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

Running title: Genomic characterization of CRKP

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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

39 **Introduction**

40 Antibiotic resistance is amongst the extremely severe public health challenges nowadays.
41 Carbapenem-resistant *Enterobacteriaceae* (CRE) is reported as a consequence mainly due to
42 acquisition of carbapenemase genes, and CRE is inferred as an urgent threat to human health by
43 the Centers for Disease Control and Prevention (CDC), USA in 2013[1]. Carbapenems such as
44 imipenem, meropenem, and biapenem represent the first-line treatment of serious infections
45 caused by multi-resistant Enterobacteriaceae including *Klebsiella pneumoniae* (*K. pneumoniae*)
46 and *Escherichia coli* (*E. coli*)[2]. Whereas carbapenems can be hydrolyzed by carbapenemase in
47 carbapenem-resistant *K. pneumoniae* (CRKP)[3], which results in resistance to β-Lactam
48 antibiotics including carbapenem. Carbapenemases can be divided into Ambler class A β-
49 lactamases (e.g. *Klebsiella pneumoniae* carbapenemases (KPC)), class B metallo-β-lactamases
50 (MBLs), verona integrin-encoded metallo-β-lactamase (VIM), New Delhi metallo-β-lactamase
51 (NDM) type, and Class D Enzymes of the OXA-48 type[4]. Among Ambler class A β-lactamases,
52 plasmid-mediated KPC has been identified in all gram-negative members of the ESKAPE
53 pathogens[5], and KPC is the most clinically indispensable enzyme due to its prevalence in
54 Enterobacteriaceae[6]. Moreover, pathogens harboring KPC-2 are resistant to all β-lactams and β-
55 lactamase inhibitors except ceftazidime/avibactam, which extremely limit treatment options as
56 well as lead to high mortality rates[7]. Additionally, NDM has become a serious threat to public
57 health due to the rapid global dissemination of NDM-bearing pathogens and the presence on
58 mobile genetic elements in an extensive series of species[8]. Consequently, it is imperative and
59 urgent to investigate the CRKP characteristics for better controlling pathogens and diagnosing as
60 well as treating patients.
61

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

73 **Materials & Methods**

74 **Patient clinical information**

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

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

85 **Bacterial Isolates and Antimicrobial Resistance**

86 Single patient isolates are obtained from specimens that received from inpatients admitted to
87 Mengchao Hepatobiliary Hospital of Fujian Medical University in 2017. A total of eight CRKP
88 isolates (**Supplementary Table 1**), which are resistance to all the antibiotics tested, such as
89 cephalosporins, penicilins, quinolones, aminoglycosides and carbapenems (Imipenem with MICs
90 ≥16 μg/ml) (Table 1), were processed following standard operating procedures: the isolates are
91 extracted according to the aseptic operating procedures and cultured in the bacterial culture
92 medium with Columbia Agar + 5% sheep blood. The study has been performed in accordance
93 with the Institutional Ethical Committee of the Faculty of Medicine, Mengchao Hepatobiliary
94 Hospital of Fujian Medical University, which approved this study.
95
96 *K. pneumoniae* isolates are confirmed by Matrix-assisted Laser Desorption Ionization-time of
97 Flight Mass Spectrometry (MALDI-TOF-MS) mass spectrometry (BioMerieux SA, BioMerieux
98 Inc., France). The resistance of pathogenic bacteria is identified by Automatic Microbial
99 Identification & Drug Sensitivity Analysis System (VITEK-2 Compact, BioMerieuxInc., France)
100 with Gram-Negative identification card (VITEK2 AST-GN13, BioMerieuxInc., France). The
101 results of antimicrobial susceptibility testing are interpreted based upon Clinical and Laboratory
102 Standards Institute (CLSI) M100-S24[9]. The standard strain under quality control is *K.*
103 *pneumoniae* isolates ATCC700603 (American Type Culture Collection, ATCC).
104

105 **Whole Genome Sequencing (WGS) and Assembly**

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

114

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

124 **Detecting Prophages in the CRKP Genomes**

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

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

129 **Carbapenemase-resistance Gene Identifications**

130 To predict the protein-coding genes and functional proteins in the CRKP genomes, all assembled
131 sequences are annotated by a web-based package RAST (Rapid Annotations using Subsystems
132 Technology)[13]. The antibiotic resistance and virulence genes, plasmids, phenotyping and
133 genotyping of CRKP genomes are scanned using the Bacterial Analysis Pipeline[14].
134 Carbapenemase-resistance genes are further identified from above annotated sequences
135 according to Simner et al.[15].

136 The protein-coding genes of long-read assembled genome are predicted using GLIMMER (Gene
137 Locator and Interpolated Markov ModelER) v3.02[16]. To functionally annotate the predicted
138 genes and perform the pathway analysis, we align them to NR, COG, Swiss-Prot, GO and KEGG
139 databases using blastX (E-value: 10^{-5}). The annotated genes serve to improve the completeness
140 of some important carbapenemase-resistance genes.

141 Comparisons of strain similarity are performed using the Harvest Tools Suite[17] (version 1.1.2).
142 For all of the isolates sequenced on a particular platform, parsnp is utilized to compare all the
143 assembled isolates against each other and known reference strain. Results are visualized using
144 EvolView.

145 **SNP Identification and Validation**

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

150 Tool Kit) software v3.8.0[21] is utilized to detect SNPs, which is described as following: (1)
151 duplicated reads are removed; (2) reads around insertions/deletions are realigned; (3) base
152 quality is recalibrated using default parameters; (4) all variants are identified using
153 HaplotypeCaller method in GATK with emitting and calling standard confidence thresholds at
154 10.0 and 30.0, respectively. To validate the detected SNPs in the seven CRKPs, we select 20 loci
155 within each sample that are located in exonic regions and sequence them with high read depth.
156 All chosen markers are designed primers for amplification using Sequenom MassARRAY
157 iPLEX platform.

158 **Results**

159 **Antimicrobial Susceptibilities of the CRKP Strains**

160 The source of isolates is supplied in **Table 1**, which denotes the infectious type and the result of
161 susceptibility testing during the patients' hospitalization. All eight strains involved in the study
162 are confirmed to be *K. pneumoniae*, with five strains from sputum, one from bile, one from blood,
163 and one from the environment (**Supplementary Table 1**). Clinical data demonstrate that seven
164 of the eight patients are referred due to pulmonary infection, and another one is referred due to
165 abdominal infection. The susceptibility testing data in **Table 1** reveals that all the *K. pneumoniae*
166 strains are resistant to almost all antibiotics, such as cephalosporins, penicilins, quinolones and
167 carbapenems (imipenem with MICs ≥16 μg/ml). For aminoglycosides antibiotics, except that
168 1567D isolate is sensitive to amikacin and tobramycin, all other isolates are resistant to
169 aminoglycosides antibiotics. The strains including 1566D, 2038D, 2039D and 2040D are
170 resistant to sulfamethoxazole/trimethoprim with MICs ≥320, and the other strains (1567D,
171 2035D, 2036D, 2037D) are sensitive to sulfamethoxazole/trimethoprim with MICs ≤ 20.

172 **Genome assembly and annotation**

173 The short-read sequenced seven CRKP strains are assembled into contigs. As listed in **Table 2**,
174 the assembled genome size of all trains ranged from 5.4 Mb to 5.8 Mb, with mean length of 5.7
175 Mb and average contigs numbering 199. The N50 length of genomes is from 176.6 kb to 251.6
176 kb with an average N50 length of 220.4 kb and mean GC content of 57.2%. To obtain a more
177 complete genome, the 1567D strain is resequenced via long-read sequencing technology and
178 assembled into three contigs with size of 5.6 Mb (**Supplementary Figure 1**). A total of 5,841
179 protein-coding genes are predicted with length between 37 to 1,649 bp (**Supplementary Figure**
180 **2**). Totals of 4,657, 5,097, 4,714, 3,179 and 3,099 predicted genes are functionally annotated in
181 NR, COG, Swiss-Prot, GO and KEGG databases, respectively (**Supplementary Figures 3, 4, 5**).

182 **Characteristics of the CRKP Isolates**

183 The isolated seven CRKP bacteria are sequenced through Illumina MiSeq platform and
184 assembled into whole genomes. To understand genetic diversity, mobile genetic elements of 24
185 prophages are identified in seven CRKP genomes, with sizes ranging from 8.4 kb to 49.3 kb
186 (**Figure 1**). According to the criterion that the length of an intact prophage should be more than
187 20 kb[22]. Prophages detected in most strains (except for 2036D) are complete with a size of at
188 least 20.2 kb with an average GC percentage of 52.7%. Additionally, three prophages are
189 respectively identified in 3 strains at the same time, revealing the genomic sequence homology
190 among all isolates. The 2036D strain is comprised of just one prophage probably because of the
191 small genome size and distinct sequence characteristics, which is expected to have less neutral
192 targets for prophage integration[22].

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

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

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

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

218 is observed in 1567D strain and *bla*$_{CTX-M-14}$ is observed in 1566D and 2040D strains. *bla*$_{CTX-M-65}$
219 is detected in the other four (2035D, 2037D, 2038D, 2039D) *K. pneumoniae* strains. *bla*$_{LEN12}$
220 gene is exclusively found in 1566D strain, and there is no *bla*$_{SHV}$ gene in it. Nevertheless, *bla*$_{SHV-}$
221 $_{93}$ and *bla*$_{SHV-11}$ genes are detected in 2036D strain and the other five *K. pneumoniae* strains,
222 respectively. Except for the 2036D strain, *bla*$_{TEM-1B}$ gene is observed in all the other six *K.*
223 *pneumoniae* strains. *Aac(3)-IId* and *rmtB* encoding fluoroquinolone resistance are observed
224 among all strains. *fosA* resulting in fosfomycin resistance[24] is also informed among all strains.
225
226 **Characterizing CRKP SNPs**
227 The SNP markers are identified for all strains that sequenced using the short-read MiSeq data.
228 The data demonstrate that 33,716 markers are detected in the 2036D strain, which is more
229 significant than the other strains with an average of 8,289 SNPs. The cSNPs located in exonic
230 regions are in slightly higher amounts among all detected SNPs of a minimal ratio of 85.5%
231 (**Supplementary Table 11**). In addition, the pairwise comparison analysis reveals that 2036D
232 isolate is disparate with the other strains based on clusters of sequence similarities using
233 subprogram of Trinity[25] (**Figure 2a**). Furthermore, the 2036D strain share few SNP loci with the
234 others, which coincides with strain clusters (**Figure 2b**).
235
236 For validations, all strains have a high detection rate in that approximately 153 out of 200 SNPs
237 (76.4%) that have amplifications, which demonstrate the analysis accuracy (**Supplementary**
238 **Table 11**). After filtering SNP loci that are not located in exome regions, containing no-alleles
239 locus, and comprise all-wild SNP loci in each isolate, we eventually obtain 92 SNPs among 200
240 validated loci. A total of 40 out of 92 SNPs are all-variation loci in all isolates, which could be

241 utilized for recognizing CRKP strain from ordinary *K. pneumoniae* (**Supplementary Table 12**).
242 In addition, 24 SNPs of strain's unique loci, including strains of 2036D (18 loci), 2035D (3 loci),
243 1566D (2 loci) and 2037D (1 loci), would be helpful resources for specific strain identification of
244 clinical analysis.
245
246 **Phylogeny analysis results**
247 The phylogenic tree shows that 1567D strain is most distantly related to the other strains, and
248 2036D is more closely related to the reference strain comparing with all the other strains
249 (**Supplementary Figure 6**).
250
251 **GWAS analysis**
252 To further identify significant SNPs and genes, we perform genome-wide association study
253 (GWAS) analysis. The patients' body temperature and counts of leukocyte are selected as
254 phenotypic character. The short-sequencing reads of six strains (**Figure 3**) are aligned to the
255 1567D genome using BWA v0.7.17 software. We call SNPs using Platypus v0.8.1[26], and then
256 filter the SNPs through plink v1.9 according to the following conditions: (i) missing loci, (ii)
257 minor allele frequency (MAF) < 0.05 and (iii) significant deviation from the Hardy-Weinberg
258 equilibrium (HWE) ($P < 0.01$). A total of 698 SNP markers are remained and utilized for GWAS
259 analysis. As a result, 9 loci are identified ($P < 0.05$). Two loci (ygbI and murB) are related with
260 temperature and the other seven loci (IsrD, SufD, yrkF, fabI, sppA, entF and ttuB) are relevant to
261 leukocyte (**Figure 3**).

262 **Discussion**

263 Data of current study confirm that all CRKP strains hold two types of plasmids with no virulence
264 gene whereas harbor an abundance of associated resistance genes such as ESBLs and
265 carbapenemases. One genotype of carbapenemases with *bla*$_{KPC-2}$ and two ST types with ST11
266 and ST2632 are identified in the study, and the ST11 with KPC-2-positive is a prevalent strain
267 accounting in all the six strains. The plasmid with IncR, ColRNAI and pMLST type with
268 IncF[F33:A-:B-] co-exist in all ST11 with KPC-2-producing CRKP strains. The initial detection
269 of a KPC-2-producing *K. pneumoniae* isolate from a hospital in China is reported in 2007[27].
270 Since then, *bla*$_{KPC-2}$-bearing *K. pneumoniae* isolates have become more prevalent and reported in
271 China as well as other countries and areas [28]. Recently, one patient is found to have susceptible *K.*
272 *pneumoniae* bacteraemia in US[15]. While that case is relatively specific since the patient might be
273 affected during the visit and hospitalization in India, which would add more complex
274 environmental factors to confound the results. CRKP of ST11 associated with *bla*$_{KPC-2}$ is
275 disseminated widely across China[18,29], which is concordant with the results of our study. These
276 findings all suggest that the CRKP-mediated infections in our hospital result from ST11 with
277 KPC-2-positive *K. pneumoniae* isolates. Continuous monitoring will be necessary to prevent
278 further dissemination of carbapenemase-resistance genes.
279 Besides carbapenemases, a variety of ESBLs such as *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{LEN}$, *bla*$_{TEM}$ are present
280 in CRKP strains of this study. *K. pneumoniae* is one of the most indispensable infectious agents
281 in the ICU[30]. There are "classic" and hypervirulent strains of *K. pneumoniae*[31,32,33]. The "classic"
282 non-virulent strain of *K. pneumoniae* (C-KP) can produce ESBLs related to nosocomial
283 infectious outbreaks especially in the ICU of a hospital. C-KP more easily acquires antimicrobial
284 resistance such as ESBLs. *bla*$_{CTX-M}$ with different type is found among all the CRKP strains.
285 Chromosome-mediated *bla*$_{SHV}$ and plasmid-mediated *bla*$_{TEM}$ are also positive for ESBLs

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

309 2036D strain, *oqxA* and *oqxB* genes are identified in it. To our knowledge, these two genes are
310 mainly reported to be responsible for the resistance to fluoroquinolones. They do have been
311 previously reported to be associated with the nitrofurantoin resistance.
312
313 The genome sequences of the seven strains include massive contigs which are highly fragmented.
314 Upon further investigation, we sequence the 1567D strain using long-read sequencing platform,
315 which could help us assemble the genome with considerable improvement in completeness and
316 contiguity. The carbapenem-resistant genes including *fosA*, *oqxA* and *oqxB* and 40 all-variation
317 SNP loci are also identified in the above genome demonstrating the high-quality assembly. The
318 assembly and annotation information will be beneficial in understanding the whole genomic
319 characterization of CRKP strain for future study.

320 **Conclusions**

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

332 **Declarations**

333 **Ethics approval**

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

336 **Consent to participate**

337 Not applicable.

338 **Consent to publish**

339 All authors read and approved the final manuscript.

340 **Availability of data and materials**

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

343 **Competing interests**

344 The authors declare that they have no competing interests.

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351 **Authors' Contributions**

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

the article with inputs and feedbacks from all the other authors. All authors read and approved the final manuscript.

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493 **Figures**

494 **Figure 1. Intact prophages identified in seven CRKP strains.**

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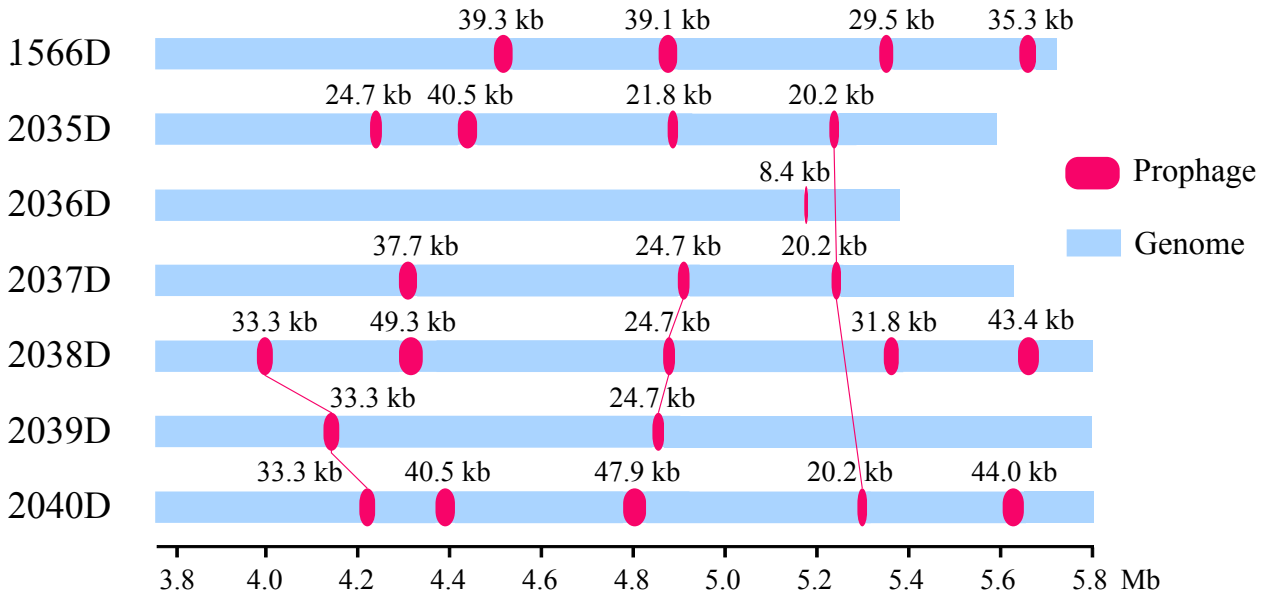

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499 **Figure 2. Assessing the genetic relatedness of the CRKP by WGS.** (a) Clustering of all isolates based on

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

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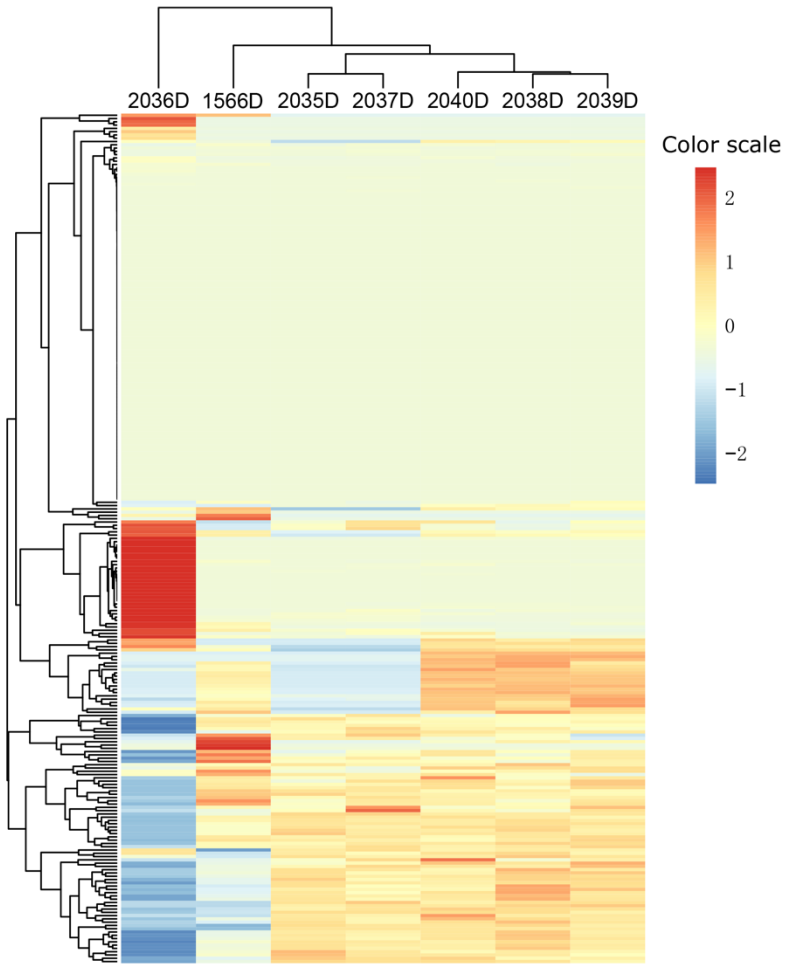
(a)



(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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506 **Figure 3. GWAS results of the analysis.**

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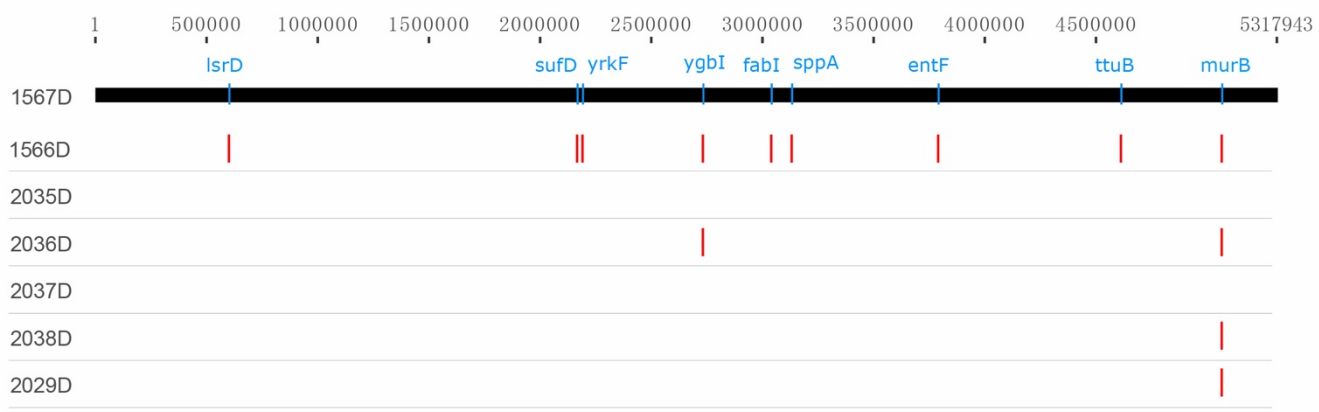

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Tables

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*