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ORIGINAL RESEARCH

Genomic Characteristics of a Carbapenem-Resistant *Klebsiella pneumoniae* Co-Carrying bla_{NDM-5} and bla_{KPC-2} Capsular Type KL25 Recovered from a County Level Hospital in China

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Background: Hypervirulent carbapenem-resistant *K. pneumoniae* (hv-CRKP) has been spreading rapidly worldwide. Here, we investigated the genomic characteristics of ST11 *K. pneumoniae* isolate SM117 with capsular serotype KL25, co-carrying bla_{NDM-5}, two copies of bla_{KPC-2} and multiple plasmid-borne virulence genes from a county level hospital in China.

Methods: Antimicrobial susceptibility of *K. pneumoniae* SM117 was evaluated. The Illumina NovaSeq 6000 and Oxford Nanopore MinION platforms were applied to sequence the genome and then *de novo* assembled. The genome sequence was annotated using the NCBI Prokaryotic Genome Annotation Pipeline and further subjected to identify the sequence type (ST), capsular type, antibiotic resistance genes, plasmid replicon types and virulence genes. The phylogenetic analysis was performed based on the core genome single nucleotide polymorphisms (cgSNPs) using CSI Phylogeny 1.4, and further visualized by Interactive Tree of Life (iTOL) V5 web server.

Results: The whole-genome sequence of *K. pneumoniae* SM117 is made up of eight contigs totaling 6,104,486 bp, contain a 5,612,620 bp single chromosome and seven plasmids. The isolate was assigned to ST11 with capsular serotype KL25, co-carrying including bla_{NDM-5}, bla_{KPC-2} and multiple plasmid-borne virulence genes including *rmpA2* and aerobactin genes *iucABCD-iutA*. The coexistence of bla_{KPC} and bla_{NDM} in *K. pneumoniae* strains exhibit a high degree of resistance to β-lactam antibiotics. The strain SM117 also carries multiple antibiotic resistance genes, making it resistant to all antibiotics except polymyxin. The closest relative of *K. pneumoniae* C793 was identified in 2023 from a hospital surface sample in Zhejiang, China, with just 52 SNPs difference.

Conclusion: This study reported the genomic characteristics of a multidrug-resistant ST11 *K. pneumoniae* with capsular serotype KL25, co-carrying bla_{NDM-5}, two copies of bla_{KPC-2} genes and multiple plasmid-borne virulence genes in China. These findings will provide important knowledge of the antibiotic resistance mechanisms, genomic epidemiological characteristics and transmission dynamics of multidrug-resistant *K. pneumoniae*.

Keywords: whole-genome sequencing, *K. pneumoniae*, multidrug-resistance, bla_{NDM-5}, bla_{KPC-2}, *rmpA2*, *iucABCD-iutA*, hvCRKP

Introduction

Given the elevated mortality rates linked to severe infections and the restricted therapeutic options at hand, the spread of carbapenem-resistant gram-negative bacteria has emerged as a major worry within hospital environments globally.[1] The two main carbapenemases in the Enterobacteriaceae family are *K. pneumoniae* carbapenemase (KPC) and New Delhi metallo-β-lactamase (NDM), encoded by the bla_{KPC} and bla_{NDM} genes, respectively.[2] Carbapenem-resistant

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Enterobacteriaceae (CRE) that produce KPC and NDM type enzymes in particular exhibit a high degree of resistance to nearly all presently available β-lactam antibiotics, poses a significant threat to global public health.

Klebsiella pneumoniae is a significant bacterial pathogen associated with both hospital- and community-acquired illnesses such as pneumonia, sepsis, urinary tract infection, bacteraemia, meningitis, and pyogenic liver abscess.[3,4] *K. pneumoniae* has historically caused serious infections, mainly in those with impaired immune systems, however, due to the recent appearance of *K. pneumoniae* strains that have acquired new genetic features and are either hypervirulent or antibiotic resistant, the rate of infection has increased. Carbapenem-resistant *K. pneumoniae* (CRKP) is recognized as a significant public health concern by the World Health Organization. In the European Union and China, CRKP strains account for 70–90% of clinical carbapenem-resistant Enterobacteriaceae infections.[5] A recent meta-analysis projected a mortality rate of 42% for CRKP in healthcare-associated infections (HAI), compared to 21% for carbapenem-susceptible strains of *K. pneumoniae*.[6] In recent years, most nations have reported the coexistence of bla_{KPC} and bla_{NDM} in *K. pneumoniae* strains.[7] What is worse, *K. pneumoniae* strains coharboring bla_{KPC} and bla_{NDM} can acquire or transmit additional antimicrobial resistance genes, such as extended-spectrum β-lactamase (ESBL) genes, fluoroquinolone resistance genes, and aminoglycoside resistance genes, causing high level of resistance to the majority of routinely used antibiotics, posing serious difficulties for therapeutic treatment.[2] The genomic features and antimicrobial resistance of bacterial pathogens in low-income and middle-income countries might be underestimated due to the paucity of genomic data.[8] Here, we present the genomic characteristics of a KL25-ST11 *K. pneumoniae* strain co-carrying bla_{KPC-2} and bla_{NDM-5} from a county-level hospital in China.

Material and Methods

In January 2023, a carbapenem-resistant *K. pneumoniae* strain was discovered from a sputum sample of a 72-year-old man hospitalized with unconsciousness after intracerebral hemorrhage in a county hospital in China. The strain was initially identified by Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS VITEK, bioMérieux), and then subjected to 16S rRNA gene sequencing. Antimicrobial susceptibility testing was performed for the following antimicrobial agents: amikacin, gentamicin, cefuroxime, ceftazidime, ceftriaxone, cefepime, cefoperazone/sulbactam, piperacillin/tazobactam, amoxicillin/clavulanate, imipenem, ertapenem, ciprofloxacin, levofloxacin, tigecycline, tetracycline, trimethoprim-sulfamethoxazole and polymyxin. The minimum inhibitory concentrations (MICs) were determined according to the Clinical and Laboratory Standards Institute (CLSI) 2023 guidelines. The broth microdilution method was employed, using cation-adjusted Mueller–Hinton broth as the culture medium. The procedure involved preparing serial dilutions of the antimicrobial agent in 96-well microtiter plates, inoculating with a standardized bacterial suspension (5×10^5 CFU/mL), and incubating at 35°C ± 2°C for 16–20 hours. The MIC was recorded as the lowest concentration of the drug that visibly inhibited bacterial growth. The breakpoint of tigecycline was based on the standards of the European Committee for Antimicrobial Susceptibility Testing (EUCAST 2019). *Escherichia coli* ATCC 25922 was used as a quality control strain.

A Genomic DNA Purification Kit (QIAGEN, Valencia, CA, USA) was used to extract genomic DNA from the isolate and NanoDrop™ spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to assess the purity and concentration of DNA.[9,10] Whole-genome shotgun sequencing of *K. pneumoniae* SM117 was undertaken using both short-read Illumina NovaSeq 6000 (Illumina Inc., San Diego, CA, USA) and long-read Oxford Nanopore MinION (Oxford Nanopore Technologies, Oxford, UK) platforms to study the mechanisms of antimicrobial resistance. Unicycler 0.4.8 was used to accomplish hybrid assembly of Illumina and Nanopore sequence reads.[11] The genome sequence was automatically annotated by the NCBI Prokaryotic Genomes Annotation Pipeline (PGAP). In silico multilocus sequence typing (MLST) and Plasmid replicon types were identified by BacWGSTdb 2.0 (http://bacdb.cn/BacWGSTdb) webserver.[12,13] The virulence genes were identified in the Virulence Factors of Pathogenic Bacteria Database (VFDB) (http://www.mgc.ac.cn/VFs/main.htm). Capsule (K) and O serotype was predicted by Kaptive (https://kaptive-web.erc.monash.edu). ABRicate 1.0.1 was used in conjunction with ResFinder 4.4.3 (http://genepi.food.dtu.dk/resfinder) and CARD 2020 (https://card.mcmaster.ca) to identify the antimicrobial resistance genes (ARGs) in the genome.[14] Genomic sequences and the associated metadata of 81 *K. pneumoniae* strains (co-carrying bla_{NDM-5} and bla_{KPC-2}) currently deposited in the NCBI GenBank were obtained. The bacterial core genome single nucleotide polymorphism (cgSNP) analysis between *K. pneumoniae* SM117

and those deposited in the public database were determined to construct a phylogenetic tree using CSI Phylogeny 1.4 (https://cge.food.dtu.dk/services/CSIPhylogeny/). Phylogenetic tree was visualized and annotated by Interactive Tree of Life (iTOL) V5 web server.[15] The genome sequences of the chromosome and plasmids of *K. pneumoniae* SM117 have been deposited in NCBI GenBank under accession numbers CP130659-CP130666.

Results and Discussion

K. pneumoniae SM117 formed a white, moist colony after 24 hours of cultivation, and non-hypermucoviscous phenotype. The whole-genome sequence of *K. pneumoniae* SM117 is made up of eight contigs totaling 6,104,486 bp, contain a 5,612,620 bp single chromosome and seven plasmids including pSM117-1 (15,598 bp), pSM117-2 (19,458 bp), pSM117-3 (195,798 bp), pSM117-4 (87,083 bp), pSM117-5 (41,758 bp), pSM117-KPC (45,135bp), and pSM117-NDM (87,036 bp). A total of 5,800 protein-coding sequences, 87 tRNAs, 25 rRNAs, 12 non-coding RNA genes and 301 pseudo genes were identified. The G + C content of the chromosome is 56.8%. SM117 was categorized as ST11 according to the *K. pneumoniae* MLST scheme and the capsular serotype of SM117 was identified as KL25. In recent years, ST11 has emerged as the most transmissible clone, playing a significant role in the escalating prevalence of carbapenem-resistant *K. pneumoniae* in China.[16,17]

The resistome of *K. pneumoniae* SM117 is made up of genes that are responsible for resistance to aminoglycosides [*aadA16, aadA2* and *aph*(*3'*)-*Ia*], β-lactams [$bla_{\text{LAP-2}}$, $bla_{\text{TEM-1}}$, two copies of $bla_{\text{SHV-12}}$, $bla_{\text{SHV-182}}$, $bla_{\text{CTX-M-65}}$, two copies of $bla_{\text{KPC-2}}$, and $bla_{\text{NDM-5}}$], tetracyclines [*tet*(A)], fluoroquinolones [*qnr*S1, *aac*(*6'*)-*Ib-cr* and *qnr*B2], macrolides [*mph*(A)], phenicols (*cat*A2), trimethoprim (*dfrA14* and *dfrA27*), rifampicin (*arr*-3) and sulphonamide (*sul*2 and two copies of *sul*1). The multidrug resistance trait to aminoglycosides, third-generation cephalosporins, carbapenems, quinolone and tetracyclines were adequately explained by these findings (Table 1). Particularly, the coexistence of bla_{KPC} and bla_{NDM} in *K. pneumoniae* strains exhibits a high degree of resistance to nearly all presently available β-lactam antibiotics, increased the burden of medical health care. The strain is only susceptible to polymyxin (MIC = 0.5 μg/mL), making polymyxin the last line treatment choice.

Table 1 Genotyping and Phenotypic Resistance Profile of the K. Pneumoniae SM117

	MIC (mg/L)	Susceptibility	Antimicrobial Resistance Genes
Aminoglycosides			*aadA16*, *aadA2* and *aph*(*3'*)-*Ia*
Amikacin	≥32	R	
Gentamicin	≥128	R	
β-lactams			$bla_{\text{LAP-2}}$, $bla_{\text{TEM-1}}$, $bla_{\text{SHV-12}}$, $bla_{\text{SHV-182}}$, $bla_{\text{CTX-M-65}}$, $bla_{\text{KPC-2}}$, and $bla_{\text{NDM-5}}$
Cefuroxime	≥64	R	
Ceftazidime	≥64	R	
Ceftriaxone	≥64	R	
Cefepime	≥64	R	
Piperacillin/tazobactam	≥64	R	
Amoxicillin/clavulanate	≥32	R	
Cefoperazone/sulbactam	≥64	R	
Ertapenem	≥8	R	
Imipenem	≥16	R	
Cefoxitin	≥64	R	
Fluoroquinolones			*qnr*S1, *aac*(*6'*)-*Ib-cr* and *qnr*B2
Ciprofloxacin	≥2	R	
Levofloxacin	≥4	R	

(*Continued*)

Table 1 (Continued).

	MIC (mg/L)	Susceptibility	Antimicrobial Resistance Genes
Tetracyclines			*tet*(A)
Tigecycline	≥8	R	
Tetracycline	≥64	R	
Macrolide			
Azithromycin	≥32	/	*mph*(A)
Erythromycin	≥32	/	
Others			
Trimethoprim/sulfamethoxazole	≥512/16	R	*sul*1, *sul*2, *dfr*A14 and *dfr*A27
Phenicols	≥32	R	*cat*A2
Rifampicin	≥32	/	*arr*-3
Polymyxin	0.5	S	/

Notes: /, indicates no defined standards for the susceptibility or resistance of the strain to the specified antibiotics.
Abbreviations: MIC, minimum inhibitory concentration; R, resistance; S, susceptible.

Analysis of the genetic environment indicates the IS*Kpn6* upstream of the $bla_{\text{KPC-2}}$ gene, with IS*Kpn27* situated downstream as a truncated fragment within the pSM117-KPC plasmid (Figure 1 A). The genetic environment of the $bla_{\text{NDM-5}}$ revealed that IS*1*, Tn*3*, IS*3000* and IS*5* lies upstream, and IS*26* situated downstream as a truncated fragment (Figure 1 B). Interestingly, another $bla_{\text{KPC-2}}$ gene was located in the chromosome and a resistance gene cluster was formed (Figure 1). The genetic environment of the cluster revealed that IS*Kpn6* lies upstream of $bla_{\text{KPC-2}}$ and IS*Kpn27* situated downstream as a truncated fragment, while the $bla_{\text{SHV-12}}$ was located upstream of the $bla_{\text{CARB-2}}$ gene and the $bla_{\text{CTX-M-65}}$ situated downstream (Figure 1 C). Plasmids are often key vectors in the dissemination of multidrug-resistant phenotypes, as they can carry multiple antibiotic resistance genes and be readily transferred between bacteria via conjugation. The presence of resistance genes on plasmids in this strain indicates that plasmid-mediated lateral gene transfer may have played a significant role in its ability to survive in the presence of multiple antibiotics. A large number of virulence genes, including *rmpA2* (regulator of mucoid phenotype) and *rscAB* (Regulator of mucoid phenotype), type 1 and type 3 fimbriae, aerobactin (*iucABCD* and *iutA*), enterobactin (*entABCE, fes* and *fepABCDG*), salmochelin (*iroE*), yersiniabactin (*irp*12, *fyuA* and *ybtAEPQSTUX), arcAB* (acriflavine resistance protein), type VI secretion system were identified. The plasmid pSM117_3 contain *rmpA2* and aerobactin genes *iucABCD-iutA*, showing that the emergence of hypervirulent *K. pneumoniae* SM117 was due to the acquisition of a roughly 196 kbp pLVPK-like virulence plasmid by classic ST11 carbapenem-resistant *K. pneumoniae* strains. A BLAST analysis revealed that pSM117_3 had 97–99% query coverage and over 99% identity with several *rmpA2* and aerobactin genes *iucABCD-iutA* bearing plasmids, ie, 99%

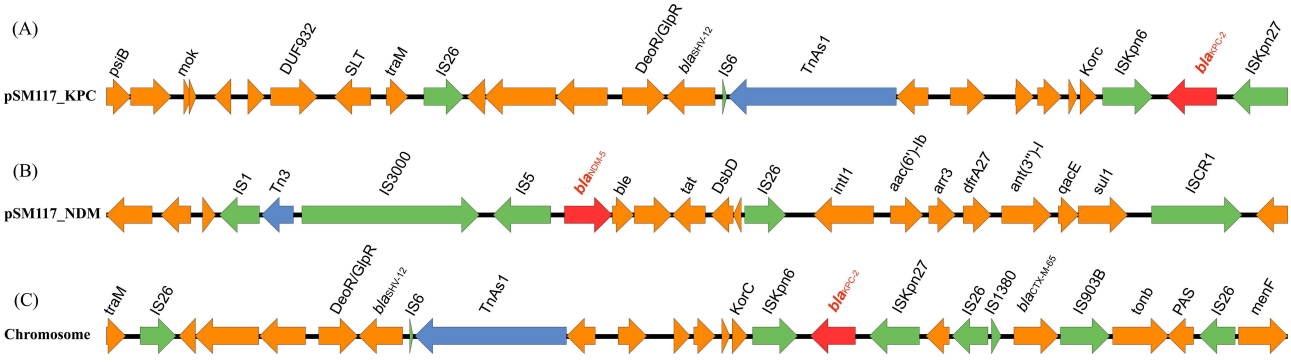

Figure 1 (**A-C**) Genetic environment of carbapenem resistance genes in *K. pneumoniae* SM117. The red arrows represent the carbapenem resistance genes, the green arrow represents the IS truncated fragment, the blue arrow represents the Tn truncated fragment, whereas the Orange arrows represent additional coding sequences (CDSs).

query coverage and 99.97% identity with pK1023_1 in *K. pneumoniae* strain KPN1023 isolated from a sputum sample in China Hefei (Figure 2). The plasmid carried several virulence factors, including *rmpA2* and aerobactin (*iucABCD-iutA*) indicates that CRKP can acquire virulence gene-carrying plasmids to become hypervirulent CRKP.[2]

The phylogenetic relationship between *K. pneumoniae* SM117 and a total of 80 $bla_{\text{NDM-5}}$ and $bla_{\text{KPC-2}}$ co-carrying *K. pneumoniae* strain currently deposited in the NCBI GenBank database were examined to evaluate the genomic epidemiological features of *K. pneumoniae* strains in a global context (Figure 3). These *K. pneumoniae* strains were most isolated from human (n = 72, 88.9%) and environment (n = 8, 9.9%), while just one strain has unknown sources. Strains isolated range from 2015 to 2024 and were recovered from various countries, including China (n = 65, 80.2%), the United States (n = 11, 13.6%), Bangladesh (n = 4, 4.9%) and Vietnam (n = 1, 1.2%). Furthermore, 47 *K. pneumoniae* isolates harbored genes encoding ESBLs (mainly CTX-M-65 and SHV-12) (Supplementary Table 1). It is concerning that, 95.7% (45/47) of the strains were isolated from China, including 26 isolates co-harbored *iut*A, *iuc*ABCD, and *rmpA2* virulence genes. This study reveals the prevalence of CRKP co-harboring bla_{KPC} and bla_{NDM}-carrying plasmids, as well as pLVPK-like virulence plasmids in China, demanding our utmost attention.

Various sequence types were identified among those isolates, including 62 isolates of ST11 (76.5%), followed by ST258 (n = 5, 6.2%), ST307 (n = 5, 6.2%), ST15 (n = 3, 3.7%), ST1451 (n = 2, 2.5%), ST22 (n = 1, 1.2%), ST3617 (n = 1, 1.2%), ST4855 (n = 1, 1.2%) and ST556 (n = 1, 1.2%). The clonal group (CG) 258 *K. pneumoniae* strains are the most

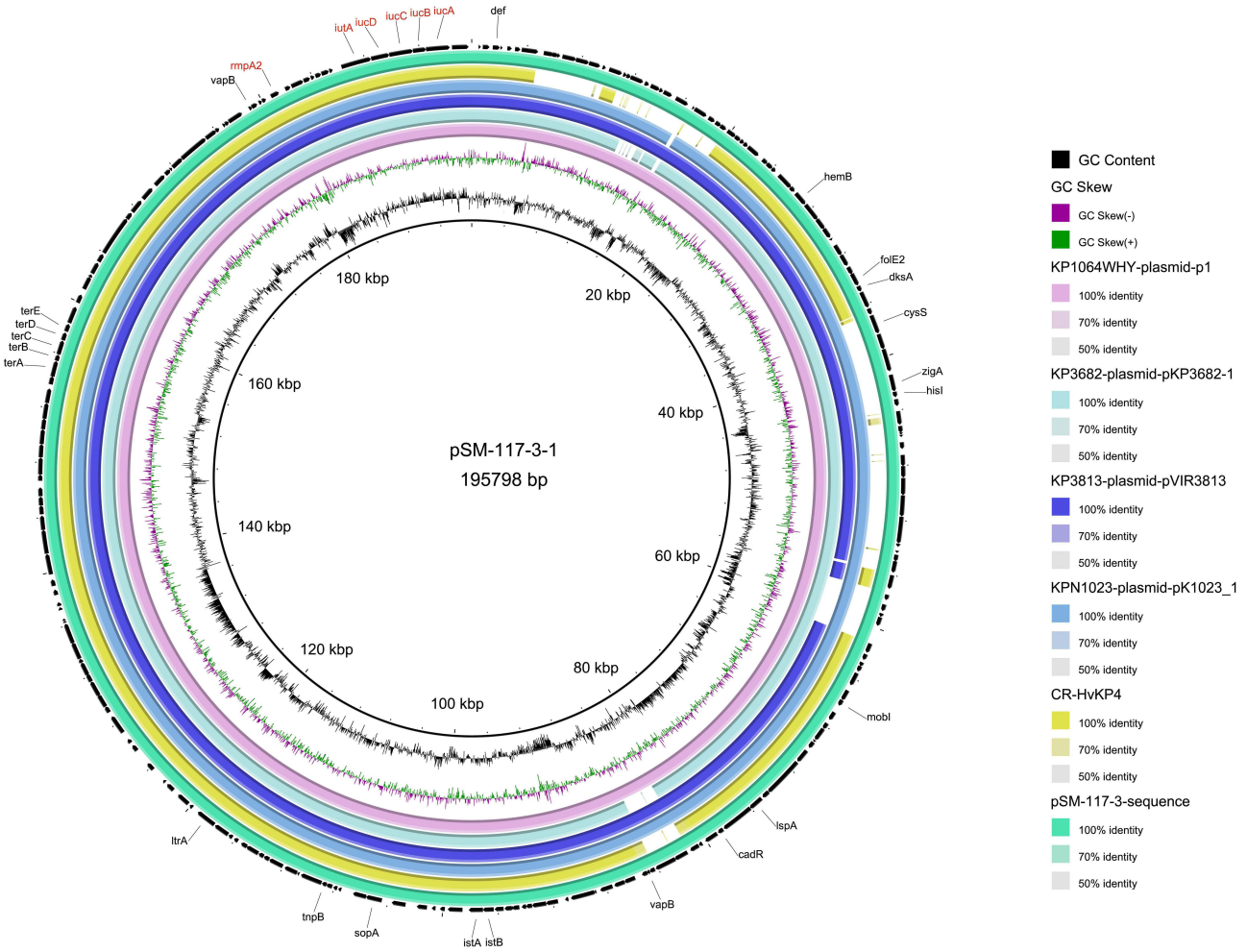

Figure 2 Circular comparative analysis of the *rmpA2* and aerobactin genes *iucABCD-iutA* bearing plasmids characterized in this study and deposited in GenBank database. Virulence genes were labeled red at the outmost ring.

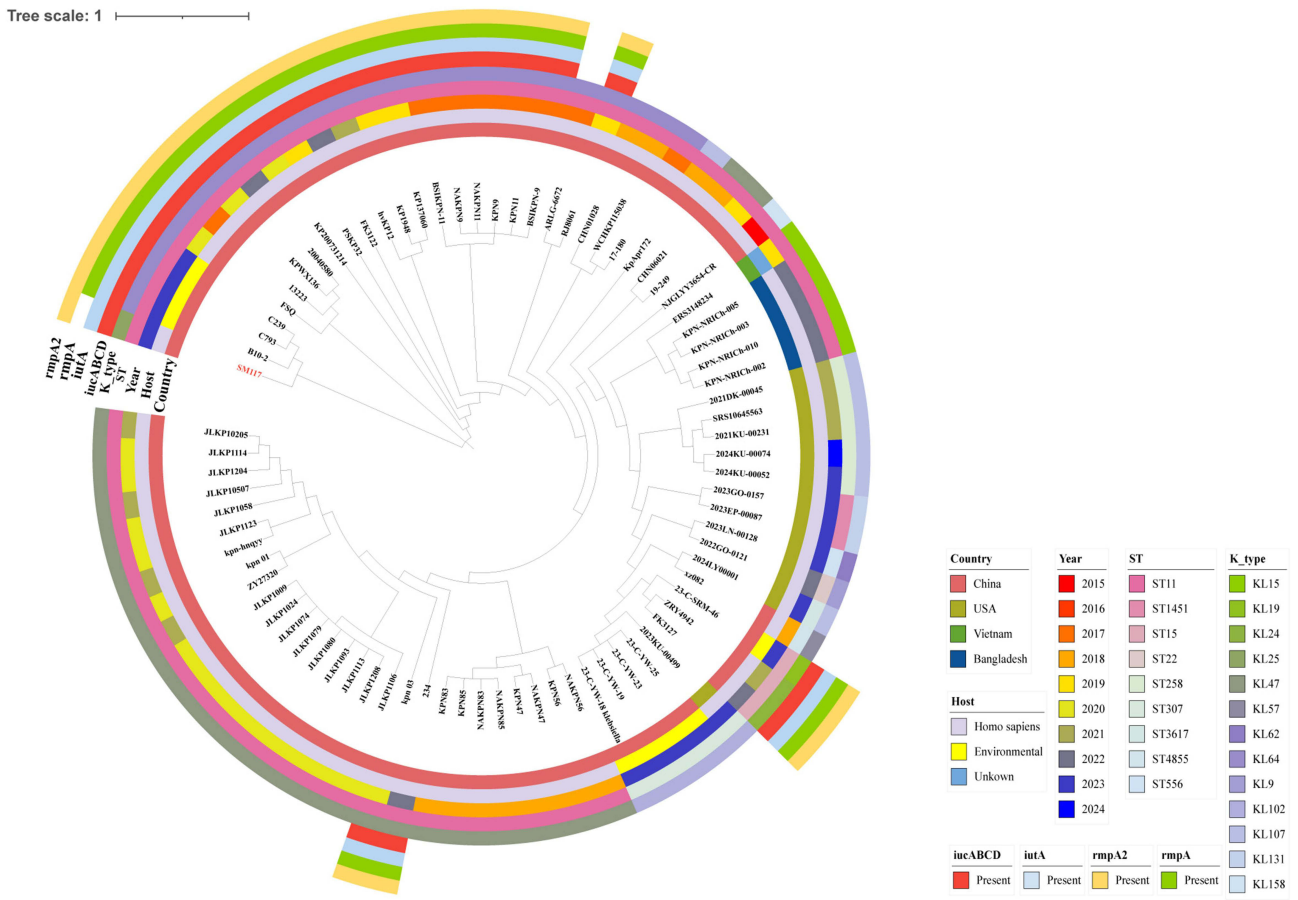

Figure 3 The phylogenetic relationship between *K. pneumoniae* SM117 and a total of 81 *K. pneumoniae* strains currently available in the NCBI GenBank database (Data as of March 18, 2024). The distance of SNPs is represented by the branch length. The positions corresponding to the five circles represent *rmpA2*, *iutA*, *iucABCD*, the country, host, the isolation date, sequence type (ST), and capsular type respectively.

prevalent clinical carbapenem-resistant strains, with ST258 and ST11 being the most common lineages globally.[18] The most prevalent clone of CRKP in Asia is ST11, which makes up to 60% of the strain in China.[16]

Additionally, various capsular serotypes were identified, including KL47 (n = 30, 36.6%), KL64 (n = 24, 29.3%), KL107 (n = 7, 8.5%), KL102 (n = 5, 6.1%), KL15 (n = 6, 6.1%), KL131 (n = 2, 2.4%), KL24 (n = 2, 2.4%), KL9 (n = 1, 1.2%), KL62 (n = 1, 1.2%), KL19 (n = 1, 1.2%), KL158 (n = 1, 1.2%), KL25 (n = 1, 1.2%) and KL57 (n = 1, 1.2%). KL47 and KL64 were the most common *K. pneumoniae* capsular serotypes. Capsular serotypes were identified among those 26 isolates which harbored *iut*A, *iuc*ABCD, and *rmpA2* virulence genes, KL64 was the most common serotype (n=20, 76.9%), followed by KL47 (n=2, 7.7%), KL24 (n=2, 7.7%), KL25 (n=1, 3.8%) and KL19 (n=1, 3.8%) (Supplementary Table 1). This study showed that KL64 had a higher virulence gene carriage rate than KL47, which is in line with another study.[19] However, CRKP strains co-harbouring $bla_{\text{NDM-5}}$ and $bla_{\text{KPC-2}}$ with KL25-ST11 type have rarely been reported. To our knowledge, in 2020, Li et al firstly reported *K. pneumoniae* isolate KSH203 with capsular serotype KL25 belonging to ST11 co-carrying $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-2}}$ from a patient in China.[7]

The strains varied widely, with an average of 13095 SNPs, ranging from 1 SNPs to 223952 SNPs (Figure 4). Close linkages were observed among the strains isolated from China, with an average of 8158 SNPs between each pair, and 214 pairs with less than 20 SNPs, indicating possible clonal spread between them. According to the results of phylogenetic analysis, the closest relative of *K. pneumoniae* C793 was identified in 2023 from a hospital surface sample in Zhejiang, China, with just 52 SNPs difference. Followed by *K. pneumoniae* C239 and

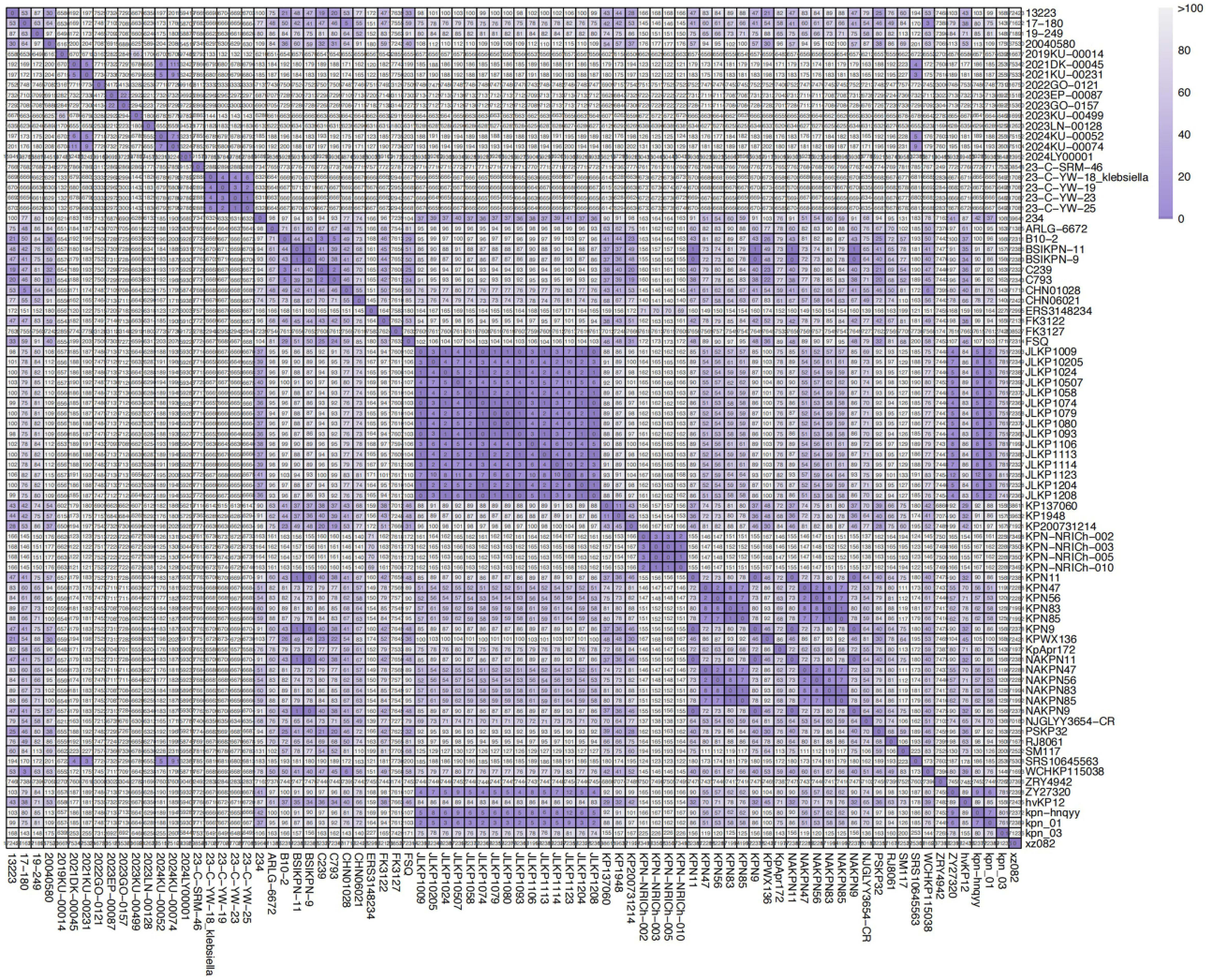

Figure 4 The single nucleotide polymorphisms (SNPs) numbers between each isolate. The strains varied widely, with an average of 13095 SNPs, ranging from 1 SNPs to 223952 SNPs.

B10, with 54 and 57 SNPs difference, respectively. All these strains were isolated from the Zhejiang province, which indicates that the strain may be actively spreading in this region.

Conclusion

To summarize, we present the whole-genome sequence of ST11 *K. pneumoniae* isolate SM117 with capsular serotype KL25, co-carrying including $bla_{\text{NDM-5}}$, $bla_{\text{KPC-2}}$ and multiple plasmid-borne virulence genes from a county level hospital in China. The superbug that combines carbapenem resistance with hypervirulence will provide significant difficulties for clinical detection and therapy. These findings will provide important knowledge of the antibiotic resistance mechanisms, genomic epidemiological characteristics and the global transmission dynamics of *K. pneumoniae* carrying multiple carbapenemase genes.

Ethics Approval

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Sanmen People's Hospital, Taizhou, China (2024-077). The isolates were collected as part of routine hospital laboratory procedures. Written informed consent from the patients was exempted because the present study only focused on the genomic characteristic analysis of bacteria.

Author Contributions

All authors made a significant contribution to the work reported, whether 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

We declare that we have no conflicts of interest in connection with this paper, and that we received no payment or services from a third party in relation to this study.

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