Original Article

Molecular typing and drug resistance analysis of carbapenem-resistant Klebsiella pneumoniae from paediatric patients in China

Xian Zhang1 #, Jian Xue2 #, Mei-Jing Shen3, Wen-Hong An1, Ze-Qiong Chen1, Kai-Feng Wu3

1 Department of Hospital Infection, The Third Affiliated Hospital of Zunyi Medical University, The First People’s Hospital of Zunyi, Guizhou, China
2 Department of Health Management, Zunyi Medical And Pharmaceutical College, Guizhou, China
3 Department of Clinical Laboratory, The Third Affiliated Hospital of Zunyi Medical University, The First People’s Hospital of Zunyi, Guizhou, China

# Authors contributed equally to this work.

Abstract

Introduction: There are few studies on paediatric carbapenem-resistant Klebsiella pneumoniae (CRKP) in China. The present study investigated the molecular epidemiological and drug resistance characteristics of CRKP from paediatric patients in China to provide a reference for the prevention and control of CRKP infection.

Methodology: In total, 20 nonrepetitive clinical CRKP isolates were collected between February 2019 and February 2020 in a tertiary hospital in China. Strain identification and drug susceptibility testing were carried out using the VITEK® 2 Compact Bacterial Identification and Monitoring System. Sequence typing, phylogenetic relationships, and antibiotic resistance-associated genes were analysed by whole genome sequencing (WGS).

Results: sequence typing (MLST) and Core genome multilocus sequence typing (cgMLST) analysis revealed the most frequently represented were ST2407-CT3536 (30%), ST76-CT5893 (25%), and ST309-CT7864 (25%). All 20 CRKP isolates were divided into three clusters. All isolates were highly resistant to a variety of β-lactams and were highly susceptible to quinolones, aminoglycosides, and sulphonamides. All isolates mainly carried the carbapenem resistance genes \( \text{bla}_{NDM-1} \) and \( \text{bla}_{KPC-2} \), among which 10 isolates carried both \( \text{bla}_{NDM-1} \) and \( \text{bla}_{KPC-2} \) simultaneously.

Conclusions: Sequence typing, phylogenetic relationships, and antibiotic resistance genes can be determined using WGS technology. This can guide CRKP infection control and clinical treatment for paediatric patients.

Key words: Whole genome sequencing; paediatrics; carbapenem-resistant Klebsiella pneumoniae; molecular typing; drug resistance.


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Introduction

Since the emergence of carbapenem-resistant Klebsiella pneumoniae (CRKP) isolates in 2001 from North Carolina, United States, the isolation rate of this strain has continued to rise worldwide [1]. CRKP is largely associated with hospital-acquired infections, especially in children. The China Antimicrobial Surveillance Network (CHINET) showed that CRKP in children increased from 2.2% in 2005 to 25.4% in 2017 and then showed a gradually decreasing trend year by year until 2020 [2, 3]. Due to its high pathogenicity and limited antibiotic treatment, CRKP infection has a significantly higher mortality rate than infection with carbapenem-susceptible Klebsiella pneumoniae (CSKP) [4]. In 2017, the World Health Organization recognized CRKP as a serious threat to human health and listed it as one of the priority pathogens for which new antibiotics urgently need to be developed [5].

Although there are several mechanisms in CRKP, carbapenemase production is the most frequent. Many carbapenemase-encoding genes (\( \text{bla}_{KPC}, \text{bla}_{NDM}, \text{bla}_{OXA-48}, \text{or bla}_{VIM} \)) are plasmid-borne and can be horizontally transferred through plasmids, prompting the rapid development of CSKP into CRKP [6]. CRKP subsequently leads to a wider resistance spectrum and a higher resistance rate in the population, and the prevention and control of nosocomial infection becomes more difficult. According to the report, silent colonization of patients may result in nosocomial infection outbreaks in CRKP [7]. In view of the easy diffusion and strong concealment of CRKP, accurately identifying the source and route of CRKP transmission...
in hospitals is an important tool for nosocomial infection prevention and control. With the promotion, progress, and decreased costs of sequencing technology, whole genome sequencing (WGS) is increasingly used in nosocomial infection control. It can provide information on pathogen identification, epidemiological analysis, and drug resistance genes [8]. Due to the immaturity of paediatric patients, the disease progresses rapidly after CRKP infection. Therefore, a large amount of basic data analysis is required to prevent and control CRKP. At present, there are few studies on paediatric CRKP in China. Thus, this study collected CRKP isolates from a paediatric department of a tertiary hospital in Guizhou, China, and used WGS technology to deeply understand the molecular epidemiology and drug resistance mechanism of paediatric CRKP. Thus, this study provides data support for clinical treatment and infection control measures of paediatric CRKP.

**Methodology**

**Bacterial isolates**

All 20 CRKP isolates from paediatric patients were obtained from a tertiary-care university hospital in Guizhou, China, from February 2019 to February 2020. Duplicate strains in the same part of the same patient were excluded.

**Antimicrobial susceptibility testing**

The identification and drug sensitivity testing of the 20 CRKP isolates were carried out by the VITEK® 2 Compact Bacterial Identification and Monitoring System. The judgement standard was in accordance with the 2022 standard of the American Clinical and Laboratory Standards Institute (CLSI) [9]. *K. pneumoniae* resistant to any carbapenems was identified as CRKP. The control strain was *Escherichia coli* ATCC 25922.

**Extraction of bacterial genomic DNA**

A single colony of CRKP was picked and added to Luria-Bertani medium for shaking overnight at 37 °C and 220 r/min. Then, 4 mL of bacterial solution was collected, and genomic DNA was extracted according to the manufacturer’s instructions of the TIANamp Bacteria DNA Kit (Tiangen Biotech, Beijing, China). The recovered product was verified by agarose gel electrophoresis, and its concentration was determined.

**Whole genome sequencing**

Second-generation DNA library construction and sequencing were completed by Shanghai Sangon Biological Company. WGS of 20 CRKP isolates was performed on the Illumina HiSeq 2500 sequencing platform.

**Sequence typing**

Core genome multilocus sequence typing (cgMLST) is a high-resolution typing method constructed with thousands of core genes as sequence typing markers based on a large number of strains. The multilocus sequence typing (MLST) was identified using the PubMLST database. To further distinguish the isolates, cgMLST was performed with the SeqSphere+ software to assign complex types (CTs).

**Phylogenetic tree construction**

Taking *K. pneumoniae* MGH78578 as the reference strain, the genomes of 20 CRKP isolates were compared with the reference strain to determine the site differences of core genome single nucleotide polymorphisms (cgSNP). The phylogenetic tree was constructed by IQtree software based on the cgSNP differences between strains [10].

**Drug resistance gene analysis**

The genome sequences of 20 CRKP isolates were compared with the Comprehensive Antibiotic Resistance Database (CARD) database using BLAST. The annotation results were obtained by combining genes and their corresponding drug resistance function annotation information.

**Results**

**Patients and Isolates**

Among the 20 CRKP isolates, 13 strains were from neonates (13/20, 65%), and the rest were from 2- to 5-month-old infants. Among the specimens, 13 strains were isolated from sputum (13/20, 65%); 2 strains were isolated from throat swabs, 2 from blood, and 2 from urine (2/20, 10% each); and 1 strain was isolated from oral secretions (1/20, 5%).

**CRKP susceptibility**

For β-lactam antibiotics, the resistance rate to ampicillin-sulbactam, cefazolin, ceftazidime, ceftriaxone, imipenem, meropenem and ertapenem was 100%; to aztreonam and cefepime was 90% (18/20); to piperacillin-tazobactam was 80% (16/20); and to cefotetan was 55% (11/20). For aminoglycoside antibiotics, the resistance rate to gentamicin was only 10% (2/20), and was completely sensitive to tobramycin and amikacin. About quinolone antibiotics, the strains were completely sensitive to levofloxacin.
and intermediate to ciprofloxacin. The resistance rate to trimethoprim-sulfamethoxazole was 25% (5/20).

**Whole genome sequencing**

The amount of data obtained from each sample was not less than 1 G, the data quality was Q30 > 90%, and the average sequencing depth was > 150x.

**CRKP sequence typing**

The 20 CRKP isolates were divided into 5 distinct sequence types (STs), including 6 strains belonging to ST2407 (6/20, 30%), 6 strains to ST76 (6/20, 30%), 5 strains to ST309 (5/20, 25%), 2 strains to ST414 (2/20, 10%), and 1 strain to ST534 (1/20, 5%). Typing by cgMLST revealed 6 distinct CTs, including four new CTs (CT7864, CT7865, CT7866, and CT7868). The 6 distinct CTs, including 6 strains belonging to CT3536 (6/20, 30%), 5 strains to CT7864 (5/20, 25%), 5 strains to CT5893 (5/20, 25%), 2 strains to CT7866 (2/20, 10%), 1 strain to CT7865 and 1 strain to CT7868 (1/20, 5% each), were shown in Figure 1.

**CRKP phylogenetic tree**

As shown in Figure 1, the 20 CRKP isolates were divided into three clusters. Among them, cluster 1 contained 7 strains, KP-zy10, KP-zy4, KP-zy6, KP-zy7, KP-zy5, KP-zy12 and KP-zy11; cluster 2 contained 7 strains, KP-zy17, KP-zy14, KP-zy1, KP-zy2, KP-zy20, KP-zy16 and KP-zy18; and cluster 3 contained 6 strains, KP-zy19, KP-zy15, KP-zy13, KP-zy8, KP-zy3 and KP-zy9.

**Distribution and characteristics of drug resistance genes**

By comparing the genome sequences of 20 CRKP isolates with the CARD database, the drug resistance genes carried by all strains were identified. As shown in Figure 1, the CRKP strains mainly carried the carbapenem resistance genes \( \text{bla}_{NDM-1} \) (15/20, 75%) and \( \text{bla}_{KPC-2} \) (14/20, 70%); the aminoglycoside resistance genes \( \text{aadA} \) (5/20, 25%), \( \text{amrA} \) (20/20, 100%), \( \text{aac(6')-IId-cr} \) (4/20, 20%), \( \text{aac(3')-Ilb} \) (3/20, 15%) and \( \text{aac(3')-Ila} \) (3/20, 15%); the \( \beta \)-lactam resistance genes \( \text{bla}_{TEM-1} \) (19/20, 95%), \( \text{bla}_{CTX-M-15} \) (16/20, 80%), \( \text{bla}_{CTX-M-65} \) (19/20, 95%), \( \text{bla}_{SHV-1} \) (20/20, 100%), \( \text{bla}_{SHV-4} \) (6/20, 30%), \( \text{bla}_{DHA-1} \) (8/20, 40%) and \( \text{bla}_{LEN-12} \) (20/20, 100%); the fluoroquinolone resistance genes \( \text{qnrB} \) (40%), \( \text{qnrS} \) (40%), \( \text{gyrB} \) (19/20, 95%), \( \text{parC} \) (20/20, 100%), and \( \text{parE} \) (20/20, 100%); the sulfonamide resistance genes \( \text{suI} \) (8/20, 40%) and \( \text{suL} \) (8/20, 40%); the fosfomycin resistance gene \( \text{fosA} \) (20/20, 100%); the trimethoprim resistance genes \( \text{dfrA} \) (20/20, 100%) and \( \text{dfrA22} \) (20/20, 100%); the tetracycline resistance gene \( \text{tet(D)} \) (20/20, 100%); and the macrolide resistance genes \( \text{mph(A)} \) (3/20, 15%), \( \text{mac(B)} \) (20/20, 100%) and \( \text{msr(E)} \) (3/20, 15%).

**Discussion**

CRKP is one of the most harmful nosocomial pathogens, accounting for more than 70% of carbapenem-resistant Enterobacteriaceae (CRE) in Chinese hospitals [11, 12]. The burden of CRKP increased 6.16 times during 2007–2015 in terms of the number of infections and deaths in a European study of
attributable deaths caused by infections with antibiotic-resistant bacteria [13]. CRKP spreads rapidly around the world, and its resistance rate to carbapenems continues to increase. According to CHINET surveillance from 2005 to 2017, the resistance rate of K. pneumoniae to carbapenems continued to rise, and its resistance rates to imipenem and meropenem increased rapidly from 3.0% and 2.9% in 2005 to 25.3% and 26.8% in 2019, respectively [12]. The increasing drug resistance rate has led to limited drugs for the treatment of CRKP infection in the clinic, and fewer types of drugs can be used for children [14]. Therefore, the prevention and control of CRKP infection in children deserves more attention. At present, nosocomial infection managers mainly track and trace the outbreaks and transmission routes of nosocomial pathogens by homology analysis through pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) [15]. These traditional methods are time-consuming, complicated, and low-resolution. When highly similar clones appear, it is often impossible to distinguish the small differences between them. However, the emergence of WGS technology has solved this problem. Using this technology, the difference between nearly a thousand SNP sites can be found between the genomes of strains with very similar homology, and the possibility and route of transmission among these strains can be accurately determined [16].

In this study, the main STs of 20 CRKP isolates were ST2407, ST76, and ST309, which was consistent with the reports from other children’s hospitals in China [17, 18]. By combining clinical epidemiological data and a phylogenetic tree, it was found that the 20 CRKP isolates belonged to three clusters. All seven isolates in cluster 1 were from neonatology, and the separation time of KP-zy4, KP-zy5, and KP-zy6 samples differed by only two days at most. Cluster 2 contained seven isolates, of which five were from the general paediatric ward, and the other two were from the paediatric intensive care unit. All six isolates in cluster 3 were from the paediatric intensive care unit. There is a possibility of spatiotemporal cross-infection within the cluster. Studies have shown that the most common sources of nosocomial infections are patients, medical staff and the environment, among which CRKP-contaminated environments and the hands of medical staff can lead to infection epidemics [19, 20]. Regardless of the cluster, most patients were in the same department, and there was overlap between cleaning staff and medical staff, thus increasing the risk of CRKP transmission.

The results of the phenotypic drug resistance in this study showed that, although CRKP was highly resistant to β-lactam antibiotics, it was most sensitive to aminoglycosides, quinolones, and sulfonamides. This was consistent with previous literature reports; thus, the above drugs can be selected for paediatric CRKP infection in hospitals [21, 22]. The results of drug resistance genes showed that all 20 CRKP isolates carried carbapenem resistance genes, of which five isolates only carried \( \text{bla}_{\text{KPC-2}} \), six isolates only carried \( \text{bla}_{\text{NDM-1}} \), and the remaining nine isolates carried both carbapenem resistance genes. This indicated that carbapenemase production is the main drug resistance mechanism of CRKP in the hospital, consistent with reports in China [23, 24]. The combined analysis of phenotypic drug resistance and the presence of drug resistance genes showed that the presence of aminoglycoside, quinolone, and sulfonamide drug resistance genes was not completely consistent with the results of phenotypic drug resistance analysis, and similar results were obtained in other studies [8, 25]. In vitro drug-susceptibility test results can guide which antibiotic to be used clinically, but the presence or absence of resistance genes confirmed by WGS did not exactly match the resistance phenotype results. The EUCAST committee also mentioned that, for most bacterial species, there is insufficient evidence to support the use of drug susceptibility phenotypes to guide clinical anti-infection decisions [26]. Therefore, the relationship between drug resistance gene information and clinical medication guidance still needs to be verified with more samples to obtain reliable data support.

As a special group, the high morbidity and mortality caused by CRKP infection in children have been a concern around the world. Most of the CRKP isolates that cause nosocomial infections in China carry \( \text{bla}_{\text{KPC-2}} \) or \( \text{bla}_{\text{NDM-1}} \). They can lead to outbreaks of CRKP through plasmid-mediated horizontal transfer between different strains. To strengthen the control and spread of paediatric CRKP infection, we should accurately and timely grasp the actiological information of patients, shorten the application time of antibiotics, reduce the types of antibiotics, and carry out the precise targeted treatment. Numerous studies have shown that carbapenem use is an independent risk factor for CRKP infection [27, 28]. Therefore, medical institutions should strictly control the use of carbapenem antibiotics. WGS can comprehensively grasp the drug resistance and drug resistance mechanism of bacteria. It can be used to track nosocomial infections and determine transmission routes, providing an important
method for preventing the spread of bacteria and reducing drug resistance in the clinic, which should be applied in the daily work of hospital infection management.

Conclusions
In this study, all 20 CRKP isolates carried carbapenem-resistance genes. They were divided into three clusters, implying three different common ancestors in the hospital. Sequence typing, phylogenetic relationships, and antibiotic resistance genes could be obtained using WGS technology. This can guide CRKP infection control and clinical treatment for paediatric patients.

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Corresponding author
Professor Kai-Feng Wu, PhD
Director of clinical laboratory
The Third Affiliated Hospital of Zunyi Medical University,
The First People's Hospital of Zunyi,
No. 98 Feng Huang North Road,
Guizhou, China.
Tel: 86-0851-23230015
Fax: 86-0851-23230015
Email: xuejian912@163.com

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