First description of increased resistance in carbapenem-susceptible Klebsiella pneumoniae with imipenem treatment driven by outer membrane remodelling in China

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Abstract
The emergence of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) posed threats to human health. Although there are numerous studies regarding porin alteration in association with the production of ESBLs and/or AmpC β-lactamase, a systematic research about the treatment-emergence of porins alteration in antibiotic resistance does not exist yet. The aim of this study was to investigate the underlying mechanism and evolution of resistance of *K. pneumoniae* during carbapenem treatment. Here, we reported three strains (FK-2624, FK-2723 and FK-2820) isolated from one patient before and after imipenem treatment during hospitalization. Antibiotic susceptibility testing indicated that FK-2624 was susceptible to almost antimicrobials but fosfomycin; FK-2723 and FK-2820 were MDR. After imipenem therapy, FK-2820 was evolved to carbapenem-resistant. PCR and Whole-Genome sequencing (WGS) indicated that resistance genes *bla SHV*, *oqxA* and *fosA5* were detected in FK-2624, in addition, FK-2723 and FK-2820 harbored *bla DHA*, *qnrB*, *aac (6')-Ib*. Virulence factors *K57*, *ybtA*, *mrkD*, *entB* and *iroN* were detected simultaneously in all of three strains. The results of pairwise comparisons, multi-locus sequencing typing (MLST) and pulsed-field gel electrophoresis (PFGE) revealed high homology among the isolates. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) results showed that isolate FK-2820 lacked OmpK 36 as there was a premature stop codon of the outer membrane porin encoding gene *ompk36* confirmed by sequencing. Real-time RT-PCR revealed that the expression of *ompK36* in FK-2820 was 0.093 times the control isolate ATCC 13883. Our study highlighted that the alteration of outer membrane porins due to the 14-day use of imipenem clinically play a potential role in leading to the carbapenem-resistance of FK-2820.

Introduction
*Klebsiella pneumoniae* is a serious hospital-acquired pathogen causing many infections including urinary tract infections, pneumonia, bloodstream infections [1, 2]. The abuse of expanded-spectrum cephalosporins for the remedy of these organisms has contributed to the appearance of Extended-spectrum β-Lactamases (ESBLs)/AmpC-producing *K. pneumoniae*. From then on, carbapenems were considered as the last resort for the treatment of infections caused by multidrug-resistant (MDR)
isolates due to their broadest antibacterial spectrum compared to other β-lactams. However, carbapenem-resistant *K. pneumoniae* (CRKP) have increasingly emerged under the antibacterial drug selection pressure. The production of carbapenemases is the mainly mechanism of CRKP since the first isolation of CRKP in America in 1996 [3]. And it could also result from porin alteration in association with the production of ESBLs and/or AmpC β-lactamase [4]. OmpK36 is a nonspecific porin in *K. pneumoniae*, which belongs to the OmpC porin group with small channel size [5]. Many reports suggest that loss of Ompk36, coupled with ESBL and/or AmpC production, plays an important role in conferring carbapenem resistance in *K. pneumoniae* [6, 7]. But the evolution of outer membrane porin still lacks of research. The current study focused on the evolution of carbapenem resistance determinants of *K. pneumoniae* isolated from one inpatient and emphasis on determining appropriate antimicrobial course of treatment.

**Materials And Methods**

**Bacterial isolates**

During October 25, 2015 to February 14, 2016, *K. pneumoniae* strains FK2624, FK-2723 and FK-2820 were isolated from the sputum samples of a patient in ICU of the First Affiliated Hospital in Wenzhou, China. FK-2624 was the first strain of *K. pneumoniae* isolated from the patient. After that, seven strains were separated successively. FK-2624 was chosen to conduct this study due to the drug resistance profiles were the same as the other seven. FK-2723 was the carbapenem-susceptible strain isolated from the same patient before carbapenem treatment. Subsequently, and after a 14-day treatment of imipenem, FK-2820 was recovered. All of the investigation protocols in this study were approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (ethical number 2019-75). Informed consent was waived because this study mainly focused on bacteria and did no interventions to patients.

**Antimicrobial susceptibility testing, detection of resistance genes and virulence factors**

The modified carbapenem inactivation method (mCIM) was carried out according to CLSI recommendations for phenotypic screening of carbapenemase producers.[8] Antimicrobial
susceptibility test was determined by the agar dilution method and interpreted according to the recommendations in the latest CLSI guidelines, including ceftriaxone, ceftazidime, cefotaxime, imipenem, meropenem, ertapenem, levofloxacin, ciprofloxacin, tobramycin, gentamicin, amikacin and fosfomycin. The minimum inhibitory concentrations (MICs) of colistin and tigecycline were determined by the broth microdilution method. The latest EUCAST breakpoints (available at http://www.eucast.org/clinical breakpoints/) were used for colistin and tigecycline. E. coli ATCC 25922 was used as the quality control strain.

A variety of antimicrobial resistance genes were amplified by PCR, and the positive amplicons were further confirmed by DNA sequence. These resistance genes included those encoding the extended spectrum β-lactamase genes (bla\textsubscript{CTX-M}, bla\textsubscript{PER}, bla\textsubscript{SHV}, bla\textsubscript{TEM} and bla\textsubscript{VEB}) [9], carbapenemase genes (bla\textsubscript{GES}, bla\textsubscript{IMI/NMC-A}, bla\textsubscript{SME}, bla\textsubscript{KPC}, bla\textsubscript{VIM}, bla\textsubscript{IMP}, bla\textsubscript{NDM} and bla\textsubscript{OXA-48}) [10, 11], AmpC β-lactamase genes (bla\textsubscript{CMY}, bla\textsubscript{FOX}, bla\textsubscript{MOX}, and bla\textsubscript{DHA}) [12], plasmid-mediated quinolone resistance (PMQR) genes (qnrA, qnrB, qnrC, qnrD, qnrS, aac(6')-lb-cr) [13], oqxAB multidrug efflux pump genes [14] and the aminoglycoside resistant genes (aac(6')-lb, aac(3')-lb, rmtB, armA, APH, ANT, rmtA, rmtB rmtC and rmtD) [15-17]. DNA sequences were identified by sequence comparisons using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [18].

To check for the presence of genes that associated with virulence in K. pneumoniae [19] [20, 21], virulence profile was detected by PCR, namely capsular serotype K1, K2, K5, K20, K54, K57, hypermucoviscosity phenotype (magA), allantoin metabolism (allS), regulator of mucoid phenotype A (rmpA), iron system capture (iroN), adhesion type 3 fimbriae (mrkD), iron transport and phosphotransferase function (kfu), siderophore (entB) and siderophore yersiniabactin (ybtA).

**Whole-Genome Sequencing (WGS)**

Genomic DNA of K. pneumoniae FK-2624, FK-2723 and FK-2820 were purified using the Bioflux DNA purification kit (Bioflux BSC12S1, Beijing) as recommended by the manufacturer. A total amount of 1μg DNA per sample was used as input material for the DNA sample preparations. Sequencing
libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB E7645S, USA) following manufacturer’s recommendations and index codes were added to attribute sequences to each sample. At last, PCR products were purified (AMPure XP system A63880, Beckman, USA) and libraries were analyzed for size distribution by Agilent2100 Bioanalyzer and quantified using real-time PCR. The genomes were sequenced by Illumina NovaSeq PE150. Sequence reads for each isolate were assembled individually. All good quality paired reads were assembled using the SOAP denovo [22, 23], SPAdes [24] and ABYSS [25] into a number of scaffolds, and annotated using the National Center for Biotechnology Information’s Prokaryotic Genome Annotation Pipeline [26]. To identify potential antibiotic resistance genes in the genomic sequence of the isolates, sequence alignment of the protein sequences of antibiotic resistance genes in the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/). Virulence genes were identified using the virulence factor database (http://www.mgc.ac.cn/VFs/) and PathogenFinder (https://cge.cbs.dtu.dk/services/PathogenFinder/). The nucleotide sequence of the genome of FK-2624, FK-2723 and FK-2820 have been submitted to GenBank with accession no. VIGL00000000, VIGM00000000 and VIGK00000000, respectively.

**Determination of genetic relatedness of same-patient isolates**

Pairwise comparisons method, pulsed-field gel electrophoresis (PFGE) and multi-locus sequencing typing (MLST) were used to establish relatedness between same-patient isolates. The sequences of the three complete genomes of *K. pneumoniae* isolates were independently used in sequence alignments. The sequence reads were mapped to the reference genomes using the Bowtie2 software, which is good for mapping short sequence reads to medium-sized and large genomes. The alignment of clean data of 3 isolates with reference *K. pneumoniae* MGH 78578 (MDR bacterium isolated from a patient [27], accession number CP000647) was performed with the default settings of programs. Finally, the alignment percentage (supplementary material Table S1) was showed by Bowtie2, revealed a high degree of genetic conservation was observed between the three *K. pneumoniae* strains.

PFGE was carried out on our strains according to the method described previously with minor
Genomic DNA was extracted from the *K. pneumoniae* isolates, followed by restriction enzyme Xba I (Takara 1093A, Japan) digestion for 2 h. PFGE was performed using a CHEF-Mapper XA PFGE system (Bio-Rad, USA) for 18 h with a switch time 6–36 s. Then DNA fingerprints were revealed by GelRed staining. The banding patterns were visualized by GelDoc XR gel imaging system (Bio-Rad, USA) and cluster analysis of similarity values of the PFGE profiles were finally performed by Quantity One program (BioRad Laboratories, USA). The sequence types (STs) of FK-2624, FK-2723 and FK-2820 were determined by MLST. Seven housekeeping genes (*gapA, infB, mdh, pgi, phoE, rpoB*, and *tonB*) were amplified and sequenced according to Diancourt *et al.* [29]. Alleles and sequence types were assigned by the MLST database (http://www.pasteur.fr/mlst/Kpneumoniae.html).

**Outer membrane protein isolation and SDS-PAGE**

FK-2624, FK-2723 and FK-2820 were cultured in Muller Hinton broth with shaking overnight at 37°C. Outer membrane porins (OMPs) were recovered by centrifugation (4200 rpm for 15 min), washed with 10 mM Tris-HCl, 5 mM MgCl₂ (PH 7.3), and lysed by sonication as described [30]. The supernatants were treated with 2% solution of sodium lauroylsarcosinate for 30 min at room temperature, centrifuged 30 min at 17000 rpm and the pellets containing the OMPs were suspended in 10 mM Tris-HCl, 5 mM MgCl₂ (PH 7.3).

Samples mixed with loading buffer (TaKaRa 9173, Japan) were first denatured by heating at 100°C for 3 min, then they were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% of separation gel and 5% of concentration gel. Bands were visualized by dyeing the gels with 0.2% Coomassie brilliant blue (Solarbio C8430, China) in 10% acetic acid and 45% methanol. *K. pneumoniae* ATCC 13883 (an isolate with known expression of OmpK35 and OmpK36) served as a control strain for OMPs profiling.

**Analysis of ompK35 and ompk36 genes**
The coding sequences of the *ompK35* and *ompK36* genes for the representative isolates were amplified and sequenced using primers listed in Table 1. Amplification was carried out with the following thermal cycling conditions: 5 min at 94°C and 35 cycles of amplification consisting of 30 s at 94°C, 30 s at 58°C, and 1 min at 72°C, with 10 min at 72°C for the final extension. DNA fragments were analyzed by electrophoresis in a 1% agarose gel at 120 V for 20 min, stained with GelRed (Biotium 41003, USA). DNA sequence analysis compared with those of *K. pneumoniae* NTUH-2044 (NCBI accession number AP 006725) was conducted.

Isolates were examined by real-time RT-PCR for expression of *ompK35* and *ompK36*. For RNA isolation, *K. pneumoniae* isolates were grown in fresh LB medium at 37 °C overnight. Total RNA was extracted from 3 mL of culture using a RNeasy Mini Kit (Qiagen 74524, Germany) according to the manufacturer’s instructions. The extracted RNA samples were stored at −80°C. Purified RNA was reverse transcribed into cDNA for RT-PCR analysis using a cDNA synthesis kit (TaKaRa 6210A, Japan) according to the manufacturer’s instructions. Gene expression levels were measured with RT-PCR using TB Green® Premix Ex Taq™ (TaKaRa RR420Q, Japan). Primers were designed on the basis of the nucleotide sequence in GenBank (Table 1). Expression of each gene was normalized to that of a housekeeping gene (*rpoB*), the primers used for RT-PCR were *rpoB*-F (5′-AAGGCGAATCCAGCTTGTTCAGC-3′) and *rpoB*-R (5′-TGACGTTGCATTTGCACCACCATCA-3′). The relative expression of *ompK35* and *ompK36* was then calibrated against the corresponding expression by *K. pneumoniae* ATCC 13883. For quality-controlling strain, relative expression was equal to 1.0. All experiments were performed in triplicate and mean of the Ct values were used for analysis. Gene expression levels were calculated using $2^{-\Delta\Delta Ct}$ method [31].

**Statistical Analysis**

The statistical correlation of the expression of *ompk35* and *ompk36* was calculated by Student’s *t* test. SPSS (version 17; IBM, USA, IL) was used for statistical analysis. A *P* value lower than 0.05 was considered statistically significant.

**Results**
Clinical characteristics of the patient

This case was a 62-year-old male patient admitted to the department of neurosurgery in the First Affiliated Hospital of Wenzhou Medical University on October 26, 2015 with headache. A diagnosis of cerebral aneurysm with subarachnoid hemorrhage was made, then, secondary pulmonary infection occurred after surgery. Treatment was started with 2 g of cefoperazone/sulbactam every 8 h (q8h), 0.5 g levofloxacin two times a day (bid.). On day 26, *K. pneumoniae* FK-2624, it was only resistant to fosfomycin, isolated from sputum sample (Table 2). After that, due to pneumonia, he was switched to tigecycline via vein infusion (ivgtt.) loading dose of 100 mg. On Day 50, chest CT suggested atelectasis, which required a tracheotomy. Then, day 52, *Pseudomonas aeruginosa* was detected in purulent sputum, so he was switched to fosfomycin with an ivgtt. loading dose of 8 g q12h, tobramycin with an ivgtt. loading dose of 80 mg q12h. On Day 71 after his admission, isolate FK-2723 was identified from the patient’s sputum sample on January 4, 2016, it turned into ceftriaxone, ceftazidime, levofloxacin, ciprofloxacin, tobramycin, gentamicin and fosfomycin-resistant, but still susceptible to carbapenems, colistin and tigecycline. Along with the condition developed, on February 1, 2016, he was given a combination of imipenem and fosfomycin for 14 days before being discharged from the hospital. On Day 113, FK-2820 was obtained, it was evolved to carbapenems-resistant (Table 2). After another 33 days hospitalization, the patient transferred to the superior hospital in a poor health condition, graded 6 by Glasgow coma scale (GCS) criterion (Figure 1).

Antimicrobial susceptibility testing, characterization of resistance genes and virulence factors

The antibiotic resistance profiles are shown in Table 2, *K. pneumoniae* FK-2624 was found to be susceptible to all the antibacterials mentioned above but fosfomycin, while isolate FK2723 was resistant to ceftriaxone, ceftazidime, cefotaxime, levofloxacin, ciprofloxacin, tobramycin, gentamicin, amikacin, and fosfomycin except carbapenems, colistin and tigecycline. Further, FK2820 was evolved to carbapenems-resistant. The MICs of imipenem and ertapenem for FK-2820 were 8 and 16 μg/mL, respectively. Although it was still susceptible to meropenem, the MIC of meropenem increased four
times, range from 0.25 $\mu$g/mL to 1 $\mu$g/mL.

Phenotypic method was used for the investigation of carbapenemase production. mCIM was negative for FK-2820. PCR and WGS was used to search for the presence of resistance genes, the $bla_{SHV}$, $bla_{DHA}$, $qnrB$, $aac (6')-Ib$, $oqxA$ and $fosA5$ genes were identified in strains FK-2723 and FK-2820. FK-2624 harbored $bla_{SHV}$, $oqxA$ and $fosA5$ genes (Table 2). In addition, sequence analysis showed that $bla_{DHA}$, $qnrB$, $aac (6')-Ib$ were co-harbored in the same plasmid. The results of BLASTn analysis revealed that the plasmid with 100% query coverage displayed 100% identity to plasmid pR47-309 (Genbank accession CP040696.1).

Of the fourteen virulence factors found in this study, $ybtA$, $mrkD$, $entB$, $iroN$ and capsular serotype K57 were found in the three isolates (Table 2). These genes were associated with high-affinity iron chelators or siderophores, iron uptake, biofilm formation and infection (such as tissue-invasive, pneumonia), potentially contributing to the increased virulence in $K. pneumoniae$.

Homology Analysis and Molecular epidemiology

Genome homology comparison suggested that over 98% homology in tested strains (Table S1). PFGE analysis revealed that the three strains isolated from the same patient to be clonally identical. In addition, they all belonged to ST 660 (Figure 2).

OMP analysis

SDS-PAGE showed that isolate FK-2820 did not express a full complement of porins compared with the positive control $K. pneumoniae$ ATCC13883 (Figure 3). Isolate FK-2820 lacked a band of ~35 kDa corresponding to the OmpK36 major porin. Sequencing of this gene and comparison with that of $K. pneumoniae$ NTUH-2044 revealed a frameshift mutation caused by the deletion of 2 nucleotides at nucleotide position 599 and 600 where created a premature stop codon, resulting in truncated porin of 164 amino acids. Further led to the resistance to imipenem (MIC=8 $\mu$g/mL) and ertapenem (MIC>16 $\mu$g/mL) but remained susceptible to meropenem (MIC=1 $\mu$g/mL). Compared with the control
strain, *K. pneumoniae* ATCC 13883, expression of *ompK35* was similar among FK-2624, FK-2723 and FK-2820 (0.93, 1.61 and 1.44, respectively). However, expression of *ompK36* in FK-2820 was decreased compared with the control isolate ATCC 13883 (0.093 times the control, *P* < 0.05) (Table 2).

**Discussion**

In last years, bacterial antimicrobial resistance has indeed emerged as one of the main concern of public health and constitutes a major challenge in the future [32]. Emergence of MDR *K. pneumoniae* has become a global health problem, posing clinical and therapeutic challenge since they exhibited resistance to the majority of commonly used antibiotics [33]. Carbapenems are a class of β-lactam antibiotics with strong activities against many Gram-positive and Gram-negative bacteria, which have begun to be utilised as a last resort in infections. However, since the first report of KPC-2 disclosed [34], carbapenemase-producing isolates spread rapidly across the globe [35]. It was remarkable that porin alteration may also play a key role in CRKP which could produce ESBLs or AmpC β-lactamases [4]. Previously reports have indicated a link between imipenem treatment and the alteration of porins in *Enterobacter aerogenes* [36, 37]. Around the world, sever outbreaks due to outer membrane alteration in *Enterobacteriaceae* have been reported [38-40]. In the current study, resistance has changed in three strains isolated from one patient, the aim of our study was to investigate the mechanism and evolution of treatment-emergent carbapenem-resistance.

FK-2624 was isolated from the patient on day 26, it was susceptible to most commonly used antimicrobial agents except fosfomycin. However, we isolated the MDR strain FK-2723 on day 71, it only exhibited susceptible to carbapenems, colistin and tigecycline. After imipenem treatment for 14 days, the isolate FK-2820 was evolved to carbapenem-resistant. WGS and molecular epidemiology analysis revealed homology among FK-2624, FK-2723 and FK-2820, the difference in resistance patterns between FK-2624 and the other two strains was due to the transfer of resistant plasmids, conferring certain resistance to FK-2723 and FK-2820. All of tested strains harbored resistant gene *fosA5*, which conferred high level resistance to fosfomycin [41]. Sparklingly, *bla*<sub>DHA</sub>, *qnrB* and *aac (6’)-Ib* were co-located in the same plasmid, the coexistence of multiple resistant genes in our isolates may contribute to high-level resistance to the majority of clinically available antimicrobial agents.
Moreover, *K. pneumoniae* strains could produce different virulence factors, such as fimbrial adhesins and siderophores, which are important in the colonization and development of the infection. To date, the delineation of hypervirulent *K. pneumoniae* (hvKp) virulence genes remains incomplete, and it remains unclear which genes are needed for maximal virulence [42]. We hold that the good markers should be critical factors in conferring the hypervirulent phenotype. If such markers are lost, then the phenotype will no longer be hypervirulent. A recent study demonstrated that *iroB*, *iucA*, *peg-344*, *rmpA* and *rmpA2* were the most accurate molecular markers for defining hvKp [43]. In current study, none of the three strains carried the hypervirulent markers, but the combination of the resistance determinants with virulence genes *ybtA*, *mrkD*, *entB* and *iroN* in MDR *K. pneumoniae* isolates may further exacerbate infections caused by these bacteria and hamper treatment. We found that ST660 remained uncommon in clinical infections. There are only two ST660 strains in the database (http://bigsdb.pasteur.fr/), which have been recovered from human blood in Vietnam and sputum in China, respectively. This case has emerged and may warrant further surveillance.

Our sequencing results showed that point mutations leading to amino acid change in FK-2820, the early termination of translation disrupting the gene coding sequence. The truncated porins were lacking the last amino acids which included a phenylalanine residue necessary for the insertion of porin into the outer membrane [44]. Our study provided interesting data on the mRNA responses of *K. pneumoniae* to carbapenem antibiotics. Compared with the control strain, *K. pneumoniae* ATCC 13883, expression of *ompK36* was decreased in FK-2820 substantially, which was consistent with the absence of the corresponding protein band in the SDS-PAGE analysis. However, the difference between FK-2624 and FK-2723 (expressions of *ompK* 36 goes from 12-fold and 1.39-fold, respectively) could not reflected on gels. In other words, transcriptional responses in *K. pneumoniae* were unpredictable. The decreasing expression of *ompK* 36 in the process of evolution might be overexpression of the negative regulation genes (such as *micF* and *ompR*) [45]. These, in turn, causing the decreasing susceptibility to carbapenem. Further studies are therefore needed to understand the pathways of mRNA regulation of resistance-related genes in *K. pneumoniae* during antibiotic pressure. Our result demonstrated the alteration of OmpK36 alone may confer a high level
of resistance to ertapenem, consistent with previous report [46].

A recently study revealed that in vitro carbapenem resistance induction could lead the loss of OmpK 36 in induced isolate after 13 days of serial passaging[47]. In this study, we reported a link between a 14-day imipenem treatment and the loss of OmpK 36 in vivo. This will give more inspiration to clinical anti-infective treatment. The limitation in our study was that there was only one patient had been investigated. A large-scale study on the inclusion of additional patients under strict antibiotic regimen is needed in further research to provide more evidence on the clinical significance of these newly emerged strains.

Here, we analyzed the three strains from a patient, data manifested that the alteration of ompk36 might due to a 14-day imipenem treatment, which further confer a high level carbapenem resistance. To our knowledge, this is the first description of a connection between imipenem treatment and alteration of OmpK36 in K. pneumoniae in China.

Conclusion
This study manifested a link between imipenem treatment of patients infected by MDR K. pneumoniae and the sequential alteration of porin of the isolates collected during the 14 days treatment, which may be the final stage of K. pneumoniae adaptation to carbapenems. This gives us more inspirations for the rational use of antibiotics during patient admission, effective surveillance should provide guidance on the utility of antimicrobial agents to treat K. pneumoniae infections.

Abbreviations
MDR: Multidrug-resistant
MALDI-TOF MS: Matrix-assisted laser desorption/ionization time of flight mass spectrometry
MIC: Minimum inhibitory concentration
PCR: Polymerase chain reaction
WGS: Whole-Genome Sequencing
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
ESBLs: Extended-spectrum β-Lactamases
CRKP: Carbapenem-Resistant K. pneumoniae
mCIM: Modified carbapenem inactivation method

PFGE: Pulsed-field gel electrophoresis

MLST: Multi-locus sequencing typing

Declarations

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

TZ, JC, and XT contributed to the design of the experiments. XT, XZ, YZ, RF, and CW performed the experiment. XT, XZ, and RF wrote the initial draft of the manuscript. TZ, JC, XT, QW, JL, YZ and HL contributed to the acquisition, analysis, interpretation of the data included in this manuscript. TZ, JC, XT, XZ, RF, JL, CW, and HL revised the manuscript. All authors approve of the final manuscript being submitted and agree to be accountable for the work detailed in the submitted manuscript.

Ethics approval and consent to participate

The work was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. The committee ruled that consent to participate was waived (ethical number 2019-75).
Consent for publication

Not applicable.

Conflict of interest

The authors have no conflict of interest to declare. The authors alone are responsible for the content and writing of the paper.

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Tables

Table 1 Primers used in this study to amplify porin genes and RT-PCR.
### Primer Sequence 5' → 3'

| Primer   | Sequence                  |
|----------|---------------------------|
| ompK35-F1 | AAGACTACTGGTGATTATCGCGACCT |
| ompK35-R1 | CGACAAAAAGCGCGAAGGTCTTT |
| ompK35-F2 | GTCGAAGCGGCAACCGATTATG    |
| ompK35-R2 | GCTTCGGGCTTTGTCGACCATT   |
| ompK36-R1 | CCGGTGAAATAGGGGTAACAGAC   |
| ompK36-F1 | CCATTAATCGAGGCTCTTACCA    |
| ompK36-F2 | GAGTTGCCTTTGAGGTCTTG      |
| ompK36-R2 | GGCGACACCTACGGTTCTGACAA   |
| RT-PCR ompK35-F | GTCTGGACCACCAATGGC        |
| ompK35-R | GATCTGAGTTTCGCTTTCA       |
| ompK36-F | GACCAGACCTACATGCGTGTA     |
| ompK36-R | GTATTCCACTGGCCGTAAC       |

**Note:** F, forward (5') primer. R, reverse primer.

Table 2. Phenotypic detection, distribution of resistance genes, MICs and virulence factors of antimicrobial agents among the *K. pneumoniae* isolates.
| Isolates codes | mCIM¹ | Resistance genes profiles | MIC (µg/ml)² |
|----------------|-------|---------------------------|-------------|
|                |       |                           | CRO | CAZ | CTX | IPM | MEM | ETP | LEV | CIP | TOB |
| FK-2624        | -     | *bla*<sub>SHV</sub>, *oqxA*, *fosA5* | 0.5 | 1   | 0.5 | 0.125 | 0.03 | 0.03 | 0.5 | 0.5 | 0.5 |
| FK-2723        | -     | *bla*<sub>SHV</sub>, *bla*<sub>DHA qnrB</sub>, *oqxA*, *aac (6')-Ib*, *fosA5* | 8   | >64 | 16  | 1    | 0.25 | 0.5 | 8   | 4   | 128 |
| FK-2820        | -     | *bla*<sub>SHV</sub>, *bla*<sub>DHA qnrB</sub>, *oqxA*, *aac (6')-Ib*, *fosA5* | 8   | >64 | 64  | 8    | 1    | >16 | 8   | 4   | 128 |

Note: ¹mCIM, modified carbapenem inactivation method; ²CRO=ceftriaxone; CAZ=ceftazidime; CTX=cefotaxime; IPM=imipenem; MEM=meropenem; ETP=ertapenem; LEV=levofloxacin; CIP=ciprofloxacin; TOB=tobramycin; GEN=gentamicin; AMK=amikacin; FOS=fosfomycin; COL=colistin; TGC=tigecycline.

Figures
Figure 1

Timeline representing the days at which the isolates were obtained from patient and the respective interventions that were performed.

Figure 2

PFGE analysis and MLST of 3 K. pneumoniae isolates. Relatedness was analyzed using QualityOne software (Bio-Rad Laboratories, USA). The phylogenetic tree was generated using UPGMA clustering. A genetic similarity index scale is indicated by the vertical line.
Figure 3

SDS-PAGE analysis of OMPs from the K. pneumoniae strains. Lanes M, protein mass marker.

ATCC 13883, an isolate with known expression of both OmpK35 and OmpK36.

Supplementary Files
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