Molecular characterization of carbapenem-resistant *Klebsiella pneumoniae* isolates with focus on antimicrobial resistance

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Abstract

The enhancing incidence of carbapenem-resistant *Klebsiella pneumoniae* (CRKP)-mediated infections in Mengchao Hepatobiliary Hospital of Fujian Medical University in 2017 is the motivation behind this investigation to study gene phenotypes and resistance-associated genes of emergence regarding the CRKP strains. In current study, seven inpatients are enrolled in the hospital with complete treatments. The carbapenem-resistant *K. pneumoniae* whole genome is sequenced using MiSeq short-read and Oxford Nanopore long-read sequencing technology. Prophages are identified to assess genetic diversity within CRKP genomes. The investigation encompassed eight CRKP strains that collected from the patients enrolled as well as the environment, which illustrate that $bla_{\text{KPC-2}}$ is responsible for phenotypic resistance in six CRKP strains that *K. pneumoniae* sequence type (ST11) is informed. The plasmid with IncR, ColRNAI and pMLST type with IncF[F33:A-:B-] co-exist in all ST11 with KPC-2-producing CRKP strains. Along with carbapenemases, all *K. pneumoniae* strains harbor two or three extended spectrum β-lactamase (ESBL)-producing genes. *fosA* gene is detected amongst all the CRKP strains. The single nucleotide polymorphisms (SNP) markers are indicated and validated among all CRKP strains, providing valuable clues for distinguishing carbapenem-resistant strains from conventional *K. pneumoniae*. In conclusion, ST11 is the main CRKP type, and $bla_{\text{KPC-2}}$ is the dominant carbapenemase gene harbored by clinical CRKP isolates from current investigations.

Keywords: *Klebsiella pneumoniae*; Carbapenem-resistant; Prophage; whole-genome sequencing

Introduction

Antibiotic resistance is amongst the extremely severe public health challenges nowadays. Carbapenem-resistant *Enterobacteriaceae* (CRE) is reported as a consequence mainly due to acquisition of carbapenemase genes, and CRE is inferred as an urgent threat to human health by the Centers for Disease Control and Prevention (CDC), USA in 2013[1]. Carbapenems such as imipenem, meropenem, and biapenem represent the first-line treatment of serious infections caused by multi-resistant Enterobacteriaceae including *Klebsiella pneumoniae* (*K. pneumoniae*) and *Escherichia coli* (*E. coli*)[2]. Whereas carbapenems can be hydrolyzed by carbapenemase in carbapenem-resistant *K. pneumoniae* (CRKP)[3], which results in resistance to β-Lactam antibiotics including carbapenem. Carbapenemases can be divided into Ambler class A β-lactamases (e.g. *Klebsiella pneumoniae* carbapenemases (KPC)), class B metallo-β-lactamases (MBLs), verona integrin-encoded metallo-β-lactamase (VIM), New Delhi metallo-β-lactamase (NDM) type, and Class D Enzymes of the OXA-48 type[4]. Among Ambler class A β-lactamases, plasmid-mediated KPC has been identified in all gram-negative members of the ESKAPE pathogens[5], and KPC is the most clinically indispensable enzyme due to its prevalence in Enterobacteriaceae[6]. Moreover, pathogens harboring KPC-2 are resistant to all β-lactams and β-lactamase inhibitors except ceftazidime/avibactam, which extremely limit treatment options as well as lead to high mortality rates[7]. Additionally, NDM has become a serious threat to public health due to the rapid global dissemination of NDM-bearing pathogens and the presence on mobile genetic elements in an extensive series of species[8]. Consequently, it is imperative and urgent to investigate the CRKP characteristics for better controlling pathogens and diagnosing as well as treating patients.

In current investigation, seven CRKP strains are extracted from patients during their hospitalizations and another one CRKP strain is obtained from the dining car in Mengchao Hepatobiliary Hospital of Fujian Medical University (**Supplementary Table 1**). The whole genome of CRKP is sequenced using MiSeq short-reads and Oxford Nanopore long-reads sequencing technology. We conduct surveillance of the CRKP-mediated infection prevalence in Mengchao Hepatobiliary Hospital of Fujian Medical University, investigate the molecular epidemiological characteristics of the strains that obtained, and identify gene phenotypes as well as resistance-associated genes of the strain emergence. The detected single nucleotide polymorphisms (SNP) markers would be helpful for recognizing CRKP strain from general *K. pneumoniae*. Data of this study provide essential insights into effective strategy developments for controlling CRKP and nosocomial infection reductions.

Materials & Methods

Patient clinical information

In total, seven patients received treatments during their hospitalizations and the data of them were completely classified and studied. One bacterium was extracted from the dining car in the hospital and since the carrier was not human, there was no clinical data relating to it. All patients, except patient 1567P that was diagnosed as abdominal infection, were diagnosed as severe pneumonia or sufferred lung infections (**Supplementary Table 1**). We further give **Supplementary Table 2-8** to in detail provide all patients' treatment records as well as the phenotype measurement results and data.

All patients received systematic medical examinations such as whole blood cell test, blood routine test, blood electrolyte test, blood clotting, fungal D-glucan detection, galactomannan detection, etc. All the records are archived in detail for further investigations.

Bacterial Isolates and Antimicrobial Resistance

Single patient isolates are obtained from specimens that received from inpatients admitted to Mengchao Hepatobiliary Hospital of Fujian Medical University in 2017. A total of eight CRKP isolates (**Supplementary Table 1**), which are resistance to all the antibiotics tested, such as cephalosporins, penicilins, quinolones, aminoglycosides and carbapenems (Imipenem with MICs ≥16 μg/ml) (Table 1), were processed following standard operating procedures: the isolates are extracted according to the aseptic operating procedures and cultured in the bacterial culture medium with Columbia Agar + 5% sheep blood. The study has been performed in accordance with the Institutional Ethical Committee of the Faculty of Medicine, Mengchao Hepatobiliary Hospital of Fujian Medical University, which approved this study.

K. pneumoniae isolates are confirmed by Matrix-assisted Laser Desorption Ionization-time of Flight Mass Spectrometry (MALDI-TOF-MS) mass spectrometry (BioMerieux SA, BioMerieux Inc., France). The resistance of pathogenic bacteria is identified by Automatic Microbial Identification & Drug Sensitivity Analysis System (VITEK-2 Compact, BioMerieuxInc., France) with Gram-Negative identification card (VITEK2 AST-GN13, BioMerieuxInc., France). The results of antimicrobial susceptibility testing are interpreted based upon Clinical and Laboratory Standards Institute (CLSI) M100-S24[9]. The standard strain under quality control is *K. pneumoniae* isolates ATCC700603 (American Type Culture Collection, ATCC).

Whole Genome Sequencing (WGS) and Assembly

The isolated seven CRKP bacteria are sequenced on Illumina MiSeq (Illumina, San Diego, CA, USA) platform. MiSeq short-read sequencing library is generated with 1 ng purified DNA. Inserting a phosphate to 5' UTR end and "A" to 3' UTR end produces end-repair, and PCR fragments (300 ~ 600 bp) are collected from bar-coded adapter ligation. The library is purified via AMPure XP (Beckman Coulter) , which is then sequenced on MiSeq platform. In sum, a total of 40.5 million reads (2 × 300 bp) with a size of 1.36 Gb data are yielded (**Supplementary Table 9**). All short reads are first filtered for the low-quality sequences and then assembled into contigs using SPAdesv3.11.1 software[10].

Subsequently, we select an isolate of 1567D to perform long-read sequencing on Oxford Nanopore MinION (Oxford, UK) platform to easily sequence across repeat regions. The sequencing library is constructed with 1.5 μg purified DNA using the LSK-108 Oxford Nanopore Technologies (ONT) ligation protocol, and the prepared library is sequenced following the standard protocol of Oxford Nanopore MinION. A total of 7.48 Gb ultra-long reads are generated with N50 length of 25,890 bp (**Supplementary Table 10**). The long reads that 'passed' during the Nanopore base calling are used to assemble into complete genomic sequences via Canu software[11]. The long-read sequencing data of the same individual are used to correct base errors of assembled genome using Nanopolish (https://github.com/jts/nanopolish).

Detecting Prophages in the CRKP Genomes

The putative prophages within contigs of the CRKP genomic sequences are identified using the PHAST web server (PHAge Search Tool)[12]. The prophage completeness and categorization

126 (intact, incomplete, or questionable) are presented applying over sequences to check homology,
127 and to detect, annotate, and graphically display prophages.

128 **Carbapenemase-resistance Gene Identifications**

129 To predict the protein-coding genes and functional proteins in the CRKP genomes, all assembled
130 sequences are annotated by a web-based package RAST (Rapid Annotations using Subsystems
131 Technology)[13]. The antibiotic resistance and virulence genes, plasmids, phenotyping and
132 genotyping of CRKP genomes are scanned using the Bacterial Analysis Pipeline[14].
133 Carbapenemase-resistance genes are further identified from above annotated sequences
134 according to Simner et al.[15].
135 The protein-coding genes of long-read assembled genome are predicted using GLIMMER (Gene
136 Locator and Interpolated Markov ModelER) v3.02[16]. To functionally annotate the predicted
137 genes and perform the pathway analysis, we align them to NR, COG, Swiss-Prot, GO and KEGG
138 databases using blastX (E-value: 10^{-5}). The annotated genes serve to improve the completeness
139 of some important carbapenemase-resistance genes.
140 Comparisons of strain similarity are performed using the Harvest Tools Suite[17] (version 1.1.2).
141 For all of the isolates sequenced on a particular platform, parsnp is utilized to compare all the
142 assembled isolates against each other and known reference strain. Results are visualized using
143 EvolView.

144 **SNP Identification and Validation**

145 We download *K. pneumoniae* genome from NCBI as the reference to identify SNP markers[18].
146 All high-quality data (Q value >20, reads length > 50 bp, number of uncertain bases < 5%) of
147 eight CRKP strains are aligned to the reference genome sequences using BWA v0.7.17[19], and
148 aligned reads are sorted by coordinates via SAMTOOLS v1.4[20]. The GATK (Genome Analysis

Tool Kit) software v3.8.0[21] is utilized to detect SNPs, which is described as following: (1) duplicated reads are removed; (2) reads around insertions/deletions are realigned; (3) base quality is recalibrated using default parameters; (4) all variants are identified using HaplotypeCaller method in GATK with emitting and calling standard confidence thresholds at 10.0 and 30.0, respectively. To validate the detected SNPs in the seven CRKPs, we select 20 loci within each sample that are located in exonic regions and sequence them with high read depth. All chosen markers are designed primers for amplification using Sequenom MassARRAY iPLEX platform.

Results

Antimicrobial Susceptibilities of the CRKP Strains

The source of isolates is supplied in **Table 1**, which denotes the infectious type and the result of susceptibility testing during the patients' hospitalization. All eight strains involved in the study are confirmed to be *K. pneumoniae*, with five strains from sputum, one from bile, one from blood, and one from the environment (**Supplementary Table 1**). Clinical data demonstrate that seven of the eight patients are referred due to pulmonary infection, and another one is referred due to abdominal infection. The susceptibility testing data in **Table 1** reveals that all the *K. pneumoniae* strains are resistant to almost all antibiotics, such as cephalosporins, penicilins, quinolones and carbapenems (imipenem with MICs ≥16 μg/ml). For aminoglycosides antibiotics, except that 1567D isolate is sensitive to amikacin and tobramycin, all other isolates are resistant to aminoglycosides antibiotics. The strains including 1566D, 2038D, 2039D and 2040D are resistant to sulfamethoxazole/trimethoprim with MICs ≥320, and the other strains (1567D, 2035D, 2036D, 2037D) are sensitive to sulfamethoxazole/trimethoprim with MICs ≤ 20.

Genome assembly and annotation

The short-read sequenced seven CRKP strains are assembled into contigs. As listed in **Table 2**, the assembled genome size of all trains ranged from 5.4 Mb to 5.8 Mb, with mean length of 5.7 Mb and average contigs numbering 199. The N50 length of genomes is from 176.6 kb to 251.6 kb with an average N50 length of 220.4 kb and mean GC content of 57.2%. To obtain a more complete genome, the 1567D strain is resequenced via long-read sequencing technology and assembled into three contigs with size of 5.6 Mb (**Supplementary Figure 1**). A total of 5,841 protein-coding genes are predicted with length between 37 to 1,649 bp (**Supplementary Figure 2**). Totals of 4,657, 5,097, 4,714, 3,179 and 3,099 predicted genes are functionally annotated in NR, COG, Swiss-Prot, GO and KEGG databases, respectively (**Supplementary Figures 3, 4, 5**).

Characteristics of the CRKP Isolates

The isolated seven CRKP bacteria are sequenced through Illumina MiSeq platform and assembled into whole genomes. To understand genetic diversity, mobile genetic elements of 24 prophages are identified in seven CRKP genomes, with sizes ranging from 8.4 kb to 49.3 kb (**Figure 1**). According to the criterion that the length of an intact prophage should be more than 20 kb[22]. Prophages detected in most strains (except for 2036D) are complete with a size of at least 20.2 kb with an average GC percentage of 52.7%. Additionally, three prophages are respectively identified in 3 strains at the same time, revealing the genomic sequence homology among all isolates. The 2036D strain is comprised of just one prophage probably because of the small genome size and distinct sequence characteristics, which is expected to have less neutral targets for prophage integration[22].

Furthermore, multilocus-sequence typing (MLST) analysis reveals that there are two unrelated sequence type (ST) in *K. pneumoniae* strains isolated from different patients. 2036D *K.*

194 *pneumoniae* strain correlates with ST2632, and the other six strains are relevant to ST11 (**Table**
195 **3**). pMLST analysis reveals that all of the six ST11 *K. pneumoniae* strains are associated with
196 IncF[F33:A-:b-] and the ST2632 *K. pneumoniae* strain is relevant to IncHI1 and IncF. Majority
197 of *K. pneumoniae* strains are ST11, with IncF [F33:A-:b-] type.
198 Plasmid analysis[23] shows different circular plasmids carried by the individual strains. All strains
199 harbored IncR and ColRNAI plasmids with no virulence genes but contain several resistance-
200 associated genes that cause resistance to carbapenems, which is demonstrated in **Table 3**. The
201 IncR plasmid is identified as multidrug-resistant plasmids and has variable copy numbers of
202 certain resistance genes among *K. pneumoniae* isolates.

203 **Detection of Antibiotic Resistance Genes of CRKP Isolates**

204 The resistance-associated genes of seven CRKP bacteria (**Table 3**) are sequenced on Illumina
205 MiSeq platform among the patient and environmental isolates. As illustrated in **Table 3**, some
206 antimicrobial resistance genes are mediated by plasmid such as β-lactamase correlative genes
207 ($bla_{\text{CTX-M}}$, bla_{KPC}, bla_{LEN}, bla_{TEM}) and those genes which encoded aminoglycoside [*aac(3)-IId,*
208 *rmtB*], chloramphenicol (*catA1, catA2*), trimethoprim (*dfrA1,dfrA17*), and fluoroquinolone
209 [*QnrS1*]. The other antimicrobial resistance genes are encoded by chromosome including bla_{SHV}
210 (narrow-spectrum β-lactamasein *K. pneumoniae*), *oqxA* (1,176 bp), *oqxB* (3,153 bp) (efflux
211 pumps), and *fosA* (420 bp, fosfomycin resistance) genes.
212 Except 2036D, all the other *K. pneumoniae* strains harbor the associated carbapenemases-
213 producing resistance gene, $bla_{\text{KPC-2}}$. Extended-spectrum β-lactamases (ESBLs) resistance genes
214 such as $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{LEN} and bla_{SHV} are also informed. bla_{TEM} is one of the genes that
215 produce ESBL. $bla_{\text{CTX-M}}$ with different types ($bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-3}}$, $bla_{\text{CTX-M-55}}$ and $bla_{\text{CTX-M-65}}$)
216 is found among all the *K. pneumoniae* strains. $bla_{\text{CTX-M-3}}$ is observed in 2036D strains. $bla_{\text{CTX-M-55}}$

is observed in 1567D strain and $bla_{\text{CTX-M-14}}$ is observed in 1566D and 2040D strains. $bla_{\text{CTX-M-65}}$ is detected in the other four (2035D, 2037D, 2038D, 2039D) *K. pneumoniae* strains. bla_{LEN12} gene is exclusively found in 1566D strain, and there is no bla_{SHV} gene in it. Nevertheless, $bla_{\text{SHV-93}}$ and $bla_{\text{SHV-11}}$ genes are detected in 2036D strain and the other five *K. pneumoniae* strains, respectively. Except for the 2036D strain, $bla_{\text{TEM-1B}}$ gene is observed in all the other six *K. pneumoniae* strains. *Aac(3)-IId* and *rmtB* encoding fluoroquinolone resistance are observed among all strains. *fosA* resulting in fosfomycin resistance[24] is also informed among all strains.

Characterizing CRKP SNPs

The SNP markers are identified for all strains that sequenced using the short-read MiSeq data. The data demonstrate that 33,716 markers are detected in the 2036D strain, which is more significant than the other strains with an average of 8,289 SNPs. The cSNPs located in exonic regions are in slightly higher amounts among all detected SNPs of a minimal ratio of 85.5% (**Supplementary Table 11**). In addition, the pairwise comparison analysis reveals that 2036D isolate is disparate with the other strains based on clusters of sequence similarities using subprogram of Trinity[25] (**Figure 2a**). Furthermore, the 2036D strain share few SNP loci with the others, which coincides with strain clusters (**Figure 2b**).

For validations, all strains have a high detection rate in that approximately 153 out of 200 SNPs (76.4%) that have amplifications, which demonstrate the analysis accuracy (**Supplementary Table 11**). After filtering SNP loci that are not located in exome regions, containing no-alleles locus, and comprise all-wild SNP loci in each isolate, we eventually obtain 92 SNPs among 200 validated loci. A total of 40 out of 92 SNPs are all-variation loci in all isolates, which could be

utilized for recognizing CRKP strain from ordinary *K. pneumoniae* (**Supplementary Table 12**). In addition, 24 SNPs of strain's unique loci, including strains of 2036D (18 loci), 2035D (3 loci), 1566D (2 loci) and 2037D (1 loci), would be helpful resources for specific strain identification of clinical analysis.

Phylogeny analysis results

The phylogenic tree shows that 1567D strain is most distantly related to the other strains, and 2036D is more closely related to the reference strain comparing with all the other strains (**Supplementary Figure 6**).

GWAS analysis

To further identify significant SNPs and genes, we perform genome-wide association study (GWAS) analysis. The patients' body temperature and counts of leukocyte are selected as phenotypic character. The short-sequencing reads of six strains (**Figure 3**) are aligned to the 1567D genome using BWA v0.7.17 software. We call SNPs using Platypus v0.8.1[26], and then filter the SNPs through plink v1.9 according to the following conditions: (i) missing loci, (ii) minor allele frequency (MAF) < 0.05 and (iii) significant deviation from the Hardy-Weinberg equilibrium (HWE) ($P < 0.01$). A total of 698 SNP markers are remained and utilized for GWAS analysis. As a result, 9 loci are identified ($P < 0.05$). Two loci (ygbI and murB) are related with temperature and the other seven loci (IsrD, SufD, yrkF, fabI, sppA, entF and ttuB) are relevant to leukocyte (**Figure 3**).

Discussion

Data of current study confirm that all CRKP strains hold two types of plasmids with no virulence gene whereas harbor an abundance of associated resistance genes such as ESBLs and carbapenemases. One genotype of carbapenemases with *bla*$_{KPC-2}$ and two ST types with ST11 and ST2632 are identified in the study, and the ST11 with KPC-2-positive is a prevalent strain accounting in all the six strains. The plasmid with IncR, ColRNAI and pMLST type with IncF[F33:A-:B-] co-exist in all ST11 with KPC-2-producing CRKP strains. The initial detection of a KPC-2-producing *K. pneumoniae* isolate from a hospital in China is reported in 2007[27]. Since then, *bla*$_{KPC-2}$-bearing *K. pneumoniae* isolates have become more prevalent and reported in China as well as other countries and areas [28]. Recently, one patient is found to have susceptible *K. pneumoniae* bacteraemia in US[15]. While that case is relatively specific since the patient might be affected during the visit and hospitalization in India, which would add more complex environmental factors to confound the results. CRKP of ST11 associated with *bla*$_{KPC-2}$ is disseminated widely across China[18,29], which is concordant with the results of our study. These findings all suggest that the CRKP-mediated infections in our hospital result from ST11 with KPC-2-positive *K. pneumoniae* isolates. Continuous monitoring will be necessary to prevent further dissemination of carbapenemase-resistance genes.

Besides carbapenemases, a variety of ESBLs such as *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{LEN}$, *bla*$_{TEM}$ are present in CRKP strains of this study. *K. pneumoniae* is one of the most indispensable infectious agents in the ICU[30]. There are "classic" and hypervirulent strains of *K. pneumoniae*[31,32,33]. The "classic" non-virulent strain of *K. pneumoniae* (C-KP) can produce ESBLs related to nosocomial infectious outbreaks especially in the ICU of a hospital. C-KP more easily acquires antimicrobial resistance such as ESBLs. *bla*$_{CTX-M}$ with different type is found among all the CRKP strains. Chromosome-mediated *bla*$_{SHV}$ and plasmid-mediated *bla*$_{TEM}$ are also positive for ESBLs

285 production and are observed in six *K. pneumoniae* strains. Co-occurrence of $bla_{\text{CTX-M}}$, $bla_{\text{KPC-2}}$,
286 $bla_{\text{SHV-11}}$ and $bla_{\text{TEM-1B}}$ are observed among five *K. pneumoniae* strains. All *K. pneumoniae*
287 strains harbor two or three ESBLs-producing genes ($bla_{\text{CTX-M}}$, bla_{SHV} and bla_{TEM}), which indicate
288 all isolates contained multiple ESBLs resistance genes. Previous reports noted consistent results
289 that co-occurrence of bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$ (any two or all three) was observed among
290 *Klebsiella* isolates[34].
291
292 *fosA* is frequently identified in the *E. coli* and *K. pneumoniae* genomes [35,36]. The *fosA5* gene is
293 first found in *E. coli* in 2014[37]. In 2017, it was reported that all of 73 carbapenem-resistant *K.*
294 *pneumoniae* isolates were positive for *fosA5* in one Chinese area: Zhejiang Province[38].
295 Antimicrobial susceptibility testing about fosfomycin is not conducted in this study though *fosA*
296 is also found among all the CRKP strains, which might indicate that fosfomycin-modifying
297 enzymes account for a majority of the fosfomycin resistance, and that fosfomycin is resistant to
298 CRKP strains. It is reported that *fosA* gene is transferred from *E. coli* to *K. pneumoniae* through
299 whole plasmid transmission or mobile genetic element transmission, which raise doubts whether
300 fosfomycin can be used as a supplementary drug for urinary tract infection caused by
301 carbapenem-resistant *E. coli* in the hospital, as *fosA* exists in all CRKP strains from our study.
302 Continuous monitoring will be necessary to prevent further dissemination of fosfomycin-
303 resistant bacteria together with prudent use of fosfomycin in clinical settings.
304
305 *OqxA* and *oqxB* genes are relevant to efflux pumps, which means that antibiotics such as
306 cephalosporins, carbapenems and fluoroquinolones are almost completely expelled from *K.*
307 *pneumoniae* through its cell membrane[39]. Although there are no carbapenemases that observed in

2036D strain, *oqxA* and *oqxB* genes are identified in it. To our knowledge, these two genes are mainly reported to be responsible for the resistance to fluoroquinolones. They do have been previously reported to be associated with the nitrofurantoin resistance.

The genome sequences of the seven strains include massive contigs which are highly fragmented. Upon further investigation, we sequence the 1567D strain using long-read sequencing platform, which could help us assemble the genome with considerable improvement in completeness and contiguity. The carbapenem-resistant genes including *fosA*, *oqxA* and *oqxB* and 40 all-variation SNP loci are also identified in the above genome demonstrating the high-quality assembly. The assembly and annotation information will be beneficial in understanding the whole genomic characterization of CRKP strain for future study.

Conclusions

In conclusion, ST11 is the main CRKP type, and $bla_{\text{KPC-2}}$ is the dominant carbapenemase gene harbored by clinical CRKP isolates from present study. The plasmid with IncR, ColRNAI and pMLST type with IncF[F33:A-:B-] exist in all ST11 with KPC-2-producing CRKP strains. Besides carbapenemases, all *K. pneumoniae* strains harbor two or three ESBLs-producing genes ($bla_{\text{CTX-M}}$, bla_{SHV} and bla_{TEM}), which indicate all isolates contain multiple ESBLs resistance genes. *fosA* genes are also found among all the CRKP strains, which may infer that fosfomycin-modifying enzymes account for a majority of the fosfomycin resistance and that CRKP strains are resistant to fosfomycin. The differential expressions of *oqxA* and *oqxB* in CRKP strain might possibly result in carbapenem-resistant, but this presumption needs more solid experimental evidences. The 40 all-variation SNP loci in all isolates could be employed and referred for distinguishing CRKP strain from ordinary *K. pneumoniae*.

Declarations

Ethics approval

The study has been performed in accordance with the Institutional Ethical Committee of the Faculty of Medicine, Mengchao Hepatobiliary Hospital of Fujian Medical University.

Consent to participate

Not applicable.

Consent to publish

All authors read and approved the final manuscript.

Availability of data and materials

The genome shotgun sequencing data and long reads of Oxford Nanopore data are deposited at NCBI/GenBank as BioProject of PRJNA506754.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

X.Y., Z.Z., L.H. and H.Y. conceived of the method. H.Y. supervised the study. S.Z., S.W. and C.Y. implemented the bacteria culture. X.Y., W.Z. and Z.H. performed the bioinformatics analysis. X.Y. and Z.H. optimized and performed the sequencing. X.Y., W.Z. and Z.H. drafted

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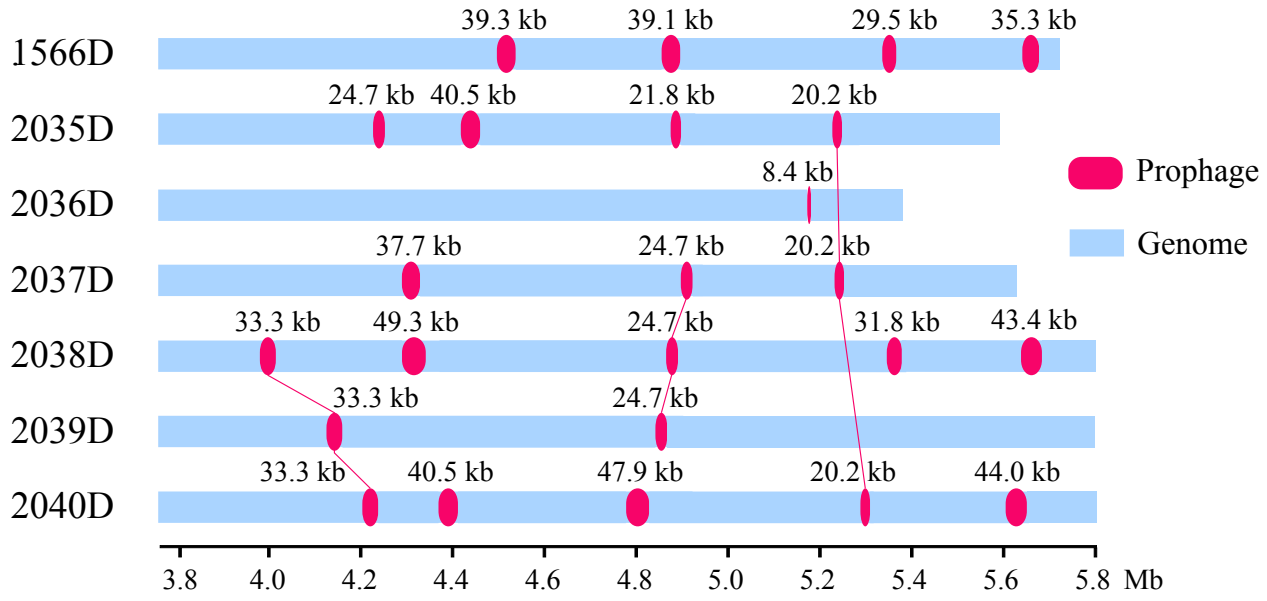
Figures

Figure 1. Intact prophages identified in seven CRKP strains.

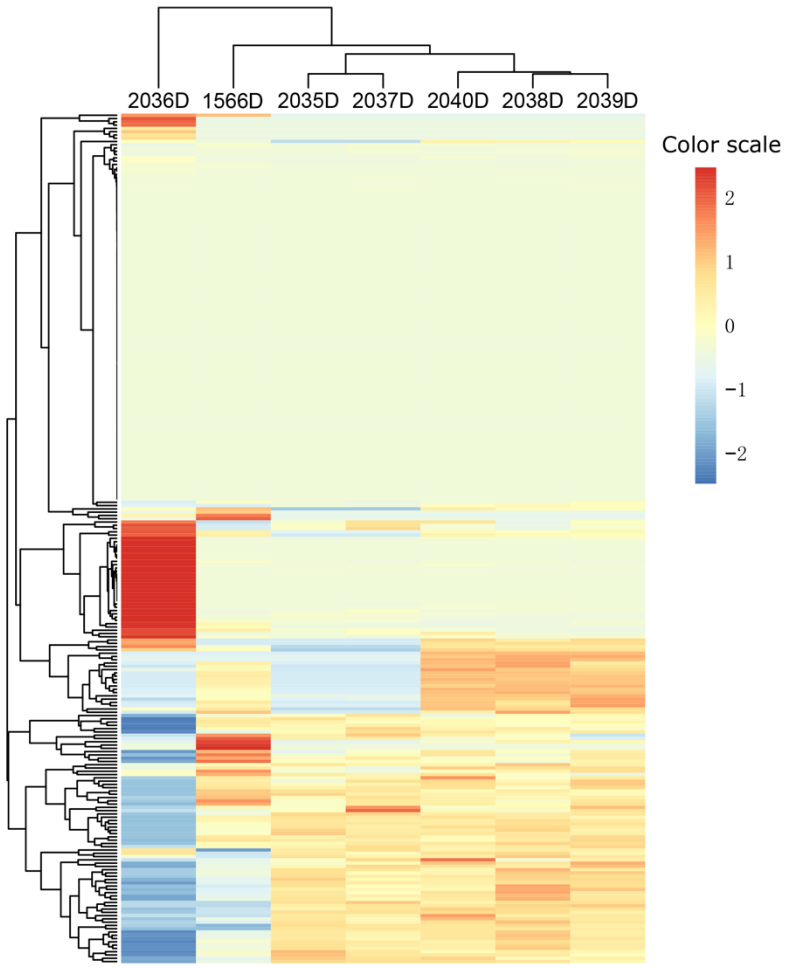


496 **Figure 2. Assessing the genetic relatedness of the CRKP by WGS.** (a) Clustering of all isolates based on

497 sequence similarity. (b) Communal SNP markers detected by pairwise comparison analysis.

498

(a)



(b)

Isolates	1566D	2035D	2036D	2037D	2038D	2039D	2040D
1566D		6922	2275	6895	7952	7848	8029
2035D	6922		2304	7249	7103	6989	7152
2036D	2275	2304		2287	2333	2342	2344
2037D	6895	7249	2287		7050	6964	7132
2038D	7952	7103	2333	7050		8417	8549
2039D	7848	6989	2342	6964	8417		8467
2040D	8029	7152	2344	7132	8549	8467	

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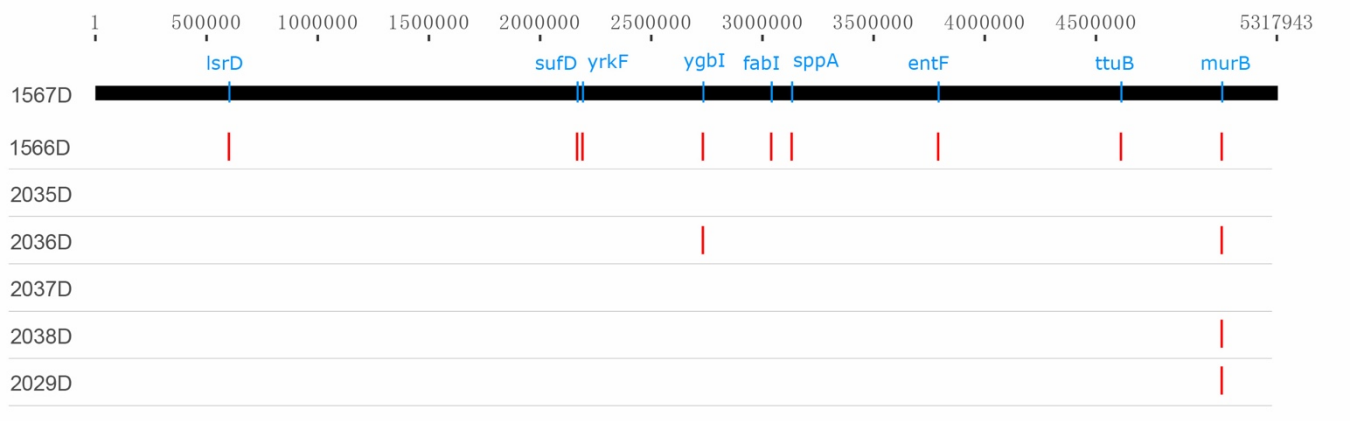
Figure 3. GWAS results of the analysis.



Tables

Table 1. Antibiotic susceptibility profiles of *K. pneumoniae*. The results of antimicrobial susceptibility testing - antibiotics MIC (mg/L) and breakpoint interpretation or epidemiological cut-off value. S: susceptible; I: intermediate; R: resistant.

Isolates	1566D	1567D	2035D	2036D	2037D	2038D	2039D	2040D
source	sputum	bile	sputum	sputum	blood	sputum	sputum	environment
Infection	Pulmonary	Abdominal	Pulmonary	Pulmonary	Pulmonary	Pulmonary	Pulmonary	N/A
ampicillin	≥32(R）	≥32(R）	≥32(R）	≥32(R）	≥32(R）	≥32(R）	≥32(R）	≥32(R）
ampicillin/ sulbactam	≥32(R）	≥32(R）	≥32(R）	≥32(R）	≥32(R）	≥32(R）	≥32(R）	≥32(R）
piperacillin/ tazobactam	≥128(R）	≥128(R）	≥128(R）	≥128(R）	≥128(R）	≥128(R）	≥128(R）	≥128(R）
cefazolin	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）
cefotetan	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）
ceftazidime	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）
ceftriaxone	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）
cefepime	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）
aztreonam	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）
imipenem	≥16(R）	≥16(R）	≥16(R）	≥16(R）	≥16(R）	≥16(R）	≥16(R）	≥16(R）
amikacin	≥64(R）	≤4（S)	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）	≥64(R）

gentamicin	≥16(R）	≥16(R）	≥16(R）	≥16(R）	≥16(R）	≥16(R）	≥16(R）	≥16(R）
tobramycin	≥16(R）	≤2（S）	≥16(R）	≥16(R）	≥16(R）	≥16(R）	≥16(R）	≥16(R）
ciprofloxacin	≥4(R）	≥4(R）	≥4(R）	≥4(R）	≥4(R）	≥4(R）	≥4(R）	≥4(R）
levofloxacin	≥8(R）	≥8(R）	≥8(R）	≥8(R）	≥8(R）	≥8(R）	≥8(R）	≥8(R）
macrodantin	256(R）	256(R）	≥512(R）	≥512(R）	≥512(R）	≥512(R）	≥512(R）	≥512(R）
sulfamethoxazole/ trimethoprim	≥320(R）	≤20（S）	≤20（S）	≤20（S）	≤20（S）	≥320(R）	≥320(R）	≥320(R)

Table 2. Assembly statistics of seven CRKP strains via short-read sequencing.

Assembly	1566D	2035D	2036D	2037D	2038D	2039D	2040D
Contig number	195	163	215	242	184	196	195
Total length (bp)	5,758,754	5,633,502	5,432,179	5,670,795	5,833,697	5,831,354	5,835,044
Largest contig (bp)	380,781	381,132	929,110	380,795	381,132	381,056	381,056
GC (%)	57.33	57.39	57.18	57.35	57.21	57.21	57.2
N50	176,606	196,479	251,620	196,479	183,862	190,289	183,862
L50	12	11	7	11	11	11	11
Total number of Ns	20	30	20	30	130	130	30

Table 3. Resistance genes among the patient and environmental isolates.

Isolates	1566D	2035D	2036D	2037D	2038D	2039D	2040D
MLST	ST11	ST11	ST2632	ST11	ST11	ST11	ST11
pMLST	IncF[F33:A-:B-]	IncF[F33:A-:B-]	IncHI1, IncF	IncF[F33:A-:B-]	IncF[F33:A-:B-]	IncF[F33:A-:B-]	IncF[F33:A-:B-]
Plasmids	IncR, ColRNAI	IncR, ColRNAI	IncR, ColRNAI	IncR, ColRNAI	IncR, ColRNAI	IncR, ColRNAI	IncR, ColRNAI
Penicillins: Ampicillin/	*blaCTX-M-14*	*blaCTX-M-65*		*blaCTX-M-65*	*blaCTX-M-65*	*blaCTX-M-65*	*blaCTX-M-14*
Narrow-Spectrum	*blaKPC-2*	*blaKPC-2*	*blaCTX-M-3*	*blaKPC-2*	*blaKPC-2*	*blaKPC-2*	*blaKPC-2*
Cephalosporins: cefazolin and	*blaLEN12*	*blaSHV-11*	*blaSHV-93*	*blaSHV-11*	*blaSHV-11*	*blaSHV-11*	*blaSHV-11*
cefotetan	*blaTEM-1B*	*blaTEM-1B*		*blaTEM-1B*	*blaTEM-1B*	*blaTEM-1B*	*blaTEM-1B*
β-lactam inhibitors/ Carbapenems	*blaKPC-2*	*blaKPC-2*	-	*blaKPC-2*	*blaKPC-2*	*blaKPC-2*	*blaKPC-2*
Extended-Spectrum Cephalosporins/ Monobactam	*blaCTX-M-14*	*blaCTX-M-65*	*blaCTX-M-3*	*blaCTX-M-65*	*blaCTX-M-65*	*blaCTX-M-65*	*blaCTX-M-65*
Aminoglycosides	*rmtB*	*rmtB*	*aac(3)-IId*	*rmtB*	*aac(3)-IId* *rmtB*	*aac(3)-IId* *rmtB*	*aac(3)-IId* *rmtB*
Fluoroquinolones	*QnrS1*	-	*oqxA* *oqxB*	-	*QnrS1*	*QnrS1*	*QnrS1*
Phosphonic Acid	*fosA*	*fosA*	*fosA*	*fosA*	*fosA*	*fosA*	*fosA*
Phenicol	-	*catA2*	*catA1*	*catA2*	*catA2*	*catA2*	*catA2*
Folate-pathway Inhibitors	*dfrA1*	-	*dfrA17*	-	*dfrA1*	*dfrA1*	*dfrA1*