-0015 fell in subclade 1, while BJ19-0013 comprised a distinct branch. Strains BJ19-0010, BJ19-0013 and BJ19-0015 had diverse plasmid replicons. Interestingly, BJ19-0010 and BJ19-0015 shared IncFII $_{(pHN7A8)}$, while BJ19-0010 carries IncFIB$_{(K)}$/IncFIB$_{(pNDM-Mar)}$ and BJ19-0015 carries IncFIB $_{(K)}$. Both BJ19-0010 and BJ19-0015 carried bla_{KPC-2}, bla_{LAP-2} and bla_{OXA-1}. The bla_{KPC-2} and bla_{OXA-1} genes were plasmid-encoded, while the bla_{LAP-2} gene was located on the chromosome. Strain BJ19-0010 also contained two copies of bla_{TEM-1B} that were located on different plasmids. However, BJ19-0013 carries IncX3 and IncFII$_{(K)}$, which are different from the other two strains. The hybrid assembly showed that the IncFII$_{(K)}$ plasmid pBJ1913-TEM had a length of 127942bp and carries the β- Lactams ARGs bla_{TEM-1B} and $bla_{CTX-M-3}$(Figure 2A). The other plasmid, pBJ1913-NDM5, had a length of 46068bp and carries the carbapenem-resistance gene bla_{NDM-5} (Figure 2B), which is nearly identical with plasmid pR15_NDM-5 in Escherichia coli strain R15 isolated from wastewater treatment plant in China.

Discussion

Carbapenems are usually effective for the treatment of drug-resistant bacterial infections. However, CRKP can express plasmid-encoded carbapenemases, which inactivate most β-lactam antibiotics and confer carbapenem resistance.[21] Our study showed that 24 CRKP strains were resistant to most tested drugs. The resistance rate to fluoroquinolones was

relatively high, which was consistent with the results of a previous study.[22] Three strains were isolated from different samples from the same patient within 4 months and carried different carbapenemase genes, which were located on separate plasmids. Genome annotation revealed that Tn3 was located upstream of the bla_{KPC-2} gene on chromosomes of BJ19-0010 and BJ19-0015, while Tn552 was located downstream of bla_{NDM-5} in BJ19-0013. The Tn3 transposon promotes the dissemination of AMR,[23] and Tn552 has a co-integrate resolution system homologous to Tn3 family elements,[24] which may have facilitated plasmid and ARG exchange among these three strains. A previous study showed that bla_{KPC-2}-positive *K. pneumoniae* isolates in China belonged primarily to ST11.[25] In this study, the majority of CRKP strains belonged to ST11, all of which contained bla_{KPC-2}. Most (68.42%, 19/24) ST11 strains were isolated from ICU patients; consequently, the monitoring of AMR strains in ICUs should be strengthened. In *K. pneumoniae*, the plasmid with an IncR replicon was identified as a multidrug resistance vector with variable ARGs.[26] ST11 strains in this study contained the IncR and IncFII $_{(pHN7A8)}$ replicons with $bla_{CTX-M-65}$, bla_{TEM-1B}, bla_{KPC-2}, and *rmtB* genes.

Conclusion

Our study revealed the prevalence of CRKP strains isolated primarily from ICU patients. All 24 strains (100%) were resistant to β- Lactams and carbapenems and carried diverse plasmids with different ARGs. The rapid dissemination of ST11 strains highlights the necessity of strengthening CRKP surveillance and may provide a theoretical basis for infection control and treatment.

Data Sharing Statement

The genomes of 24 CRKP strains have been deposited in NCBI with the BioProject accession number PRJNA874878.

Ethics Approval and Informed Consent

The institutional ethics committees of Dong zhimen Hospital Beijing University of Chinese Medicine approved the study. As all data were anonymously collected and interpreted, the institutional ethics committees waived the need for written informed consent from the participants. The study was also supervised by Chinese PLA Center for Disease Control and Prevention.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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