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Molecular epidemiological analysis of *bla*_{NDM-5}-producing *Klebsiella pneumoniae* ST2407-K25 causing infection outbreaks in pediatric patients based on whole genome sequencing

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Abstract

Background Pediatric patients are vulnerable to the threat of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) due to their limited immunity and few available antibiotics. Especially when these pathogens exhibit hypervirulent phenotypes, they are often associated with poor clinical outcomes.

Methods In this study, we investigated a CRKP outbreak in pediatric patients from 2019 to 2021 in a teaching hospital in China based on whole genome sequencing. We sequenced twenty-nine CRKP isolates isolated from unduplicated pediatric patients to understand their genetic relationships, virulence factors, resistance mechanisms, and transmission trajectories. Conjugation experiments were performed to evaluate the horizontal transfer ability of carbapenem resistance determinants in twenty-nine CRKP isolates. We then characterized these isolates for biofilm formation ability and serum resistance. Genetic relatedness, comparison of plasmids, and chromosomal locus variation of CRKP isolates were analyzed by bioinformatics.

Results All the isolates were carbapenemase-producers harbouring *bla*_{NDM-5}. Among them, twenty-eight isolates belonged to the ST2407 group, with the consistent capsular serotype K25. The virulence-related factors: *ureA*, *fim*, *ybtA*, *irp1/irp2*, and *mrkA* were prevalent in these isolates. Additionally, most CRKP isolates showed moderately adherent biofilm formation. Although the ST2407 clonal group did not exhibit serum resistance, the heterogeneous level of serum resistance was related to the disruption of *oqxR*. Conjugation and WGS revealed that the *bla*_{NDM-5} carried by the twenty-eight CRKP ST2407 isolates was located on nonconjugative IncX3 plasmids associated with

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deleting the T4SS-encoding genes. Clonal transmission of CRKP ST2407 in pediatric patients was suggested by the phylogenetic tree.

Conclusions Our study provides evidence of the clonal spread of *bla*_{NDM-5}-producing *K. pneumoniae* in pediatric patients and the necessity for the T4SS system for horizontal transfer of the IncX3 plasmid carrying *bla*_{NDM-5}. Additionally, the disruption of *oqxR* may have affected the serum resistance of CRKP. The results of this study emphasize the importance of continuously monitoring for CRKP infection in pediatric patients to prevent recurrent infections.

Keywords *Klebsiella pneumoniae*, *bla*_{NDM-5}, IncX3, ST2407_K25, Biofilm, Pediatric infection control

Introduction

Klebsiella pneumoniae is a significant pathogen of nosocomial infection causing diverse bas such as severe pneumoniae, urinary tract infections, meningitis, and even bacteremia. The unceasing increase in carbapenem-resistant *K. pneumoniae* (CRKP) infections threatens global public health and increases treatment failure in clinical settings [1]. The interspecies dissemination of carbapenemase by horizontal gene transfer is thought to be the primary cause of this increase. Especially in *K. pneumoniae*, various mechanisms, such as the toxin-antitoxin system, reduced plasmid burden, and multiple replicons, have been suggested to explain the extraordinarily rapid dissemination of plasmid-mediated antimicrobial resistance, including carbapenemases [2–4]. New Delhi metallo- β -lactamase (NDM) is one of the recently identified carbapenemases that has attracted widespread attention due to its ability to hydrolyze almost all β -lactam antibiotics except aztreonam and spread widely among *Enterobacteriaceae* [5]. NDM-5, a variant of NDM-1 with alterations V88L and M154L, was first identified in a multidrug-resistant *E. coli* isolate [6]. Reportedly, NDM-5-producers tend to be more resistant to carbapenem than those producing NDM-1 [7]. Thus, antimicrobial agents are limited in clinical settings due to multidrug-resistant carbapenem-resistant Enterobacteriaceae (CRE), including those carrying *bla*_{NDM-5} [8, 9].

In China, the IncF and IncX plasmids are important incompatibility groups carrying *bla*_{NDM} for rapid horizontal transfer [10, 11]. Successful adaptation of IncX3 plasmids carrying *bla*_{NDM} in *E. coli* has been widely described [12]. Compared with those of other metallo- β -lactamase, the protein characteristics of NDM also determine its advantages in spreading widely in the host [13]. A study reported the clonal spread of *bla*_{NDM5}- and *bla*_{NDM-1}-producing *Klebsiella* isolates in medical settings, mostly associated with IncX3 [14, 15]. However, relatively little information is available about some resource-limited regions. There is a lack of effective inhibitors of metalloenzymes, and metallo- β -lactamase propagation and evolution deserve special attention.

CRKP infection is usually associated with adverse clinical outcomes, especially for isolates with the

hypervirulent phenotype [16, 17]. In developing countries, *K. pneumoniae* is a major pathogen causing neonatal infections [18, 19]. Currently, outbreaks of CRKP infection in pediatric patients have been reported and are of broad concern. Previous studies in other parts of China have reported an epidemic of CRKP in pediatric patients [20, 21]. Due to the unique nature of pediatric settings, contact with outsiders or medical staff also increases the risk of infection in pediatric patients. In addition, unlike adults, low birthweight, toy sharing, and breastmilk were associated with infections in pediatric patients [22]. CRKP infections in pediatric patients are more challenging to treat due to weak host immunity and medication side effects that limit the choice of antibiotics [23]. These pathogens can interfere with antimicrobial treatment and colonizing infection sites through biofilm formation [24].

In this retrospective study, we investigated an outbreak of CRKP infection in pediatric patients in a university hospital in Southwest China. We focused on the molecular epidemiological and microbial characteristics of CRKP in this outbreak.

Materials and methods

Collection, identification, and antimicrobial susceptibility testing of isolates

This retrospective study was conducted at the Affiliated Hospital of Southwest Medical University (Luzhou, China). From May 2019 to February 2021, we collected twenty-nine nonrepetitive CRKP isolates isolated from hospitalized pediatric patients. The isolates were identified by MALDI-TOF (Brucker, Germany). Antimicrobial susceptibility tests were performed using modified broth microdilution tests on the MicroScan WalkAway System (Siemens, Germany), with ceftriaxone, trimethoprim-sulfamethoxazole, tigecycline, cefotaxime, piperacillin/tazobactam, aztreonam, ertapenem, meropenem, gentamicin, and ciprofloxacin. The polymyxin B (Solarbio, China) and tigecycline (Macklin, China) minimal inhibitory concentrations (MICs) were determined by broth microdilution. *E. coli* ATCC25922 and *K. quasipneumoniae* ATCC 700,603 served as the quality control. The MIC of tigecycline was determined following the guidelines of the U.S.

Food and Drug Administration (FDA), and other antimicrobial susceptibility testing results were determined using the Clinical and Laboratory Standards Institute (CLSI) guidelines (2020-M100) [25].

Modified carbapenem inactivation methods and polymerase chain reaction for detecting carbapenemases

The modified carbapenem inactivation method (mCIM) and the ethylenediaminetetraacetic acid (EDTA)-modified carbapenem inactivation method (eCIM) were used for the preliminary screening of carbapenemase production. Protocols and results were interpreted using the Clinical and Laboratory Standards Institute (CLSI) guidelines (2020-M100) [25].

Bacterial DNA extraction was performed by the boiling method. Briefly, 3~4 single purified colonies were selected, resuspended in 500 μ L of sterile distilled water, and boiled at 100 °C for 10 min. Then, after centrifugation at 12,000 rpm for 10 min, the supernatant was collected and stored at -20 °C for subsequent use. The carbapenemase-encoding genes: KPC, NDM, VIM, IMP, and OXA-48 were detected by amplicon identification, and the primers used were designed as previously described [7, 26, 27]. All primers used in this study are listed in Supplementary Table 1. The amplified sequences were analyzed and compared to the BLAST database (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Biofilm formation and serum resistance assays

As previously described, the biofilm formation ability was determined using crystal violet staining [28]. Three biological replicates were set in the experiment, and absorbance was measured at 570 nm on a microplate reader (PerkinElmer, USA). The biofilm formation ability of the isolates was classified into four grades: non-adherent (0, $OD \leq OD_C$), weakly adherent (+, $OD_C < OD \leq 2 \times OD_C$), moderately adherent (++ , $2OD_C < OD \leq 4 \times OD_C$), and strongly adherent (+++ , $4OD_C < OD$).

We evaluated the virulence potential of these isolates by serum resistance assays [29]. Briefly, fresh colonies were selected from blood agar plates, and the concentration of bacterial suspension was adjusted to 0.5 in McFarland standards and diluted to 1×10^6 CFUs/mL with sterile LB (Luria-Bertani) broth. Then 25 μ L of bacterial solution was mixed with 75 μ L of serum isolated from healthy individuals. The mixture was incubated at 37 °C by shaking, diluted at 0, 1, 2, and 3 h, and inoculated on LB agar plates for counting.

Conjugation assay

Conjugation assay was conducted to determine whether *bla*_{NDM-5}-harboring plasmids are capable of horizontal transfer. *bla*_{NDM-5}-positive *K. pneumoniae* isolates were used as donors, and rifampicin-resistant *E. coli* EC600

retained in our laboratory were the recipient isolates. The donors and recipients were co-cultured in antibiotic-free LB broth at 2:1 for 18 h at 37 °C [30]. Then, 100 μ L of the mixture was inoculated in Mueller-Hinton (MH) medium containing 1 mg/mL rifampicin (SOLARBIO, China) and 4 μ g/mL meropenem (MACKLIN, China) at 37 °C for 16–18 h.

Whole genome sequencing (WGS) and analysis

A fresh colony was selected, inoculated in LB liquid medium, and incubated at 37 °C for 16 h. Subsequently, the sediment was enriched by centrifugation at $12,000 \times g$. Total bacterial DNA was prepared according to the protocol recommended by the manufacturer of the reagent (Promega, USA). Purified genomic DNA was quantified by TBS-380 fluorometer (Turner BioSystems Inc., Sunnyvale, CA). The prepared libraries were then used for paired-end sequencing (2 \times 150 bp) on an Illumina HiSeq X Ten sequencing platform. The raw reads obtained after sequencing were filtered using FASTp (version 0.19.6) and then assembled using SOAP denovo (version 2.04). The predicted CDSs were annotated from the NR (latest version), Swiss-Prot (version 2017.04.10), and Pfam (version 31.0) databases using BLAST+ (version 2.3.0), Diamond (version 0.8.35), and HMMER (version 3.1b2) tools for sequence alignment. Additionally, genomic DNA was extracted from the selected Kp1 (LZKP00001) and Kp3 (LZKP00003) cells using a magnetic-bead-based kit (Qiagen, Germany). Purified DNA was sequenced using the Illumina NovaSeq 6000 platform with a read length of PE150 and the Oxford Nanopore platforms. De novo assembly was performed using continuous long sequence reads following the Canu workflow (version 1.7) assembly pipeline. Prokka software (version 1.10) was used to predict the coding genes. Chromosomal point mutations and acquired antimicrobial resistance genes were detected by ResFinder 4.1 (<https://cge.cbs.dtu.dk/services/ResFinder/>). The mobile elements were analyzed using PlasmidFinder 2.1 (<https://cge.cbs.dtu.dk/services/PlasmidFinder/>) and IS Finder (<https://www-is.biotoul.fr/blast.php>). Virulence factors were analyzed using the virulence factor database (VFDB) (<http://www.mgc.ac.cn/VFs/main.htm>). We used Call SNPs & Infer Phylogeny (CSI Phylogeny, <https://cge.cbs.dtu.dk/services/CSIPhylogeny/>) to determine the single nucleotide polymorphisms (SNPs) between similar genomes and construct phylogenetic tree [31]. Phylogenetic trees were visualized and polished using ITOL (<https://itol.embl.de/>). Additionally, we retrieved the genome of a *K. pneumoniae* isolate in the NCBI database (SCKP090620, GenBank: GCA_014655055.1) for comparison with the isolate in this study. BLAST Ring Image Generator (BRIG) 0.95 software was used for comparative circular genome mapping.

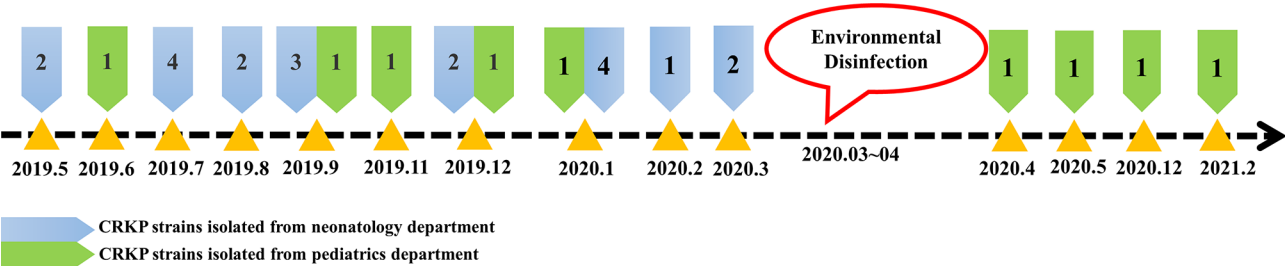


Fig. 1 Timeline of this outbreak mediated by twenty-nine CRKP isolates (2019.05–2021.02). Blue represents CRKP isolates isolated from neonatal patients. Green represents CRKP 2407 isolated from pediatric patients. The numbers in the pentagonal arrows represent the number of CRKP isolates

Results

Epidemiological investigation

From May 2019 to May 2020, we successfully isolated 27 CRKP isolates from pediatric patients (neonates: $n=20$; infants: $n=7$) at the Affiliated Hospital of Southwest Medical University (Luzhou, China). Due to COVID-19, prevention and control measures have decreased the flow of people and hospitalized pediatric patients. In late March 2020, healthcare workers disinfected the pediatric (including neonatal) settings, and the disinfection process lasted for a month. First, the health workers used 75% alcohol to wipe the surfaces of the pediatric wards, incubators, and ventilators. Then, they locked the wards and used ultraviolet (UV) radiation and ozone to disinfect the environment for 48 h. One month later, UV radiation and ozone were used again to disinfect the environment. The timeline of this outbreak is shown in Fig. 1. Between December 2020 and February 2021, two CRKP isolates were isolated again from patients. Overall, 13 and 7 CRKP isolates were isolated from neonatal patients in 2019 and 2020, respectively. Eight CRKP isolates were isolated from infant patients in 2019–2020 (Fig. 1).

Of the 19 neonatal patients in this outbreak, very low birth weight (<1500 g) and low birth weight (<2500 g) newborns accounted for 63% ($n=12$, 12/19) and 26% ($n=5$, 5/19) of the infected patients, respectively [32]. Bronchopneumonia was the most prevalent condition of other pediatric patients ($n=10$), accounting for 90% (9/10). Most patients had favorable clinical outcomes (90%, 26/29) in this outbreak, with no deaths (Table S2). This study investigated the possible causes of this outbreak by analyzing the microbiological and molecular characteristics of these pathogens.

Isolate collection, identification, and antimicrobial susceptibility test

From May 2019 to May 2021, a total of twenty-nine non-repetitive CRKP isolates were isolated from pediatric patients (neonates and infants), and susceptibility testing showed that these isolates were resistant to meropenem, ertapenem, and imipenem. Of them, 19 isolates were isolated from neonates, which accounted for 66% (19/29),

Table 1 Antimicrobial susceptibility testing of the twenty-nine carbapenem-resistant *K. pneumoniae*

Antimicrobial agents	Total (<i>n</i> = 29) <i>R</i> (%)	MIC ₅₀ (μg/ml)	MIC ₉₀ (μg/ml)
Piperacillin	29 (100%)	> 64	> 64
Piperacillin-tazobactam	29 (100%)	> 128/2	> 128/2
Cefepime	29 (100%)	> 16	> 16
Ceftriaxone	29 (100%)	> 32	> 32
Meropenem	29 (100%)	64	128
Ertapenem	29 (100%)	64	128
Aztreonam	28 (97%)	> 16	> 16
Gentamicin	0 (0)	< 1	< 1
Amikacin	0(0)	< 4	< 4
Ciprofloxacin	0 (0)	< 0.5	< 1
Tetracycline	0 (0)	< 4	< 4
Trimethoprim-sulfamethoxazole	0 (0)	< 2/38	< 2/38
Tigecycline	0 (0)	< 2	< 2
Polymyxin B	0 (0)	< 1	< 1

and the rest were isolated from infant patients (34%, 10/29). These isolates were identified by MALDI-TOF (Brucker, Germany) as *K. pneumoniae*. These bacteria were mainly isolated from the respiratory tract, including sputum ($n=18$, 62%) and endotracheal tubes ($n=5$, 17%), followed by blood ($n=4$, 14%), pus ($n=1$, 3%) and ascites ($n=1$, 3%). The isolates from pediatric patients were all isolated from sputum ($n=10$, 34%), and all the data are presented in Supplementary Table 2. Antimicrobial susceptibility testing showed that all isolates resisted cephalosporins and carbapenems ($n=29$, 100%). Except for Kp1, which is susceptible to aztreonam, the other twenty-eight isolates were resistant to aztreonam. However, this study did not find isolates resistant to aminoglycosides, tetracycline, fluoroquinolones, tigecycline, or colistin (Table 1).

Table 2 General characteristics of twenty-nine carbapenem-resistant *K. pneumoniae* isolates

Characteristics	Carbapenem-resistant <i>K. pneumoniae</i> (n,%)	
Department	Pediatric (10, 34%)	Neonatal (19, 66%)
Source	Sputum (18, 62%); Endotracheal tubes (5, 17%); Blood (4, 14%); Pus (1, 3%); Ascites (1, 3%)	
MLST	ST2407 (28, 97%), ST35 (1, 3%)	
β-lactamase genes	<i>bla</i> _{NDM-5} (29, 100%), <i>bla</i> _{DHA-1} (28, 97%), <i>bla</i> _{SHV-1} (28, 97%) <i>bla</i> _{CTX-M-14} (28, 97%), <i>bla</i> _{SHV-33} (1, 3%)	
Incompatibility group	FIB (28, 97%), X3 (29, 100%)	
Capsular serotypes	K25 (28, 97%), K22.37 (1, 3%)	
Virulence factor	ST2407 (n = 28, 97%): <i>fyuA</i> , <i>irp1/irp2</i> , <i>mrkABCDHFUI</i> , <i>ybtAEPQSTUX</i> , etc.	ST35 (n = 1, 3%): <i>kfuABC</i> , <i>mecABCDEGHUI</i> , <i>mrk-ABCDHFUI</i> , etc.
<i>ramR</i>	ST2407 (n = 28, 97%): A19V and K194* ST35 (n = 1, 3%): K194*	
other	ST2407 (n = 28, 97%): <i>qacEΔ1</i> , <i>sul1</i> , and <i>qnrB</i> (n = 28, 97%)	

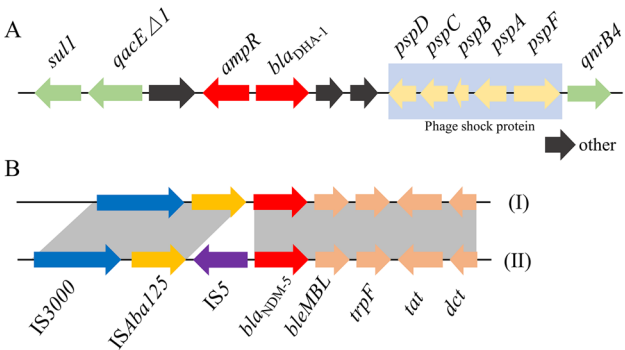


Fig. 2 Genetic structure around the *ampR* (A) and *bla_{NDM-5}* (B) genes of twenty-nine CRKP isolates in the present study and comparison with similar sequences. *sul1*: sulfonamide-resistant dihydropteroate synthase), *qacEΔ1*: quaternary ammonium compound efflux SMR transporter QacE delta 1), *ble_{MBL}*: bleomycin resistance gene, *trpF*: encodes a phosphoribosylanthranilate isomerase, *qnrB4*: quinolone resistance pentapeptide repeat protein *QnrB4*, *bla_{DHA-1}*: extended-spectrum class C beta-lactamase DHA-1, *tat*: encodes a twin-arginine translocation path-way signal sequence domain protein

Carbapenem-encoding genes in CRKP isolates

The combined mCIM and eCIM revealed that all twenty-nine isolates carried metallo-β-lactamases (*n*=29, 100%), and their activity could be inhibited by EDTA (Table S3). In addition, we demonstrated by PCR that all twenty-nine CRKP isolates carried the *bla_{NDM}* genes (Fig.S1).

Whole genome analysis

Through WGS, we obtained twenty-nine *K. pneumoniae* whole genomes, all with GC contents between 57 and 58%. The sequence type of Kp1 isolated in December 2020 belonged to ST35, while the remaining twenty-eight CRKP isolates all belonged to ST2407 (Table 2). WGS reconfirmed that all twenty-nine CRKP isolates carried *bla_{NDM-5}*. Furthermore, the plasmid replicon IncX3 was ubiquitously present in these isolates, accounting for 100% (29/29). In addition to Kp1, the remaining ST2407 isolates harbored IncFIB, accounting for 97% (28/29). Additionally, twenty-eight aztreonam-resistant ST2407

isolates also carried *bla_{SHV-1}*, *bla_{CTX-M-14}*, *bla_{DHA-1}*, *qnrB4*, *sul1* and *qacEΔ1*. The *bla_{CTX-M-14}* and *bla_{DHA-1}* genes were located on the same scaffold, and the LysR family transcriptional regulator encoding the *ampR* gene is adjacent to *bla_{DHA-1}* (Fig. 2A).

Furthermore, Kp1 carried *bla_{SHV-33}*, but *bla_{CTX-M-14}* and *bla_{DHA-1}* were not detected. We found A19V and K194* point mutations in the *ramR* gene in 28 ST2407 isolates, whereas Kp1 only had K194* point mutations in *ramR*. The 28 ST2407 isolates also exhibited consistent mutation profiles of the *acrR* gene, including A151V, G164A, P161R, F172S, F197I, R173G, L195V, and K201M, which were not found in Kp1 (Table 2).

The virulence factors of these isolates were analyzed using the VFDB. The results showed that all twenty-nine CRKP isolates carried multiple protein-encoding genes associated with adhesion (*mrkABCDHFUI*, *fimABCGEF-GHIK*), iron uptake (*entABCDEFES*, *fepABCDG*), regulation of capsule synthesis (*rcsAB*), and T6SS (*impAFGHJ*). Kp1 sequencing also identified the *pilW* gene, which can encode Type IV pili in *Yersin*, and the *farB* gene, which encodes the far efflux system in *Neisseria*. The ST2407 group also carried *papC* and *papD* genes, which encode P fimbriae belonging to *Escherichia*. Significantly different from Kp1, the ST2407 group also harbours various genes encoding yersiniabactin: *fyuA*, *irp1/irp2*, and *ybtA-EPQSTUX* (Table 2). We did not find the hypervirulent isolates-specific markers *rmpA/rmpA2* and *iucA* in the genomes of the twenty-nine CRKP isolates. Moreover, the capsular serotypes of the ST2407 group were K25, while that of Kp1 was K22.37.

To further analyze the genetic and evolutionary relationships of these isolates, we constructed a phylogenetic tree. The phylogenetic tree showed that the *K. pneumoniae* in this outbreak was divided into two branches, which was consistent with the results of the multilocus sequence typing (MLST) analysis. The only Kp1 isolate in branch 1 was ST35, while the twenty-eight ST2407 isolates were located in branch 2 without obvious

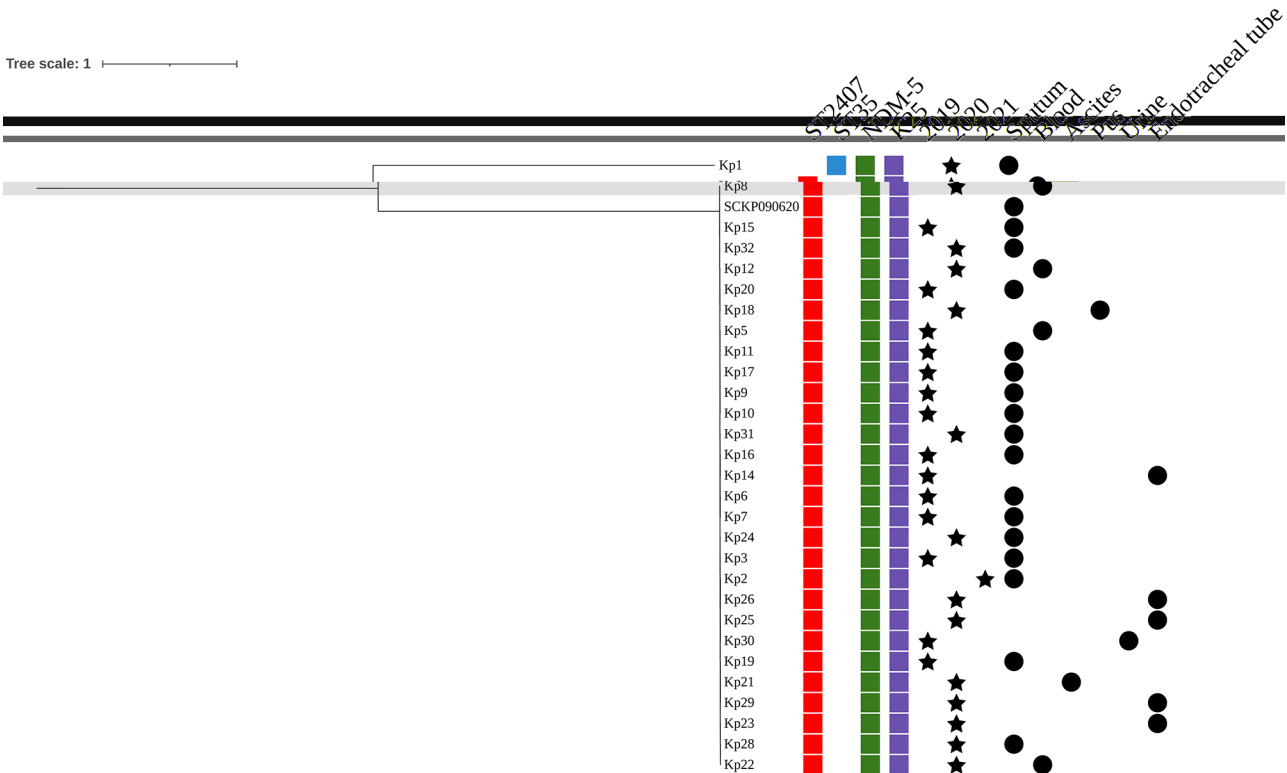


Fig. 3 Phylogenetic tree inferred with CSI Phylogeny 1.4 based on genome sequencing

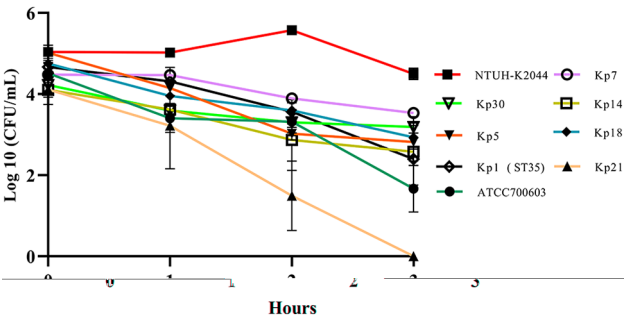


Fig. 4 Serum resistance of seven representative carbapenem-resistant *K. pneumoniae* ST2407 isolates and one carbapenem-resistant *K. pneumoniae* ST35 isolate. Positive control: NTUH-K2044; Negative control: ATCC700603

differences. All ST2407 isolates isolated from pediatric patients from 2019 to 2021 in this outbreak are closely related in terms of binding evolutionary relationships and genetic relatedness. These results also support the clonal spread of the ST2407 group in pediatric patients for up to one and a half years (Fig. 3). The ST2407 group in this study belongs to the same lineage as a *bla*_{NDM-5}-producing ST2407 isolate previously isolated from Chengdu, Sichuan Province (China), which supports the cross-regional transmission of these pathogens.

Biofilm formation ability and serum resistance

Since the isolates were mainly isolated from the respiratory tract and endotracheal tubes, we determined their biofilm formation ability. The twenty-nine CRKP isolates showed certain biofilm formation ability, with those of moderately adherent ability accounting for 41% (2+, *n*=12) and those of strongly adherent ability accounting for 55% (3+, *n*=16), while Kp1 displayed weak adherent ability (Table S4).

Considering the clonal transmission of these isolates, eight representative *K. pneumoniae* isolates were selected from different infection sites and evaluated for their serum resistance. As shown in Fig. 4, compared with that of positive control, the serum resistance of ST2407 isolates derived from blood and respiratory tract and wound secretions did not show significant differences. However, the ST2407 isolate Kp21 isolated from ascites showed sensitivity to serum, with a dramatic decrease in colony count at the second hour (Fig. 4) (Table S5). Since this phenomenon may be related to factors such as capsular defects, we further focused on our analysis of point mutations in the *rcsAB*, *csrD*, and *pal* genes. The sequence alignment results showed that the sequences of the *rcsAB*, *csrD*, and *pal* genes of the eight ST2407 isolates derived from different parts were identical. Further analysis showed that the frameshift deletion in the *oqxR* carried by Kp21 compared to the other strains.

Analysis of *bla*_{NDM-5}-harboring plasmids

In this study, conjugation experiments revealed that from the twenty-nine CRKP isolates carrying *bla*_{NDM-5} plasmids, only Kp1 was successfully transferred to *E. coli* EC600. After acquiring *bla*_{NDM-5}-harboring IncX plasmids, the recipient *E. coli* EC600 showed dramatically reduced susceptibility to β -lactam antibiotics other than aztreonam. The plasmid replicon typing results revealed that all twenty-eight CRKP ST2407 isolates carried three incompatibility (Inc) groups: namely FIB ($n=28$, 97%) and X3 ($n=29$, 100%), while Kp1 (ST35) had only one incompatibility (Inc) group, namely X3 ($n=1$, 3%).

The genetic surrounding environment of *bla*_{NDM-5} carried by these CRKP isolates consisted of a relatively conserved region: *bla*_{NDM-5}-*ble*_{MBL} (encoding bleomycin binding protein)-*trpF* (encoding phosphoribosylanthranilate isomerase)-*tat* (encoding twin-arginine translocation pathway signal sequence domain protein), followed by *umuD* (encoding SOS mutagenesis and repair protein), IS26, and *dct* (encoding divalent-cation tolerance protein CutA). Additionally, *bla*_{NDM-5} was flanked by IS3000, IS*Aba*125, and IS5. For type I, IS5 was missing. Sequence alignment revealed that most isolates had an approximately 13 kb region harboring *bla*_{NDM-5} (Fig. 2B).

In-depth sequencing indicates that Kp1 carries an IncX plasmid carrying *bla*_{NDM-5} with a total length of 46,611 bp. We searched the NCBI database and found

that p1NDM-5 was similar to the plasmid p1678-4 from a *K. pneumoniae* isolate isolated in Shanghai (China) with a query coverage and per cent identity >99.9%. The subsequent comparison of p1NDM-5 was compared by sequence alignment with similar *bla*_{NDM}-carrying plasmids isolated from other parts of China, and it was found that the sequence structure of p1NDM-5 is consistent with these plasmid sequences. The most apparent difference between p1NDM-5 and pNDM5_020042 was that IS*Aba*125 of pNDM5_020042 was truncated. In particular, p1NDM-5 exhibited 100% query coverage and 99.98% identity with the plasmid p25NDM-5 isolated from an *E. coli* strain isolated in Luzhou (Fig. 5A).

The acquired antimicrobial resistance genes of Kp3 were mainly on the plasmids p3NDM-5 and p3IncF. The p3IncF plasmid was 190,701 bp in length with a GC content of 51.07%, and its replication type was IncFIB. The p3IncF carried *bla*_{CTX-M-14}, *sul1*, *bla*_{DHA-1}, *qnrB4* and *qacEΔ1*. The plasmid backbone of p3IncF was similar to that of pNH25.1 (GenBank accession number: CP024875.1), with coverage and percentage identity of 89 and 99.97%, respectively. The multiple sequence alignment revealed that pA2359-IMP, pNH25.1, and p59062CZ_IncFIB were missing a region containing the phage shock protein compared with p3IncF (Fig. 5B). The p3NDM-5 plasmid is also an IncX3 plasmid carrying *bla*_{NDM-5}, with a length of 27,668 bp. The p3NDM-5

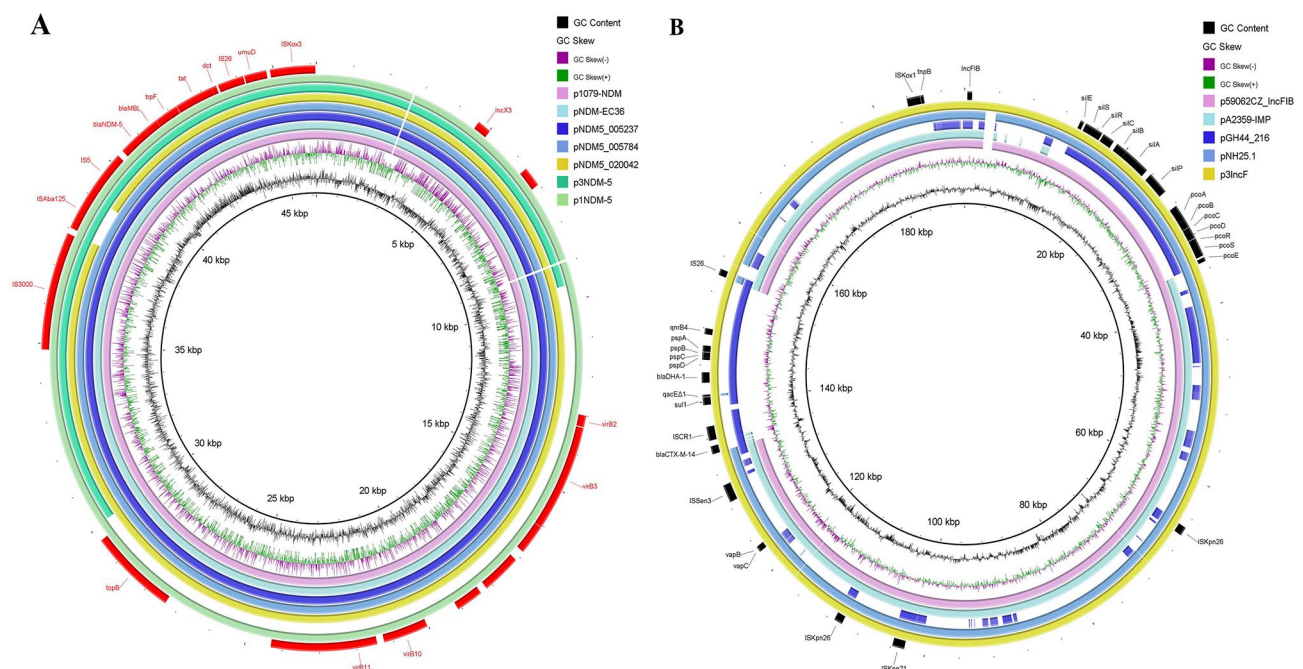


Fig. 5 (A) Circular comparison of p1NDM-5 (GenBank accession number: CP089987.1) and p3NDM-5 (GenBank accession number: CP089991.1) with other IncX3 plasmids carrying *bla*_{NDM-5}. pNDM-EC36 (GenBank accession number: MG591703.1), pNDM5_005784 (GenBank accession number: CP028577.1), pNDM5_005237 (GenBank accession number: CP026577.1) and p1079-NDM (GenBank accession number: MG825384.1) were isolated from *E. coli* isolates. pNDM5_020042 was isolated from an *E. hormaechei* isolate. (B) Circular comparison of p3IncF with other similar plasmids. The blank parts represent areas that are missing from each other

plasmid exhibited 99.94% identity compared to the p1NDM-5 plasmid under a 55% query coverage. For the *bla*_{NDM-5} genetic surrounding environment, p3NDM-5 was consistent with p1NDM-5, and its *ISAbi125* was not truncated. In-depth analysis revealed that some *virB11*, *virB10*, *virB2*, and *virB3* genes responsible for encoding the T4SS system of p3NDM-5 were lost (Fig. 5A).

Discussion

This study describes an outbreak of CRKP ST2407 among pediatric patients in a university hospital in Southwest China. Continuous monitoring revealed that these pathogens were prevalent in pediatric patients for nearly a year. Most of the newborns in this outbreak had low birth weight (<2500 g) or very low birth weight (<1500 g) and usually had a higher risk of infection than healthy children [33] (Table S2). However, most patients in this study had good clinical outcomes and survived even after developing bloodstream infections. These isolates showed hypovirulence in serum resistance assays and may not produce an adequate number of infectious bacteria in sterile body fluids and are eliminated. Previous studies in neonates showed that septicemia caused by NDM-producing isolates did not result in higher mortality [34]. The mortality rate of *bla*_{NDM}-producing *K. pneumoniae* also appears to be significantly lower than the high mortality rate of *bla*_{KPC}-producing *K. pneumoniae* causing clinical infections [35]. In the clinical treatment of NDM-producing isolates, although many drugs showed resistance in vitro, they achieved good results in vivo [36]. Additionally, in this study, all pediatric patients were treated with beta amide antibiotics after infection with CRKP, and most patients were discharged with improvement (Table S2). One of the reasons for this phenomenon may be the zinc ion dependence of metalloenzyme-mediated resistance, in which the difference in ex vivo zinc ion concentration affects the hydrolytic activity of NDM on β -lactam antibiotics [54]. Therefore, there is a need to further evaluate the clinical significance of infections with metalloenzyme-producing isolates.

The prevalence of *K. pneumoniae* ST2407 has also been previously reported in pediatric patients in Shenzhen and Hunan, China. Nevertheless, no *bla*_{NDM-5}-carrying ST2407 isolates were identified, and WGS was not performed at that time [37, 38]. The phylogenetic tree revealed that the ST2407 group in this study is highly homologous to a completely sequenced ST2407 isolate from a medical setting in Chengdu, Sichuan (China), which indicates that the clone has the possibility of cross-regional transmission and long-term colonization in the environment (Fig. 2) [39]. However, a larger WGS study is still necessary to gain insight into the prevalence and evolutionary history of the ST2407 group in China. However, *K. pneumoniae* ST35 is widely found in humans,

animals, and the environment (<https://bigsdbs.pasteur.fr/>). An ST35 CRKP isolate isolated from pediatric patients was previously reported in Jiangsu, China [40]. Based on those findings and the phylogenetic tree results, we believe that Kp1 was a sporadic case and had no apparent relationship with the ST2407 group in this study. However, the homology of the *bla*_{NDM-5}-producing plasmid carried by Kp1 and a locally resistant plasmid from *E. coli* also suggests horizontal antimicrobial resistance transfer.

In developing countries, contaminated environmental reservoirs may be the primary source of these resistant microorganisms [19]. In this study, healthcare workers took timely disinfection measures for surfaces in pediatric and neonatal settings, which effectively controlled the outbreak's development and supported the notion that the hospital setting might have been the source of this outbreak. Additionally, inappropriate and prolonged use of antibiotics also exacerbates the antimicrobial resistance rate of bacteria isolated from pediatric patients [19]. To our knowledge, no previous study has reported the isolation of such a large number of metallo-beta-lactamase-producing *K. pneumoniae* isolates in pediatric patients. IncX3 was the incompatibility group in these *bla*_{NDM}-carrying plasmids, consistent with previous studies suggesting that IncX3 plasmids are significant carriers of *bla*_{NDM} transmission in China [41]. However, we did not find the transfer of IncX3 plasmids carrying *bla*_{NDM-5} to the recipient in conjugation experiments. This may be due to the loss of the T4SS system responsible for conjugation and mobilization. A previous study has shown that inhibition of type IV secretion traffic ATPase VirB11 activity significantly reduces the transfer frequency of X3 plasmids carrying *bla*_{NDM-5} [14]. In another study, the horizontal transformation of IncX plasmid carrying *bla*_{NDM-1} isolated from *K. pneumoniae* into *E. coli* was demonstrated by transformation [15]. Therefore, whether the *bla*_{NDM-5} plasmids carried by the isolates in this study can be transferred horizontally by other means needs to be further investigated.

Almost all of the twenty-nine CRKP isolates in this study carried the identical gene environment *bla*_{NDM-5}. For the deletion of IS5 of type A, we speculated that can be affected by the limitation of sequencing technology. CTX-M-14, a variant of CTX-M-9, exhibited strong hydrolytic activity against cefotaxime and weak hydrolytic activity against ceftazidime and aztreonam [42]. Furthermore, *bla*_{DHA} is a class of plasmid-mediated AmpC β -lactamases, and *bla*_{DHA-1} was previously shown to be associated with aztreonam resistance [43]. We concluded that the aztreonam resistance of these twenty-eight NDM-5-producing ST2407 isolates is mainly mediated by *bla*_{DHA-1}. The *ramR* gene in the twenty-eight ST2407 isolates also identified mutations previously present in tigecycline-resistant isolates, but these mutations did not

appear sufficient to confer the level of clinical resistance to tigecycline. Tigecycline is a relatively rare antibiotic for treating infections in pediatric patients. It is unclear whether these mutations are pre-existing or are caused by other selection pressures.

Our results revealed that the biofilm formation ability of the ST2407 group may promote its long-term spread or colonization in pediatric patients for a long time (Table S4). The biofilm formation ability of *K. pneumoniae* is often related to factors such as fimbriae, lipopolysaccharides, and capsules. Type I and III fimbriae coding genes are usually located at core chromosomal loci and regulate adhesion and biofilm formation [44, 45]. In particular, twenty-eight ST2407 isolates carried plasmid-mediated AmpC enzymes and were controlled by *ampR* (Fig. 4). *AmpR* is involved in the expression of various virulence factors in bacteria. *K. pneumoniae* can be involved in capsular synthesis, antiserum killing, biofilm formation, fimbriae synthesis, and intestinal colonization [46]. In addition to adhering tightly to cellular and abiotic surfaces and not being efficiently removed, biofilms can also resist the penetration of antibiotics and promote the transfer of resistance genes, ultimately leading to treatment failure [47]. Additionally, the *qacEΔ1* and *sul1* genes are usually located on an integron and can mediate resistance to some clinically common disinfectants (Table 2) [48]. Thus, the presence of these antiseptic resistance genes also contributes to these microorganisms to colonize hospital settings in the long term. Although the twenty-eight ST2407 isolates showed consistent capsular serotypes and virulence gene patterns and did not show to serum, the results of serum resistance assays were still heterogeneous (Fig. 4). Further analysis showed that the disruption of the *oqxR* gene of Kp21 might be an essential reason for this result. *OqxR* is a transcriptional regulator that can regulate the expression of the multidrug efflux pump *OqxAB* [49]. In *K. pneumoniae*, the disruption of *oqxR* is associated with its outer membrane protein production and membrane permeability [50]. Outer membrane integrity is also one of the essential factors of immune phagocytosis mediated in *K. pneumoniae* [51]. The *rarA-oqxABR* locus was also shown to be associated with the maintenance of *K. pneumoniae* virulence in a previous study [52]. How the disruption of *oqxR* occurs is still unclear, and the contribution of *oqxR* disruption to Kp21 virulence needs to be further confirmed.

We have not observed any CRKP outbreaks in pediatric patients since March 2020, indicating the effectiveness of these disinfection measures. We have continued surveillance and found that a CRKP ST2407 isolate was isolated from an infant patient in February 2021 (Fig. 1). A possible explanation for this is that most newborns are in isolation, and infant patients tend to be in an environment where patients move more frequently. After the complete

disinfection of the pediatric and neonatal wards, the genetic phenotypic isolate did not appear again in the neonatal ward, and the resistant isolate with the same genetic phenotype soon appeared in the pediatric ward, indicating that these genetic phenotypic drug-resistant isolates spread in the hospital ward and community, especially in the community. Although the virulence of this phenotypic isolate may be weak, great attention must be paid to its transmission route. Therefore, continuous monitoring of the transmission of this phenotypic isolate in hospitals and communities will continue to be the focus of our ongoing investigation and research in the future.

The limitations of this study must be acknowledged. First, this is a retrospective study. Thus, we did not determine the source of the epidemic. Second, we did not conduct an epidemiological survey in other hospital wards. Third, we did not investigate the molecular epidemiology of related isolates in the community. The above shortcomings will be addressed in later research. However, in this study, we cannot exclude the possibility that the hospital environment may be the source of these infections. Healthcare facilities still need strict and effective infection control measures, including hand hygiene, contact isolation, and active screening culture, to address the spread of CRKP in pediatric patients [53].

Conclusion

Overall, we described the complete process of a CRKP outbreak in pediatric patients from beginning to end at a teaching hospital in Southwest China. Among the twenty-nine CRKP isolates, twenty-eight belonged to ST2407 and had highly similar resistance profiles, virulence characteristics, and genetic backgrounds, supporting the notion of clonal spread of these microorganisms in pediatric patients. Therefore, strict monitoring and infection control measures should be taken to prevent the outbreak of CRKP in pediatric patients.

Abbreviations

CRKP	Carbapenem resistant <i>Klebsiella pneumoniae</i>
MIC	Minimum inhibitory concentration
WGS	Whole genome sequencing

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12941-024-00747-7>.

Supplementary Material 1: Additional file 1. Supplementary Table 1 PCR primers for detecting carbapenemase-encoding genes used in this study.

Supplementary Material 2: Additional file 2. Supplementary Table 2 Individual characteristics of twenty-nine non-repeat patients with CRKP infections.

Supplementary Material 3: Additional file 3. Supplementary Table 3 The results of mCIM and eCIM tests.

Supplementary Material 4: Additional file 4. Supplementary Table 4 The

biofilm formation capacity of twenty-nine CRKP isolates in this study.

Supplementary Material 5: Additional file 5. Supplementary Table 5 Serum resistance of 7 representative carbapenem-resistant *K. pneumoniae* ST2407 isolates and 1 carbapenem-resistant *K. pneumoniae* ST35 isolate.

Supplementary Material 6: Additional file 6. Supplementary Fig. 1 *bla*_{NDM} genes detected by agarose gel electrophoresis.

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Not applicable.

Author contributions

ZZ, CY, JH, and MT isolated isolates and designed the study. JH, XX, and CJ performed the assays. ZZ, JH, YD, and CY analyzed the data. ZZ, JH and YD wrote and revised the manuscript. JG, YD, and JL contributed reagents and materials. JL supervised the study. All authors contributed to manuscript revision, read, and approved the submitted version.

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Data availability

All data supporting the findings of this study are available either within the article or in the supplemental material. All sequenced genomes in this study were uploaded to the NCBI database under the project accession number: PRJNA787439.

Declarations

Ethics approval and consent to participate

The work design has been approved by the Affiliated Hospital of Southwest Medical University Ethics Committee (KY2022267).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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