Genomic a Nalysis and Molecular Characteristics in Carbapenem R Esistant *Klebsiella Pneumoniae* Strains

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Research

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Abstract

Background

Klebislla pneumoniae is an important bacterium and responsible for both infections acquired in hospital and community because of its multidrug resistance and the virulence. The aim of this research was to investigate clonal lineages, antibiotic resistance profiles and virulence factors of the hospital isolated Carbapenem Resistant strains.

Methods

Whole-genome sequencing of one strain was performed using the Illumina Hiseq and PacBio *RS* systems. Genomic information was comprehensively analyzed by various bioinformatics approaches. Molecular typing was based on multi locus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) analysis. Antimicrobial susceptibility testing, drug resistance and virulence genes were determined.

Results

K. pneumoniae KPX comprised one circular chromosome and four circular plasmids. This strain harbors a variety of antimicrobial resistance and virulence determinants. The closest relative of *K. pneumoniae KPX* was another ST11 clinical isolate recovered Sichuan. Fifty isolates were phenotypically confirmed extended-spectrum beta-lactamases ESBLs producers. MLST analysis revealed 97% sequence type 11. Fifty Carbapenem Resistant isolates mainly belong to three clones according to the PFGE DNA patterns. PFGE patterns has more variety than ST profiles. In addition, *KPC-2* (98.0%), *SHV-11* (98.0%), *TEM-1* (76.0%), *CTX-M* (76.0%), *Oqxb1*(66%), *qnrS* (70%), *Int1* (42.0%), *sul1* (82.0%), *sul2* (96.0%), *iutA* (88%), *iucABCD*(10%), and *rmpA2* (100%) genes were presented in multiple drug resistant strains.

Conclusion

The dataset presented in this study provided the genomic and epidemiological analysis of Carbapenem Resistant *K. pneumoniae* in hospital settings. Antimicrobial-resistance profiles suggested the presence of significant selective antibiotic pressure. Appropriate surveillance is essential to development of effective control strategies in the prevention of nosocomial infection.

Background

Klebsiella pneumoniae is considered to be one of the most common nosocomial pathogens that lead to a wide ranger of infections, including, but not limited to, pneumonia, urinary tract infections and bacteremia [1]. With the spread of the multiple drug resistance or extensively-drug resistant due to AmpC and extended-spectrum beta-lactamases (ESBL) producing, Carbapenem has been commonly adopted by clinicians for the treatment of severe bacterial infections. However, according to the report from CHINET 2018, surveillance data revealed that the resistance rates of *K. pneumoniae* to carbapenems (imipenem, meropenem and ertapenem) were up to 10.1%, while 4.9% in 2013[2]. The emergence of carbapenem-resistant *K. pneumonia* (CRKP) and New Delhi metallo-β-lactamase (NDM) have resulted in the limited therapeutic options to treat such infections and shed a thread to public health [3,4]. *K. pneumoniae* carbapenemase (KPC)-type carbapenemase, an Ambler class A β-lactamase, is one of the most important carbapenemases commonly found in *K. pneumonia* drug-resistant strains [5]. What's more, *K. pneumoniae* and *Escherichia coli* are the predominant carriers of bla_{NDM}[6]. Most bla_{KPC}-and bla_{NDM}-carrying *K. pneumoniae* strains are extensively-drug resistant (XDR) bacteria that are often co-resistant to all β-lactams, quinolones, aminoglycosides and macrolides except tigecycline and colistin [7]. If Carbapenem- resistant *K. pneumoniae* are co-resistant to tigecycline and colistin, it often indicates the emergence of pandrug-resistant (PDR) bacteria [8]. Although pandrug-resistant *K. pneumoniae* in China is still rare, the transformation from XDR to PDR are more likely to arouse more public attention in clinic. In addition, about 420 of recorded *K. pneumonia* drug-resistant strains in China were sequence type (ST) 11, which were widespread in SiChuan and HangZhou[9,10]. In view of this, to reveal changing trends in the prevalence of extensively-drug resistant *K. pneumoniae* strain, in the present study, one Carbapenem-resistant *K. pneumoniae* strain, KPX, was collected from a sputum specimen of an inpatient in a comprehensive hospital in Jiangsu province, China. The whole genome and plasmids sequence of this strain was sequenced and comprehensively analyzed. Then molecular typing and drug resistant mechanism of other Carbapenem-resistant *K. pneumoniae* strains were further analyzed.

Materials And Methods

Strains

Fifty *K. pneumoniae* clinical isolates were collected from a hospital of Nanjing, Jiangsu, China. The *K. pneumoniae* isolates were grown on Columbia Blood Agar at 37°C for 24 h. One of *K. pneumoniae* strains, KPX was sequenced from a sputum sample of an eighty-year-old male patient hospitalized with symptoms of severe pneumonia and fever.

The species identification and antimicrobial susceptibility of K. pneumoniae isolates were determined with an automated VITEK®2 AST-GN13 system (bioMérieux, Marcy-l'Étoile, France) or the Kirby-Bauer method using antibiotic panels with the following antimicrobial agents: Piperacillin, Amoxicillin, Ampicillin/Sulbactam, Piperacillin /Tazobactam, Cefoxitin, Cefoperazone/sulbactam, Cefuroxime, Ceftriaxone, Cefotaxime, Cefepime, Ceftazidime, Meropenem, Imipenem, Gentamicin, Levofloxacin, Ciprofloxacin, Aztreonam, Trimethoprim/Sulphamethoxazole.

Whole-Genome analysis for K. pneumonia KPX

Two genomic DNA libraries were set up according to the instructions of the Illumina Hiseq and PacBio *RS* systems for producing the complete genome sequence of *K. pneumonia* KPX. For Illumina Hiseq sequencing, the genomic DNA was fragmented and set up a paired-end library with an average insert size of ~300 bp. The library was sequenced on an Illumina Hiseq platform (Illumina, San Diego, CA, USA) using the 150-bp paired-end sequencing mode. For PacBio sequencing, 8-10k insert whole genome shotgun libraries were generated and sequenced on a Pacific Biosciences RS instrument (Pacific Biosciences, CA). The complexity of the genome was evaluated by using the Illumina data, which was tried to be assembled using Velvet assembler (v1.2.09) with a kmer length of 17. Contigs with length less than 200bp were removed to obtain the reliable assembled results. Both the PacBio reads and Illumina reads were used to assemble the complete genome sequence. The assemblies were conducted using a hybrid *de novo* assembly method modified by Koren, S., et al., in which a *de-Bruijn* based assembly algorithm and a CLR reads correction algorithm were integrated in "PacBioToCA with Celera Assembler" pipeline [11,12]. The complete genome sequence of *K. pneumoniae* KPX was annotated using Glimmer version 3.02 (http://cbcb.umd.edu/software/glimmer/). tRNA and rRNA were identified using the tRNAscan-SE (v1.23, http://lowelab.ucsc.edu/tRNAscan-SE) and the RNAmmer (v1.2, http://www.cbs.dtu.dk/services/RNAmmer/), respectively. Plasmid replicons, antimicrobial resistance genes, and virulence genes were identified using PlasmidFinder 2.1 databases, CARD （http://arpcard.Mcmaster.ca）Version 1.1.3）, and VFDB （http://www.mgc.ac.cn/VFs/）. The integrons and gene cassettes within the genomes were identified according to the INTEGRALL database [13]. The circular maps of chromosome and plasmids of *K. pneumoniae* KPX were displayed using CGView Server, and multiple plasmid alignments were compared using BLAST Ring Image Generator (BRIG) [14]. Both core genome multilocus sequence typing (cgMLST) and core genome single nucleotide polymorphism (cgSNP) strategies were used to analyze bacterial whole-genome sequence typing and phylogenetic tracking using the BacWGSTdb server [10].

MLST typing

Multilocus sequence typing （MLST） was performed according to protocols provided on the MLST web site for *K. pneumoniae* (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). The PCR products of 7 house-keeping genes were sequenced by Qingke biotechnology Co., Ltd, Nanjing. The sequences were given different allele numbers, which will define sequence type (ST).

PFGEtyping

Pulsed-field gel electrophoresis (PFGE) of all isolates were carried out as previously described methods [15]. In brief, the pretreated bacterial genomic DNA in gel was digested by the restriction enzyme *XbaI*(TaKaRa, Japan) for 2 h, and the fragments were separated in a CHEF Mapper system (Bio-Rad Laboratories, Hercules, CA, USA) with pulses ranging from 5 to 35 seconds at a voltage of 6 V/cm and switch angle of 120° for 15 hours at 14°C. And then gels were stained with GelRed (Beyotime, Shanghai) and DNA patterns were acquired by Bio-Rad Gel Doc XR. We used hierarchic clustering with the Between-groups linkage method to analyze correlation among digitized PFGE outputs by SPSS software (version 18.0, SPSS Inc. USA). The PFGE profiles were interpreted as followed according to Tenover et al. [16]. For example, Clone A means the isolates have only 1 to 3 different bands and they can be defined as subtype (Clone A_1…Clone A_n). If there are over 3 different bands, the isolate will be defined as another clone, for example, Clone B, Clone C, and Clone D etc.

Antimicrobial resistance and virulence-associated genes PCR analysis

DNA was extracted by using Tianamp Genomic DNA Kits (Tiangen Biotech, Beijing) and SanPrep Column Plasmid Mini-Preps kits (Sangon Biotech, Shanghai). The PCR mixture was prepared with a final volume of 25 μl, containing of template DNA and primers. The specific primers for detecting antimicrobial resistance genes and virulence-associated genes were shown in **Table 1**. The amplified PCR products were separated by electrophoresis in 1.2% agarose gels and visualized after staining with Goldview dye (Beyotime, Shanghai).

Ethical Approval

This study was approved by the Ethics Committee of Nanjing Medical University, Nanjing, China. Written informed consent was obtained from the patient.

Results And Discussion

The comprehensive whole-genome analysis

The complete genome sequence of *K. pneumoniae* KPX comprises a circular chromosome (5,468,925 bp) and four plasmids (179,972 bp, 141,377 bp, 85,181 bp, and 20,247 bp) which annotated 5984 protein coding genes and identified 85 tRNA genes and 25 rRNA operons. KPX harbors multiple antimicrobial resistance determinants including *aadA2*, *rmtB*, $bla_{CTX\text{-}M\text{-}65}$, $bla_{KPC\text{-}2}$, $bla_{TEM\text{-}1}$, $bla_{SHV\text{-}64}$, $bla_{SHV\text{-}11}$, *qnrS1, carA, sul1, sul2,* and *catII,* which confer resistance to aminoglycosides, β-lactams, fluoroquinolones, macrolides, sulphonamides and phenicols. The quinolone resistance-determining region (QRDR) mutations in GyrA (S83I and D87G) and ParC (S80I) were detected. The overexpression of efflux pump genes (i.e., *acrA*, *acrB*, *marA*, *soxS*, and *acrAB*) were determined by qRT-PCR, which was associated with tigecycline resistance in *K. pneumonia*. The genes, *intI1*, *aacA4*, *cmlA1*, *qacEΔ1*, *tnpA*, and $bla_{CTX\text{-}M\text{-}19}$ were clustered in integron Class Ⅰ. The segment between the two IS26 elements was carried with *aadA* and *sul1* genes. The virulence-related factors, aerobactin siderophore receptor and yersiniabactin encoding genes *iutA* and *irp1/2* and *YbtU/Q/T*, were present. *K. pneumoniae* KPX was identified as sequence type (ST11) by MLST. By setting a minimum sequence homology of 95% and a minimum length of 60% in PlasmidFinder 2.0 databases, we also identified three plasmids belonging to incompatibility (Inc) group F [IncHI1B, IncFII/IncR] and ColRNAI.

The phylogenetic relationship between *K. pneumoniae* KPX and close ST11 *K. pneumoniae* strains was assessed by cgSNPs and cgMLST analysis. The analysis of cgSNPs showed that the phylogenetic tree was grouped into the same clade according to geographically related isolates (**Figure 1**). The most of these isolates were found from Sichuan and Hangzhou. Our results showed that SCKP020029 (NCBI Accession number: CP029384), another ST11 strain collected from a human sample in Sichuan is the most closely related strain to *K. pneumoniae* KPX (**Figure 2**).

Among the plasmids of *K. pneumoniae* KPX, pKPX-A carried with a set of virulence genes including *iucBCD*, *iutA*, *rmpA*, and *rmpA2* on plasmid (179,972 bp), which belonged to an IncFIB/IncHI1B plasmid. Multiple plasmid comparisons by BRIG showed that pKPX-A plasmid was similar with two virulence plasmids, i.e. pVir_020079 (99.99% identity) and pKPN-QL24 (99.01% identity) (**Figure 3**). pKPX-B, the IncFII/ IncR plasmid (141,377 bp), carried the carbapenem resistance gene $bla_{KPC\text{-}2}$ and shared 99.88% similarity with plasmid pKPC2_020079 from the KPC-2-producing *K. pneumoniae* strain SCKP020079 isolated in Sichuan, China. The resistance gene cassettes on plasmid pKPX-B was organized to IS26-*rmtB*- $bla_{TEM\text{-}1}$-IS26, IS26-$bla_{CTX\text{-}M\text{-}65}$-IS26-Tn3, and IS26-$bla_{SHV\text{-}64}$-$bla_{KPC\text{-}2}$-IS26. In addition, pKPX-C, IncFII-type plasmid (85,181 bp), harbored the antimicrobial resistance genes, such as *sul2*, *qnrS1*, *cat*Ⅰ, and *tet(R/G)*, which confer resistance to sulphonamide, fluoroquinolone, chloramphenicol, tetracycline, respectively.

The characteristics of drug-resistant K. pneumoniae infection subjects

Fifty strains were isolated from patients aged from 47 to 98 years old. The mean age of the patients was 78.5 ± 10.2 years, and the male to female ratio of the patients was 2.1:1. The distribution of age were shown in Table S1. The isolates were mainly collected from ICU/emergency ICU and neurosurgery department, accounting for 46.0% and 24.9%, respectively. The strains were mainly isolated from sputum (39, 78.0%), and the others were part from blood, urine, endotracheal tube suction, bronchoalveolar samples and so on.

Antimicrobial susceptibility patterns of K. pneumonia

Fifty *K. pneumoniae* isolates were tested for its susceptibility against 18 types of antibiotics. The overall susceptibility, intermediate and resistance were determined and the results were shown in **Figure 4**. Most *K. pneumoniae* strains showed a high percentage of resistance against Piperacillin (100%), Amoxicillin acid(100%), Ampicillin/ Sulbactam(100%), Piperacillin /Tazobactam(97.96%), cefoxitin(100%), cefoperazone/sulbactam(100%), Cefuroxime(100%), Ceftriaxone(100%), Cefotaxime(100%), Cefepime(97.87%), Ceftazidime(100%), Meropenem(100%), Imipenem(97.87%), Gentamicin(81.63%), Levofloxacin(100%), Ciprofloxacin(100%), Aztreonam(100%), but

Trimethoprim/Sulphamethoxazole(47.92%). Fifty isolates were phenotypically confirmed extended-spectrum beta-lactamases ESBLs producers.

Molecular typing

MLST analyses revealed 4 different sequence types including ST1 (n =1), ST11 (n = 47), ST15 (n = 1), and ST258 (n =1), indicating the genetic consistency of all these isolates. ST11 was the dominant clone in our study, which has been demonstrated as a predominant clone of KPC-producing *K.pneumoniae* in China [9]. ST11 is a single-locus variant (*tonB*) of ST258. ST258 was detected in one isolate, which was reported as a dominant molecular epidemiology clone in the USA [17].

Figure 5 showed the representative PFGE results of the multidrug resistant isolates. Multiple DNA fragments were observed in each isolate, which had more than ten bands. The results of genetic correlation were presented as a dendrogram by SPSS software. The results revealed that 50 isolates had DNA diversity with multivariate clones. Fifty strains were mainly classified into three clones. That is, clone A had 12 isolates (24%), clone B had 9, and clone C had 7, respectively. The others had independent resource. Clonal characteristics and distributions were shown in **Figure 6**. Clone A, B and C strains mainly existed in ICU and EICU department, which indicated no dissemination occurred among inpatient wards. Although new DNA fingerprinting technique MLST as alternatives are widely used, the discriminatory abilities of PFGE are more robust than that of MLST.

PCR-based drug-resistant genes and virulent genes detection

According to the results of KPX strain DNA sequencing, we selected drug resistant genes- *KPC-2, SHV-11, TEM-1, CTX-M, oqxB1, qnrS, sul2, sul1, int1,* and virulent genes- *iutA, iucABCD, rmpA2* to detect. Considering the prevalence of *NDM1*gene, *NDM1*gene has also been included. The existence of these genes were shown in **Table 2.** In multidrug resistant strains, 98% (49/50) isolates were found to be positive for the presence of *KPC-2*. All three ESBL genes (*SHV-11, TEM-1* and *CTX-M*), as Amber class A of β-lactamases [18] are encoding β-lactamase enzymes which is the primary resistance mechanism in Gram-negative bacteria resulted in β-lactams degradation [19]. All these isolates had *SHV-11* out of which 49 (98.0%) isolates. 38 (76.0%) isolates carried *TEM-1* gene and 38 (76.0%) of them carried *CTX-M* gene. 30 of the isolates carried all the three genes together. None of the isolates were PCR-positive for NDM. 66% was for *Oqxb1*, encoding multidrug efflux pump, which is one of the mechanisms of quinolone resistance. *oqxAB* genes have been prevalent among Gram-negative bacteria, especially in Enterobacteriaceae. *oqxAB* genes often confer cross-resistance or reduce susceptibility to multiple agents, such as chloramphenicol, trimethoprim, ciprofloxacin, enrofloxacin, norfloxacin, and tigecycline [20,21]. 70% was for *qnrS*, mutant in gene results in quinolone resistance. 42.0% was for class 1 integron integrase (*Int1*); 82.0% and 96.0% for *sul1 and sul2*, which is related to resistance to sulfonamide. Of antimicrobial susceptibility patterns the percentage of resistance against Trimethoprim/Sulphamethoxazole was 47.92%, which was inconsistent with *sul1* and *sul2* carrying rate. This can be attributed to a decline in the use of these antibiotics. But the results need to be validated *in vivo*. The *sul1* gene is predominantly linked with class 1 integrons [22]. Of the 41 *sul1* positive isolates, 15 (36.6%) carried *Int1*. 88% was for *iutA* and 10% for *iucABCD*, *iucABCD-iutA*, encoding aerobactin, iron acquisition systems [23]. Research has demonstrated that certain virulence traits are associated with antibiotic-resistant phenotypes [24]. For example, carriage of the *iutA* gene has previously been associated with antibiotic resistance in *E. coli* of extraintestinal origin [25] and is also common among strains producing ESBLs of the CTX-M types [26]. However, in this study we did not find this correlation. 100% was for *rmpA2*, capsule up-regulation genes, which are those most associated with invasive infection [27].

In conclusion, this study presented the hybrid genome assembly and annotation of an extensively-drug resistant *K. pneumonia* strain, analyzed the molecular types and antimicrobial resistance and virulence profiles of clinical isolates in Jiangsu province, China. *KPC*-producing *K. pneumoniae* isolates is widespread in Nanjing, Jiangsu. Although NDM-producing *K. pneumoniae* isolates are rare, nationwide surveillance is absolutely necessary to the development of strategies for prevention, diagnosis and treatment of *K. pneumoniae* infections.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Nanjing Medical University, Nanjing, China. Written informed consent was obtained from the patient.

Consent for publication

All the authors agree to publish.

Availability of data and materials

All data is true and reliable,and can provide the original data at any time.

Competing Interests

The authors declare that there are no conflicts of interest.

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Authors' contributions

Conceived and designed the experiments: QW. Performed the experiments: XYQ, YTT, XTX, YLG, MX,HLC,ML and YXL. Analyzed the data: SYZ, QW, ZZ and LL. Contributed reagents/materials/analysis tools: SYZ, and LL. Wrote the paper: QW. All the authors read and approved the final manuscript.

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Not applicable

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Tables

Table 1
Primers used in this study

Primer	Sequence(5−3)	Size(bp)	Citation
rmpA2_F	AGAGTATTGGTTGATAGCCGGA	159	(1)
rmpA2_R	GAAATGTCAAGCCACATCCATTG		
iucABCD_F	CCAACTCCGTCCGTACCCTGTCA	838	(1)
iucABCD_R	CGAGGGATCGACGATGGTGTTCT		
iutA_F	AATCACCTGGGGGCTGGATGCT	683	(2)
iutA_R	CCGCACCTTCCACGCCGTAAAT		
KPC-2_F	GCTACACCTAGCTCCACCTTC	990	(3)
KPC-2_R	ACAGTGGTTGGTAATCCATGC		
NDM-1_F	GAAGCTGAGCACCGCATTAG	769	(4)
NDM-1_R	GGGCCGTATGAGTGATTGC		
TEM-1_F	ACAGCGGTAAGATCCTTGAGAG	461	(2)
TEM-1_R	GAAGCTAGAGTAAGTAGTTCG		
SHV-11_F	ACCTTTAAAGTAGTGCTCTGC	432	(2)
SHV-11_R	CACCATCCACTGCAGCAGCTG		
CTX-M_F	ATGGTTAAAAAATCACTGCGTCACTGCGYCAGTTC	876	(5)
CTX-M_R	TCACAAACCGTYGGTGACGATTTTAGCCGC		
qnrS_F	ACGACATTCGTCAACTGCAA	417	(6)
qnrS_R	TAAATTGGCACCCTGTAGGC		
int1_F	CAGTGGACATAAGCCTGTTC	161	(7)
int1_R	CCCGAGGCATAGACTGTA		
sul1_F	CGGCGTGGGCTACCTGAACG	433	(7)
sul1_D	GCCGATCGCGTGAAGTTCCG		
sul2_F	GCGCTCAAGGCAGATGGCATT	289	(8)
sul2_D	GCGTTTGATACCGGCACCCGT		
oqxB_F	CTGGATTTTCCGTCCGTTTAAC	68	(9)
oqxB_R	TTGCCTACCAGTCCCTGATAGC		

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Primer	Sequence(5− 3)	Size(bp)	Citation

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Table 2
Drug-resistant genes and virulence genes patterns among *K. pneumoniae* isolates

No.	Age	Gender	*KPC2*	*SHV11*	*TEM1*	*CTX-M*	*NDM1*	*oqxB1*	*qnrS*	*sul2*	*sul1*	*int1*	*iutA*	*iucC*	*rmpA2*
1	80	M	+	+	+	-	-	-	-	+	-	+	+	+	+
2	83	M	+	+	-	-	-	-	-	+	+	-	+	-	+
3	84	M	+	+	+	+	-	+	+	+	-	+	+	-	+
4	79	M	+	+	+	+	-	+	-	+	-	+	+	-	+
5	61	M	+	+	-	-	-	-	-	-	+	+	+	-	+
6	92	M	+	+	+	+	-	+	+	+	-	+	+	-	+
7	92	F	+	+	+	+	-	+	+	+	+	-	+	-	+
8	69	M	+	+	-	+	-	+	+	+	+	-	+	-	+
9	84	M	+	+	+	-	-	-	+	+	+	-	+	-	+
10	77	F	+	+	+	+	-	+	+	+	+	+	+	-	+
11	79	M	+	+	+	+	-	+	+	+	+	-	+	-	+
12	67	M	+	+	+	-	-	-	+	-	+	+	+	-	+
13	83	M	+	+	+	+	-	+	+	+	+	-	+	-	+
15	88	F	+	+	-	+	-	-	-	+	+	+	+	-	+
16	71	M	+	+	+	+	-	+	-	+	+	+	+	-	+
17	87	M	+	+	-	+	-	-	-	+	+	-	+	-	+
18	98	F	+	+	+	+	-	+	+	+	+	-	+	-	+
19	65	F	+	+	+	+	-	+	+	+	+	-	+	-	+
20	77	M	+	+	-	+	-	-	-	+	+	-	+	-	+
21	73	M	+	+	+	+	-	-	-	+	+	+	-	-	+
22	75	M	+	+	-	+	-	+	+	+	+	+	+	-	+
23	79	M	+	+	+	+	-	+	+	+	+	-	+	-	+
24	75	M	+	+	+	+	-	+	+	+	+	+	+	-	+
25	84	M	+	+	+	+	-	-	+	+	-	+	+	-	+
26	81	F	+	+	+	+	-	+	+	+	+	-	+	-	+
27	59	F	+	+	-	+	-	-	-	+	+	+	+	-	+
28	87	M	+	+	+	+	-	+	+	+	+	-	+	-	+
29	81	M	+	+	+	+	-	+	+	+	+	-	+	-	+
30	87	F	+	+	+	+	-	+	-	+	+	+	+	-	+
31	82	M	+	+	-	+	-	+	+	+	+	-	+	-	+
32	85	F	+	+	+	-	-	-	-	+	+	+	-	+	+
33	72	F	+	+	+	+	-	+	+	+	+	+	+	-	+
34	85	M	+	+	+	+	-	+	+	+	+	-	-	-	+
35	70	F	+	+	+	+	-	+	+	+	-	+	+	-	+
36	85	M	+	+	+	-	-	-	+	+	-	-	+	-	+

No.	Age	Gender	*KPC2*	*SHV11*	*TEM1*	*CTX-M*	*NDM1*	*oqxB1*	*qnrS*	*sul2*	*sul1*	*int1*	*iutA*	*iucC*	*rmpA2*
37	47	M	-	+	+	+	-	+	-	+	+	-	-	+	+
38	49	F	+	-	-	-	-	-	-	+	-	-	+	-	+
39	87	M	+	+	+	+	-	+	+	+	+	+	+	-	+
40	82	F	+	+	-	+	-	+	+	+	+	-	+	-	+
41	86	F	+	+	+	+	-	+	+	+	+	+	+	-	+
42	83	M	+	+	+	+	-	+	+	+	+	-	+	-	+
43	93	F	+	+	+	-	-	-	+	+	+	-	-	+	+
44	64	M	+	+	+	+	-	+	+	+	-	-	+	-	+
45	85	M	+	+	+	-	-	-	-	+	+	-	-	+	+
46	79	M	+	+	+	+	-	+	+	+	+	-	+	-	+
47	76	F	+	+	+	+	-	+	+	+	+	-	+	-	+
48	78	M	+	+	-	-	-	-	+	+	+	-	+	-	+
51	82	M	+	+	+	+	-	+	+	+	+	-	+	-	+
52	76	M	+	+	+	+	-	+	+	+	+	-	+	-	+
53	83	M	+	+	+	-	-	-	+	+	+	+	+	-	+

Supplemental Table

Table S1 Age distribution between patients with multidrug-resistant ***K. pneumoniae***

Age	No. of patients with multidrug-resistant
40~	2
50~	1
60~	5
70~	14
>80	28
Total	50

Figures

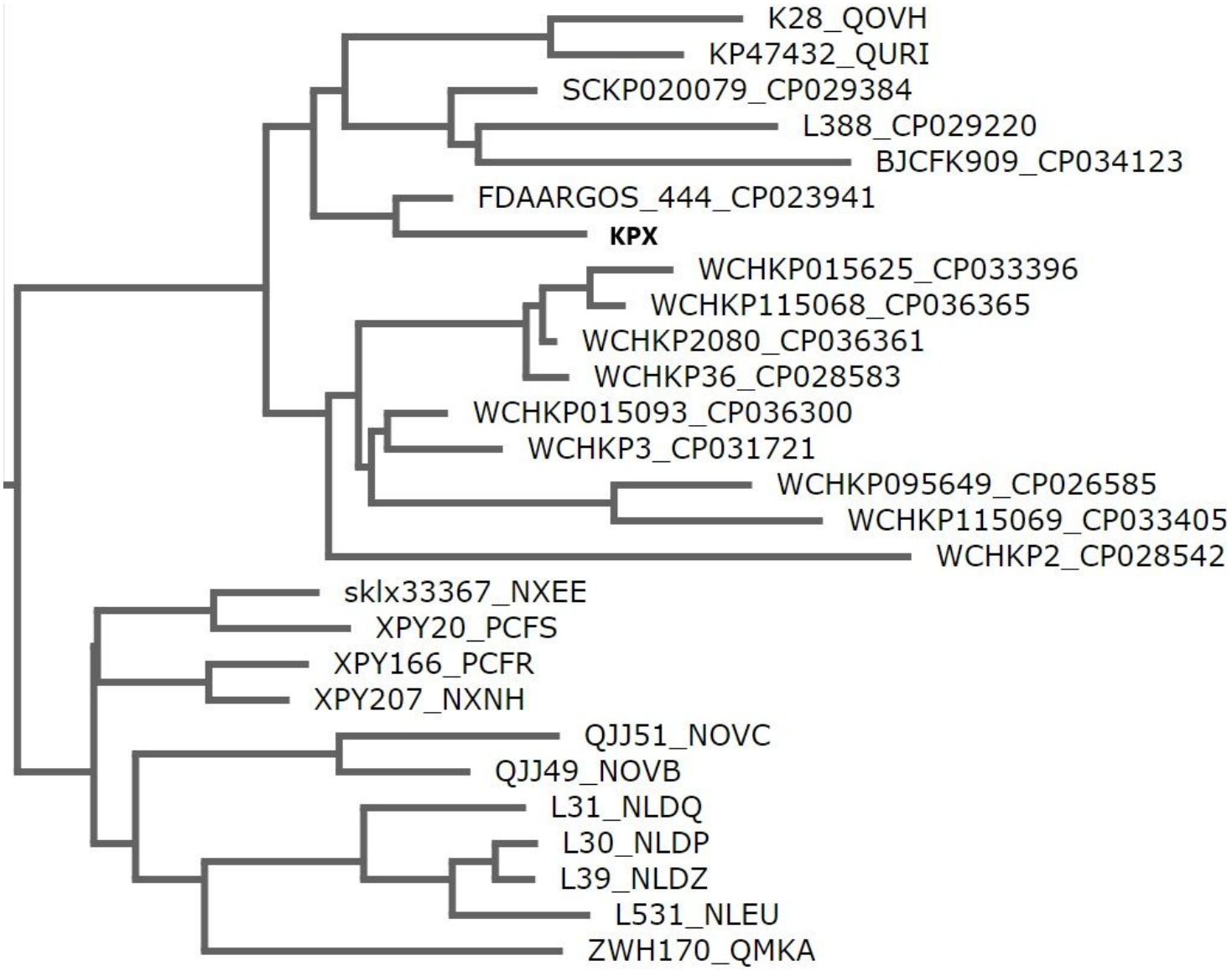


Figure 1

Neighbor-joining tree using only single nucleotide polymorphisms (SNPs) derived using BacWGSTdb. User_query represented K.pneumonia KPX.

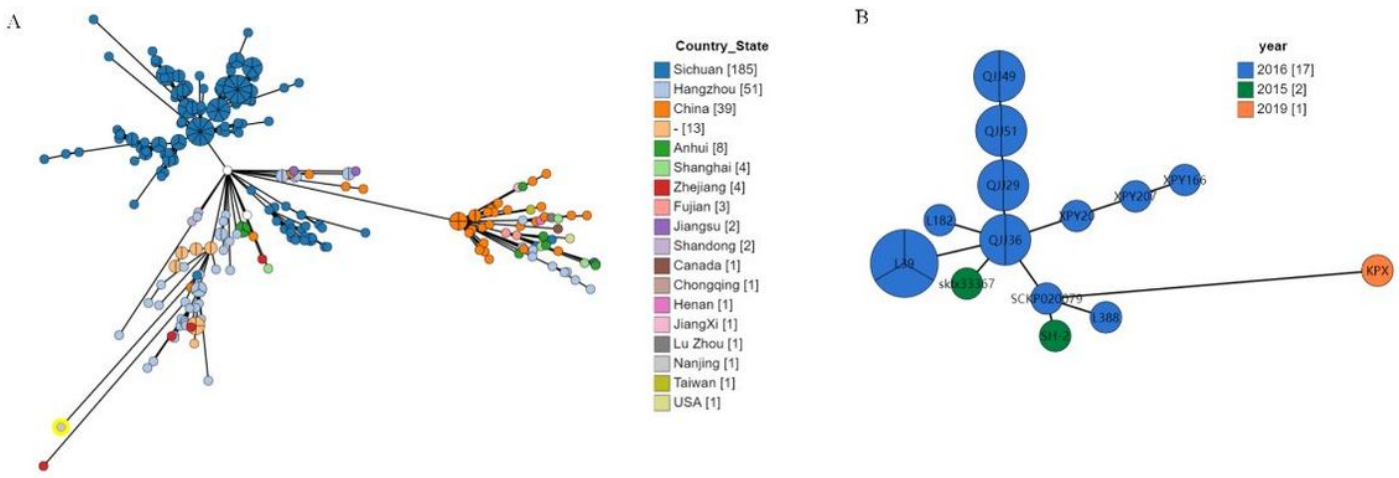


Figure 2

Phylogenetic relationship between K. pneumoniae KPX and closely related strains presently from BacWGSTdb. (A) The phylogenetic tree of ST11 K. pneumoniae strains including K. pneumoniae KPX. (B) The tree of the closely related strains with K. pneumoniae KPX. The dots represented the different isolates. The numbers given in square brackets indicated the number of isolates from each country or province.

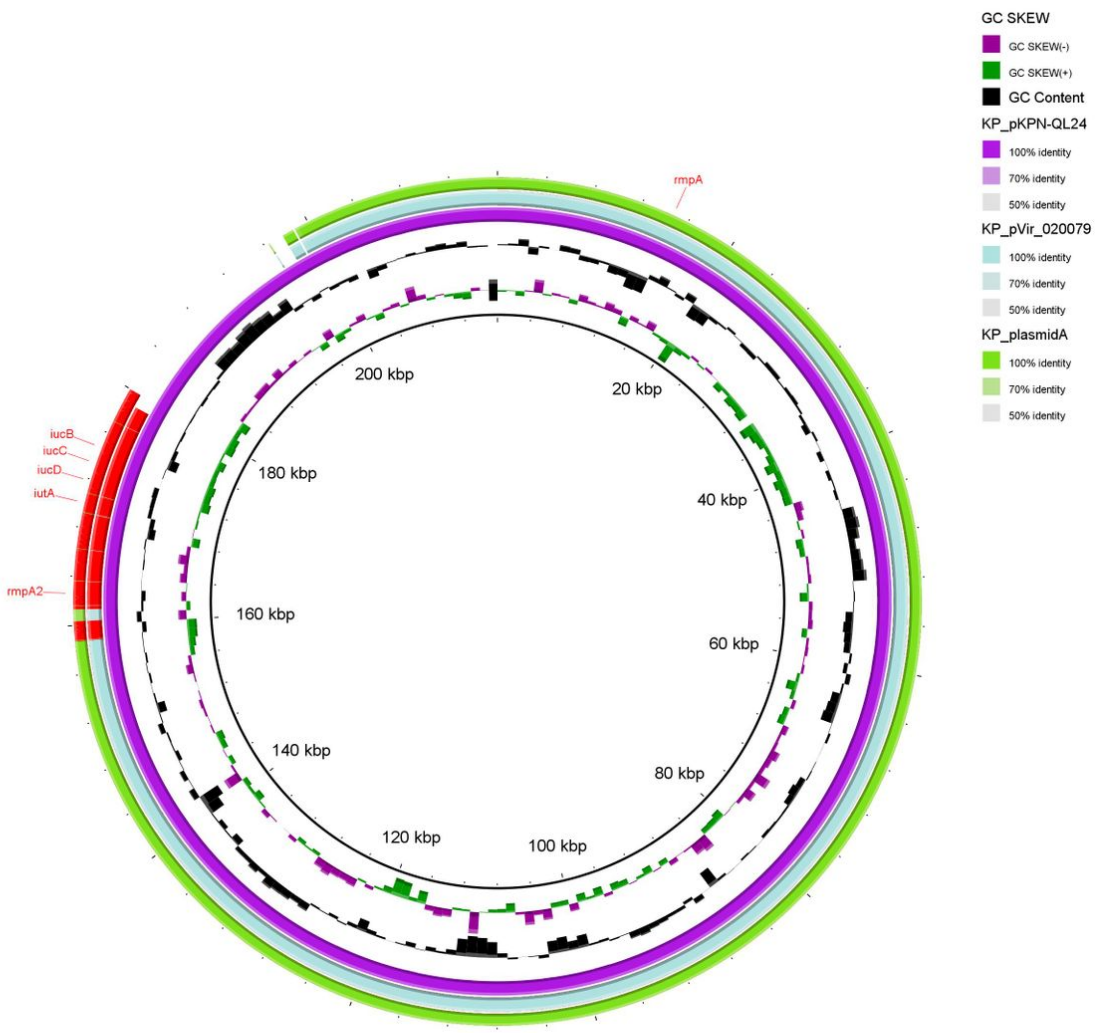


Figure 3

Genetic comparison of similar plasmids from different K. pneumoniae strains. Virulence gene, such as aerobactin (iucABCD, iutA), and mucoid phenotype (rmpA2) were annotated. The green circle represented plasmid A from KPX strain.

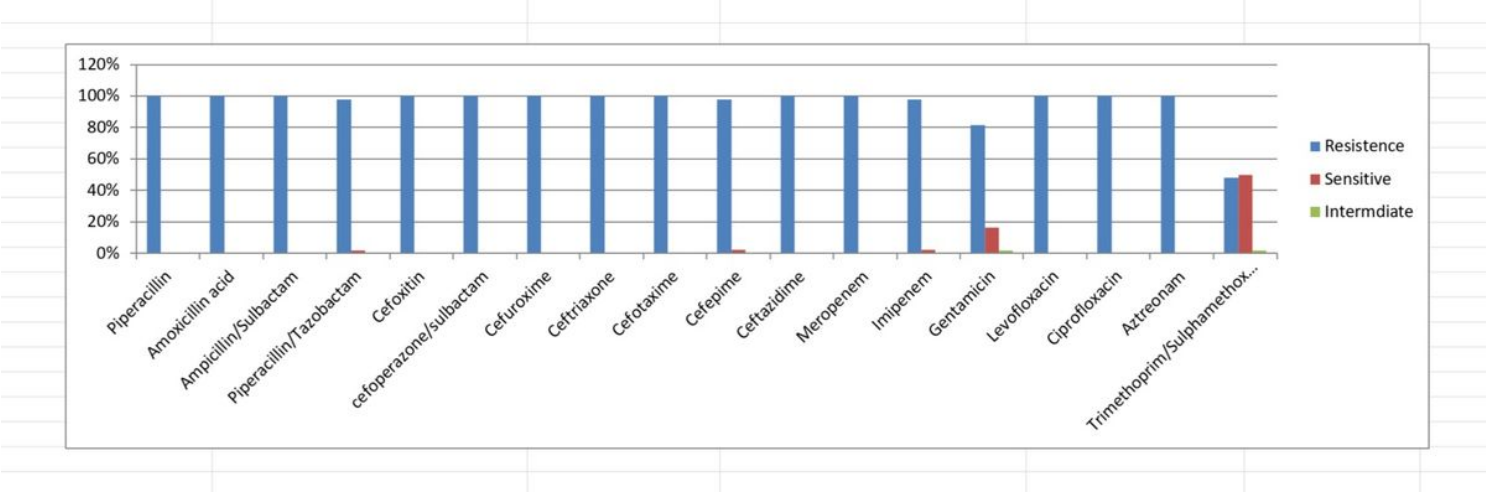


Figure 4

Antimicrobial resistance profiles of K. pneumoniae isolates against eighteen antibiotics. R: resistance, S: sensitive and I:intermediate.

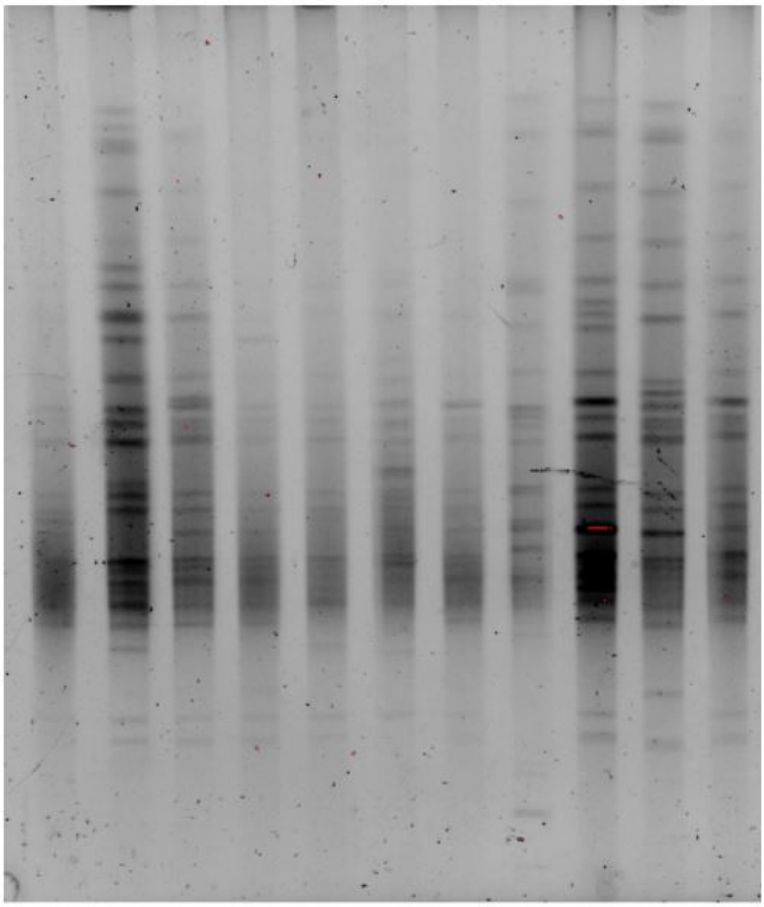
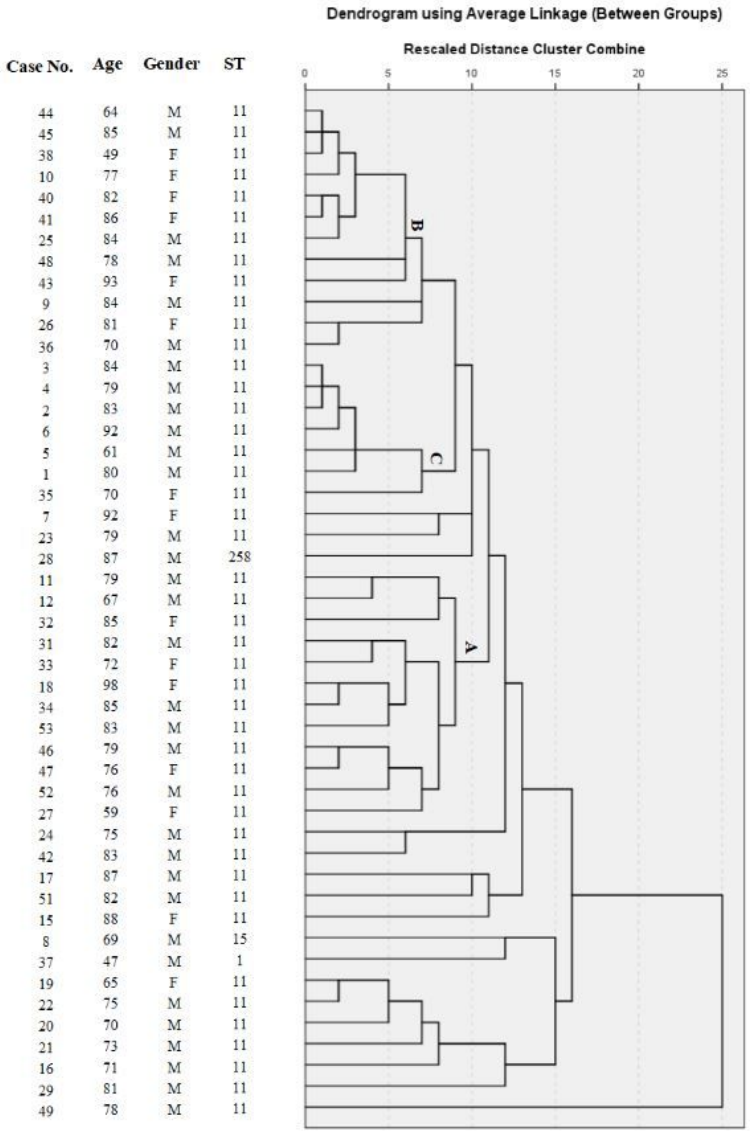


Figure 5

Molecular typing of representative multidrug resistant K.pneumoniae isolates by PFGE.



Case No.	Age	Gender	ST
44	64	M	11
45	85	M	11
38	49	F	11
10	77	F	11
40	82	F	11
41	86	F	11
25	84	M	11
48	78	M	11
43	93	F	11
9	84	M	11
26	81	F	11
36	70	M	11
3	84	M	11
4	79	M	11
2	83	M	11
6	92	M	11
5	61	M	11
1	80	M	11
35	70	F	11
7	92	F	11
23	79	M	11
28	87	M	258
11	79	M	11
12	67	M	11
32	85	F	11
31	82	M	11
33	72	F	11
18	98	F	11
34	85	M	11
53	83	M	11
46	79	M	11
47	76	F	11
52	76	M	11
27	59	F	11
24	75	M	11
42	83	M	11
17	87	M	11
51	82	M	11
15	88	F	11
8	69	M	15
37	47	M	1
19	65	F	11
22	75	M	11
20	70	M	11
21	73	M	11
16	71	M	11
29	81	M	11
49	78	M	11

Figure 6

Hierarchial clustering analysis of multidrug resistant K.pneumoniae isolates by PFGE with demographic information.