Co-transfer of plasmid-mediated blaAmpC and fluoroquinolone resistance genes in Klebsiella pneumoniae isolates causing nosocomial urinary tract infection

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Research

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Abstract

**Background:** *Klebsiella pneumoniae* is a pathogen that frequently causes nosocomial urinary tract infection (UTI), and the prevalence of plasmid-mediated resistance determinants among clinical isolates of *K. pneumoniae* leads to the appearance of resistance to antibiotics. The aim of this study was to investigate the prevalence of plasmid-mediated quinolone resistance (PMQR) genes in acquired AmpC (ac-AmpC) β-lactamase-producing *K. pneumoniae* isolates from patients with nosocomial UTI and to characterize the transmissibility of plasmids co-harbouring *bla*<sub>AmpC</sub> and PMQR genes.

**Methods:** From January 2017 to June 2018, we collected 46 AmpC-producing *K. pneumoniae* isolates causing nosocomial UTI from a tertiary care hospital in China. β-lactamase, PMQR and virulence genes were detected by PCR and sequencing. Clonal relatedness was assessed using ERIC-PCR and multilocus sequence typing (MLST). Plasmids carrying multiple *bla*<sub>AmpC</sub> and PMQR genes were characterized by PCR-based replicon typing (PBRT) and S1-PFGE. Conjugation and electroporation experiments were carried out to assess resistance transfer mediated by plasmids. Overlapping PCR was used to map the genetic context of the *bla*<sub>AmpC</sub> genes.

**Results:** In the studied isolates, non-susceptibility of third-generation cephalosporin and fluoroquinolone was very high (>80%). *bla*<sub>CMY-2</sub>, *bla*<sub>DHA-1</sub>, and quinolone resistance gene (*qnr*) were detected in 11, 41 and 33 isolates, respectively. Among the isolates, 6 strains co-harboured multiple AmpC and *qnrB* genes. The *bla*<sub>AmpC</sub> and *qnrB* genes from these six isolates were co-transferrable to recipients via conjugation or electroporation, with IncFIA, IncFIB and IncA/C being the dominant replicons (sizes from ~78 to 217 kb). Forty-six isolates were categorized into 25 ERIC types, and the 6 isolates harbouring multiple *bla*<sub>AmpC</sub> and *qnrB* genes belonged to ST1/STnew1. The conserved genetic structures in *bla*<sub>CMY-2</sub> and *bla*<sub>DHA-1</sub> were identical to those described in the pNF4656 and pSAL-1 plasmids, respectively.

**Conclusion:** This work reports that *qnrB* is highly prevalent in AmpC-producing *K. pneumoniae* isolates and illustrates the emergence of plasmids co-harbouring multiple acquired *bla*<sub>AmpC</sub> and *qnrB* genes in *K. pneumoniae* causing UTI in China. We determined that the IncFIA, IncFIB and IncA/C plasmids carrying *bla*<sub>AmpC</sub> with *qnrB* resistance genes and several mobile genetic elements mediate the local prevalence in *K. pneumoniae* UTI. The genetic context of *bla*<sub>AmpC</sub> was highly conserved.

Background

Urinary tract infections (UTIs) are one of the most frequent infections among hospitalized patients and in critical care units [1]. Gram-negative *Klebsiella pneumoniae* is an opportunistic bacteria responsible for different nosocomial infections, such as pneumonia, UTI, liver abscesses and bloodstream infections [2]. β-lactam and fluoroquinolone antimicrobials continue to play important roles in the treatment of serious infections caused by gram-negative pathogens. However, the spread of plasmid-mediated drug resistance factors among gram-negative bacteria leads to the appearance of strains resistant to antibiotics. These strains cause the development of UTIs resistant to these antimicrobial therapies [1, 3].
The most common cause of bacterial resistance to broad-spectrum β-lactam antibiotics is the production of extended-spectrum broad-spectrum β-lactamases (ESBLs) and AmpC β-lactamase [4]. Recently, the prevalence of AmpC enzymes has been increasingly reported worldwide [5–7]. AmpC-type cephalosporinases are Ambler class C β-lactamases that are poorly inhibited by clavulanic acid and confer resistance to a variety of β-lactams but not fourth-generation cephalosporins or carbapenems [8]. Although most AmpC enzymes are intrinsic and chromosomally encoded, they can also be acquired through mobile genetic elements, such as plasmids. These plasmids encode acquired AmpC (ac-AmpC) β-lactamases and have been mainly detected in *Escherichia coli*, *Klebsiella* spp., *Salmonella* spp. and *Proteus mirabilis* [5, 9–11].

Based on sequence similarities with species-specific AmpC enzymes, ac-AmpC variants are typically derivatives of the DHA, ACC, FOX, EBC, and CIT (CMY-2) families and have broadly spread geographically, particularly in Europe, Canada, and Tunisia as well as in Korea and China [5, 12–15]. Several genetic elements surrounding *ampC* genes are involved in the mobilization of ac-AmpC β-lactamase genes, e.g., the insertion sequences IS26 (CMY-13), IS*Ecp7* (CMY-2-type, ACC-1-type), and IS*CR1*, associated with complex integrons (DHA-1 type) [8, 16].

Moreover, ac-AmpC β-lactamases are highly expressed and can be transferred to other bacteria through conjugation and transformation to frequently cause resistance to non-β-lactam antibiotics such as aminoglycosides, fluoroquinolones, sulphonamides, trimethoprim, and chloramphenicol, which makes anti-infective therapy more challenging [17]. Because these *bla*AmpC genes can be located on large plasmids (> 30 kb in size) that, as principal vehicles, contain additional antimicrobial resistance genes, such as the plasmid-mediated quinolone resistance (PMQR) genes *qnr*, *aac(6')-Ib-cr*, *qepA* and *oxxAB*, they facilitate the classic mechanisms of fluoroquinolone resistance [18, 19].

The occurrence of both ac-AmpC β-lactamases and PMQR genes on transmissible elements has also been observed in companion and food-producing animals and culinary herbs, but the co-existence of plasmid-borne AmpC β-lactamases and PMQR genes in bacterial isolates collected from patients with nosocomial infections is uncommon [18, 20]. The present study was conducted to investigate the prevalence of PMQR genes in ac-AmpC β-lactamase-producing *K. pneumoniae* isolated from patients with nosocomial UTI. The transmissibility of plasmids bearing multiple ac-AmpC and PMQR genes was a focus, the characterization of plasmids was performed, and the genetic context of AmpC genes was analysed. This study also highlights the possibility of co-transmission of multiple resistance genes in large individual plasmids.

**Materials And Methods**

**Bacterial strains**

This study was performed at the clinical microbiology laboratory of a university-affiliated hospital in Dalian, China, between January 2017 and June 2018. All *K. pneumoniae* isolates were collected
consecutively from patients with nosocomial UTI (one per patient) and were selected according to a resistance phenotype compatible with AmpC production: resistance or reduced susceptibility, as determined according to the Clinical and Laboratory Standards Institute (CLSI) breakpoints, to amoxicillin/clavulanic acid (< 18 mm, > 8/4 µg/ml) and cefotaxime (< 26 mm, > 1 µg/ml), ceftazidime (< 21 mm, > 4 µg/ml) or aztreonam (< 21 mm, > 4 µg/ml). The presence of the *ampC* gene was confirmed by multiplex PCR as described by Pérez-Pérez et al. [21]. The study protocol was approved by the Ethics Committee of Dalian Medical University.

*Escherichia coli* ATCC 25922 (American Type Culture Collection, Manassas, VA, USA) served as the control strain for susceptibility testing. Azide-resistant *E. coli* J-53 (J53AzR) was kindly provided by the First Affiliated Hospital of Dalian Medical University and used as the recipient in conjugation experiments, and *E. coli* HB101 (Takara, Dalian, China) was used in transformation experiments.

**Antimicrobial Susceptibility Testing**

Susceptibility studies were carried out using a MicroScan (Siemens AG, Munich, Germany) and the standard disc diffusion method according to the CLSI guidelines for the following antimicrobials (Oxoid, Hampshire, England): ampicillin (AMP, 10 µg), cefazolin (KZ, 30 µg), cefuroxime (CXM, 30 µg), cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), ceftriaxone (CRO, 30 µg), cefepime (FEP, 30 µg), cefoxitin (FOX, 30 µg), aztreonam (ATM, 30 µg), amoxicillin/clavulanic acid (AMC, 20 µg/10 µg), imipenem (IPM, 10 µg), levofloxacin (LEV, 5 µg), ciprofloxacin (CIP, 5 µg) and amikacin (AMK, 30 µg). Quality control strains were included to monitor the performance of each test.

**Detection of β-lactamase and PMQR genes**

Total DNA preparations were obtained by thermolysis of isolates as described previously [21]. Multiplex PCR assays were performed on all isolates for the detection of *ampC* family (*bla*<sub>MOX</sub>, *bla*<sub>CMY</sub>, *bla*<sub>FOX</sub>, *bla*<sub>DHA</sub>, *bla*<sub>ACC</sub>, *bla*<sub>ACT</sub>, and *bla*<sub>MIR</sub>) and PMQR genes (*qnrA*, *qnrB*, *qnrS*, *aac(6′)-Ib* and *qepA*). The primers used for multiplex PCR amplification are listed in Table 1. *aac(6′)-Ib-cr* was differentiated from its wild-type allele by digestion with the enzyme *BtsCl* (New England Biolabs, Massachusetts, USA). Other β-lactamase genes (*bla*<sub>SHV</sub>, *bla*<sub>TEM</sub>, *bla*<sub>CTX-M</sub> and *bla*<sub>OXA</sub>) were analysed by PCR using specific primers and conditions we described previously [22].
| Target(s) | Primer | Sequence (5′ to 3′, as synthesized) | Expected amplicon size (bp) | Reference |
|-----------|--------|-----------------------------------|-----------------------------|-----------|
| MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11 | MOXMF | GCT GCT CAA GGA GCA CAG GAT | 520 | [21] |
| | MOXMR | CAC ATT GAC ATA GGT GTG GTG C | | |
| LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1 | CITMF | TGG CCA GAA CTG ACA GGC AAA | 462 | [21] |
| | CITMR | TTT CTC CTG AAC GTG GCT GCT GGC | | |
| DHA-1, DHA-2 | DHAMF | AAC TTT CAC AGG TGT GCT GGG T | 405 | [21] |
| | DHAMR | CCG TAC GCA TAC TGG CTT TGC | | |
| ACC | ACCMF | AAC AGC CTC AGC AGC CGG TTA | 346 | [21] |
| | ACCMR | TTC GCC GCA ATC ATC CCT AGC | | |
| MIR-1T ACT-1 | EBCMF | TCG GTA AAG CCG ATG TTG CGG | 302 | [21] |
| | EBCMR | CTT CCA CTG CGG CTG CCA GTT | | |
| FOX-1 to FOX-5b | FOXMF | AAC ATG GGG TAT CAG GGA GAT G | 190 | [21] |
| | FOXMR | CAA AGC GCG TAA CCG GAT TGG | | |
| qnrA | qnrAMF | ATTTCTCACGCCAGGATTTG | 516 | [40] |
| | qnrAMR | GATCCGCAAAGGTTAGGTCA | | |
| qnrB | qnrBMF | GATCGTGAAAGCCAGAAAGG | 476 | [40] |
| | qnrBMR | ATGAGCAACGATGCCTGGTA | | |
| qnrS | qnrSMF | GGGTTGTACATTTTATTGAATCG | 307 | [40] |
| | qnrSMR | CACCTACCCATTTATTTTCA | | |
Clonal relatedness among *K. pneumoniae* isolates was determined by enterobacterial repetitive intergenic consensus (ERIC)-PCR with the ERIC1R (5'-ATGTAAGCTCCTGGGGATTCA-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') primers. PCR was performed briefly using the following program parameters: denaturation at 94 °C for 30 s, annealing at 52 °C for 45 s, and extension at 72 °C for 4 min for 35 cycles, followed by a final extension at 72 °C for 10 min. Banding patterns generated by ERIC-PCR were analysed using BioNumerics 7.6 software (Applied Maths, Kortrijk, Belgium). PCR fingerprints were analysed using the Dice (similarity) coefficient. Cluster analysis was performed as previously described based on the unweighted pair group method with arithmetic averages (UPGMA) with a position tolerance of 0.05 [23].

### Resistance transfer ability of multiple AmpC-producing *K. pneumoniae* isolates

Conjugation experiments were performed using a broth mating protocol. Multiple AmpC-producing *K. pneumoniae* isolates (0.3 ml) were mated with the sodium azide-resistant *E. coli* strain J53AzR (0.7 ml). Transconjugants were selected on LB agar plates containing sodium azide (100 µg/ml) and cefotaxime (4 µg/ml).

When a multiple *ampC*-harbouring plasmid could not be transferred by conjugation, we tried electroporation to harvest transformants. *E. coli* HB101 transformants were obtained by electroporation of plasmid DNA extracted with the MiniBEST Plasmid Purification Kit (Takara, Dalian, China) in an electroporator (Eppendorf, Eppendorf AG, Germany) and selected on LB agar supplemented with cefotaxime (4 µg/ml). PCR amplification, antimicrobial susceptibility testing and plasmid replicon typing were performed for all transconjugants/transformants to identify resistance determinants, antibiotic phenotypes and incompatibility groups, respectively.

### Molecular Characterization Of Plasmids Carrying Multiple Ampc Genes

S1-nuclease (Takara, Dalian, China) digestion as well as pulsed-field gel electrophoresis (S1-PFGE) analysis was performed for donor strains and the corresponding transconjugants or transformants.
plasmid size estimation, comparison with the molecular weight marker *Salmonella braenderup* H9812 was performed. Plasmid replicons were determined using the PCR-based replicon typing (PBRT) scheme with 18 pairs in PCR for detecting F, FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y and FII replicons, as described by Carattoli *et al.* [24].

**Virulence factors of multiple AmpC-producing *K. pneumoniae* isolates**

*K. pneumoniae* isolates harbouring multiple ac-AmpC β-lactamases were screened for the presence of seven genes encoding putative virulence factors and six capsular serotypes by PCR as we previously described [25]. These virulence-associated genes included genes encoding regulators of exopolysaccharide synthesis (*rmpA*), fimbrial adhesins (*mrkD*), the ferric iron uptake system (*entB, ybtS, iutA* and *kfu*), allantoin metabolism (*allS*) and the virulent capsular serotype (K1, K2, K5, K20, K54 and K57).

**Genetic context analysis of bla\textsubscript{ac-AmpC} genes in multiple AmpC-producing *K. pneumoniae* isolates**

The flanking regions of ac-AmpC genes were identified by overlapping PCR as previously described [26]. For *bla\textsubscript{CMY-2}*, the presence of a composite genetic structure (*bla\textsubscript{CMY-2}*-*blc*-sugE) originating from the *Citrobacter freundii* chromosome and IS\textsubscript{Ecp1} gene (responsible for the transfer of the *bla\textsubscript{CMY-2}*-like*-blc*-sugE region) was explored. For the genetic organization of *bla\textsubscript{DHA-1}*, the searched genes were IS\textsubscript{CR1}, IS26, orf2 (conserved region of unknown function in *Morganella* species), *ampR, qacEΔ1* and *sul1*. PCR products were sequenced and compared with those available in GenBank (www.ncbi.nih.gov/BLAST).

**Multilocus Sequence Typing (mlst)**

MLST analysis of multiple AmpC-producing *K. pneumoniae* isolates was conducted by sequencing fragments of seven housekeeping genes (*gapA, infB, mdh, pgi, phoE, rpoB*, and *tonB*), and sequence types (STs) were assigned using the *K. pneumoniae* MLST website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae).

**Statistical analysis**

Fisher’s exact test was used to compare the distribution of PMQR genes between simplex and multiplex ac-AmpC-producing *K. pneumoniae* isolates. *P*-values < 0.05 were considered statistically significant. SPSS 13.0 Statistics software (Stats Data Mining Co., Ltd., Beijing, China) was used for analyses.

**Results**

**Isolates and antimicrobial susceptibility analysis**

Forty-six *K. pneumoniae* isolates were confirmed to produce ac-AmpC β-lactamase and were found to be resistant to AMP (100%) and KZ (100%) and to present reduced susceptibility to the second- (CXM, 97.83%) or third-generation cephalosporins tested (CTX, 100%; CAZ, 89.13%; CRO, 97.83%). Among the
isolates, only 32.61% (n = 15) exhibited diminished susceptibility to a fourth-generation cephalosporin (FEP) and 4.35% exhibited diminished susceptibility to a carbapenem antibiotic (IMP). Regarding FOX, a monobactam (ATM), and amoxicillin plus clavulanic acid (AMC), the great majority of the isolates exhibited non-susceptibility (93.48%, 91.30% and 100.00%, respectively). Among the other antibiotics tested, a high percentage of resistance to quinolones (LEV, 82.61%; CIP, 89.13%) and aminoglycosides (AMK, 78.26%) was observed (Table 2).
Table 2
Prevalence of PMQR genes and resistance profile in AmpC-lactamase-producing K. pneumoniae isolates

| AmpC β-lactamase | Isolate | PMQR | Other β-lactamases | Resistance profile (Kirby-Bauer) |
|-------------------|---------|------|--------------------|---------------------------------|
| CMY-2 (n = 5, 10.87%) | Kp4 | - | **bla**<sub>CTX-M-15</sub> | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, AMC, AMK |
| | Kp15 | qnrB, qnrS | **bla**<sub>SHV-27</sub>, **bla**<sub>CTX-M-14</sub>, **bla**<sub>TEM-1</sub>, **bla**<sub>OXA-10</sub> | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, AT, AMC, LEV, CIP, AMK |
| | Kp16 | qnrB | **bla**<sub>CTX-M-3</sub>, **bla**<sub>TEM-1</sub> | AMP, KZ, CXM, CTX, CRO, FOX, AT, AMC, LEV, CIP, AMK |
| | Kp17 | qnrB | **bla**<sub>SHV-11</sub>, **bla**<sub>CTX-M-3</sub>, **bla**<sub>OXA-10</sub> | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, AT, AMC, IPM, LEV, CIP, AMK |
| | Kp18 | qnrB | **bla**<sub>SHV-12</sub>, **bla**<sub>CTX-M-3</sub>, **bla**<sub>OXA-10</sub> | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, ATM, AMC, LEV, CIP, AMK |
| DHA-1 (n = 35, 76.09%) | Kp3 | - | **bla**<sub>SHV-12</sub>, **bla**<sub>DHA-1</sub> | AMP, KZ, CTX, CAZ, FOX, AMC, AMK |
| | Kp5 | qnrB | **bla**<sub>SHV-11</sub>, **bla**<sub>OXA-10</sub> | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, AT, AMC, LEV, CIP, AMK |
| | Kp6 | qnrB | **bla**<sub>SHV-11</sub>, **bla**<sub>OXA-10</sub> | AMP, KZ, CXM, CTX, CAZ, CRO, FEP, FOX, AT, AMC, CIP |
| | Kp8 | qnrB | **bla**<sub>CTX-M-14</sub> | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, AMC, CIP |
| | Kp9 | qnrB | **bla**<sub>CTX-M-14</sub>, **bla**<sub>TEM-1</sub> | AMP, KZ, CXM, CTX, CRO, FOX, AT, AMC, LEV, CIP, AMK |
| | Kp10 | qnrB | **bla**<sub>CTX-M-3</sub> | AMP, KZ, CXM, CTX, CAZ, CRO, AT, AMC, LEV, CIP |
| | Kp12 | qnrB | **bla**<sub>SHV-33</sub>, **bla**<sub>CTX-M-3</sub>, **bla**<sub>TEM-1</sub>, **bla**<sub>OXA-10</sub> | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, AT, AMC, LEV, CIP, AMK |
| | Kp14 | qnrB | **bla**<sub>SHV-28</sub>, **bla**<sub>OXA-10</sub> | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, AT, AMC, LEV, CIP, AMK |
| | Kp19 | qnrB | **bla**<sub>SHV-33</sub>, **bla**<sub>OXA-10</sub> | AMP, KZ, CXM, CTX, CAZ, CRO, FEP, FOX, AT, AMC, LEV, CIP, AMK |
| | Kp21 | qnrB | **bla**<sub>SHV-12</sub>, **bla**<sub>CTX-M-3</sub> | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, AT, AMC, LEV, CIP, AMK |
|   |   |   |   |   |
|---|---|---|---|---|
| Kp22 | qnrB | $bla_{CTX-M-15}$, $bla_{TEM-1b}$ | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, ATM, AMC, LEV, CIP, AMK |
| Kp23 | qnrB | $bla_{CTX-M-3}$, $bla_{OXA-10}$ | AMP, KZ, CXM, CTX, CAZ, CRO, FEP, FOX, ATM, AMC, LEV, CIP |
| Kp24 | qnrB | $bla_{SHV-12}$, $bla_{CTX-M-3}$, $bla_{OXA-10}$ | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, ATM, AMC, LEV, CIP, AMK |
| Kp25 | qnrB | $bla_{SHV-12}$, $bla_{CTX-M-3}$, $bla_{OXA-10}$ | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, ATM, AMC, LEV, CIP, AMK |
| Kp26 | qnrB | $bla_{SHV-11}$, $bla_{CTX-M-14}$, $bla_{TEM-1}$, $bla_{OXA-10}$ | AMP, KZ, CXM, CTX, CRO, FEP, FOX, ATM, AMC, CIP, AMK |
| Kp27 | qnrB | $bla_{SHV-11}$, $bla_{CTX-M-3}$, $bla_{TEM-1}$, $bla_{OXA-10}$ | AMP, KZ, CXM, CTX, CRO, FEP, FOX, ATM, AMC, LEV, CIP, AMK |
| Kp28 | qnrB | $bla_{SHV-11}$, $bla_{CTX-M-3}$, $bla_{OXA-10}$ | AMP, KZ, CXM, CTX, CRO, FEP, FOX, ATM, AMC, LEV, CIP, AMK |
| Kp29 | qnrB | $bla_{SHV-12}$, $bla_{CTX-M-3}$, $bla_{OXA-10}$ | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, ATM, AMC, LEV, CIP, AMK |
| Kp30 | qnrB | $bla_{SHV-12}$, $bla_{CTX-M-3}$, $bla_{OXA-10}$ | AMP, KZ, CXM, CTX, CAZ, CRO, FEP, FOX, ATM, AMC, LEV, CIP, AMK |
| Kp31 | qnrB | $bla_{SHV-12}$, $bla_{CTX-M-3}$, $bla_{OXA-10}$ | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, ATM, AMC, LEV, CIP, AMK |
| Kp32 | qnrB | $bla_{SHV-12}$, $bla_{CTX-M-3}$, $bla_{OXA-10}$ | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, ATM, AMC, LEV, CIP, AMK |
| Kp33 | qnrB | $bla_{SHV-12}$, $bla_{CTX-M-3}$ | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, ATM, AMC, LEV, CIP, AMK |
| Kp34 | qnrA, qnrB | $bla_{SHV-11}$, $bla_{CTX-M-3}$, $bla_{TEM-1}$ | AMP, KZ, CXM, CTX, CAZ, CRO, FEP, FOX, ATM, AMC, LEV, CIP, AMK |
| Kp35 | qnrB | $bla_{SHV-12}$, $bla_{CTX-M-3}$, $bla_{OXA-10}$ | AMP, KZ, CXM, CTX, CAZ, CRO, FEP, FOX, ATM, AMC, LEV, CIP, AMK |
| Kp36 | qnrB | $bla_{SHV-12}$, $bla_{CTX-M-3}$ | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, ATM, AMC, LEV, CIP, AMK |
| Kp   | qnr  | bla/Ampicillin, Cefotaxime, Ceftazidime, Cefoperazone, Cefoxitin, Aztreonam, Ticarcillin, Ampicillin-Sulbactam, Piperacillin, Cefotiam, Cefuroxime, Levofloxacin, Ciprofloxacin, Moxifloxacin, Aztreonam | CMY-2 + DHA-1 (n = 6, 13.04%) |
|------|------|---------------------------------------------------------------------------------|-----------------------------|
| Kp37 | qnrB | $bl_{\text{SHV-12}}$, $bl_{\text{CTX-M-3}}$, $bl_{\text{OXA-10}}$            | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, ATM, AMC, LEV, CIP, AMK |
| Kp38 | -    | $bl_{\text{SHV-12}}$                                                          | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, AMC, LEV, CIP, AMK |
| Kp39 | qnrB | $bl_{\text{SHV-12}}$, $bl_{\text{CTX-M-14}}$                                   | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, AM, AMC, LEV, CIP, AMK |
| Kp40 | qnrB | $bl_{\text{SHV-12}}$, $bl_{\text{CTX-M-14}}$                                   | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, AM, AMC, LEV, CIP, AMK |
| Kp41 | qnrB | $bl_{\text{SHV-12}}$                                                          | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, AM, AMC, LEV, CIP, AMK |
| Kp42 | qnrB, qnrS | $bl_{\text{SHV-33}}$, $bl_{\text{CTX-M-3}}$, $bl_{\text{TEM-1}}$, $bl_{\text{OXA-10}}$ | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, AM, AMC, LEV, CIP, AMK |
| Kp43 | -    | $bl_{\text{SHV-11}}$, $bl_{\text{CTX-M-3}}$, $bl_{\text{OXA-10}}$            | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, AM, AMC |
| Kp44 | -    | $bl_{\text{SHV-12}}$, $bl_{\text{CTX-M-15}}$                                   | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, AM, AMC, AMK |
| Kp45 | -    | $bl_{\text{SHV-12}}$                                                          | AMP, KZ, CXM, CTX, CRO, FEP, FOX, AM, AMC, AMK |
| Kp46 | -    | $bl_{\text{SHV-12}}$, $bl_{\text{CTX-M-3}}$                                   | AMP, KZ, CXM, CTX, CAZ, CRO, FEP, FOX, AM, AMC, IPM, LEV, CIP, AMK |
| Kp1  | qnrB | $bl_{\text{SHV-28}}$, $bl_{\text{OXA-10}}$                                    | AMP, KZ, CXM, CTX, CAZ, CRO, ATM, AMC, LEV, CIP, AMK |
| Kp2  | qnrB | $bl_{\text{SHV-33}}$, $bl_{\text{CTX-M-15}}$                                   | AMP, KZ, CXM, CTX, CAZ, CRO, FEP, FOX, AM, AMC, LEV, CIP |
| Kp7  | qnrB | $bl_{\text{SHV-11}}$, $bl_{\text{CTX-M-3}}$                                   | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, AM, AMC, LEV, CIP |
| Kp11 | qnrB | $bl_{\text{SHV-12}}$, $bl_{\text{CTX-M-15}}$, $bl_{\text{OXA-10}}$            | AMP, KZ, CXM, CTX, CAZ, CRO, FEP, FOX, AM, AMC, LEV, CIP |
| Kp13 | qnrB | $bl_{\text{SHV-12}}$, $bl_{\text{CTX-M-15}}$, $bl_{\text{OXA-10}}$            | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, AM, AMC, LEV, CIP |
Prevalence Of Various $\beta$-lactamase And Pmqr Genes

Multiplex PCR revealed two ac-AmpC-encoding genes, $bla_{\text{CMY}-2}$ ($n = 11$) and $bla_{\text{DHA}-1}$ ($n = 41$), in the 46 isolates studied. Single $ampC$ genes were detected in 40 isolates, i.e., $bla_{\text{CMY}-2}$ ($n = 5$) or $bla_{\text{DHA}-1}$ ($n = 35$). Among these 40 isolates, 33 $K. pneumoniae$ isolates also produced PMQR genes. In the 6 remaining strains (Kp1, Kp2, Kp7, Kp11, Kp13 and Kp20), two $ampC$ genes ($bla_{\text{CMY}-2}$ and $bla_{\text{DHA}-1}$) were detected, and PMQR ($qnrB$) genes were co-harboured. The prevalence of $qnrB$ and fluoroquinolone nonsusceptibility was higher in the multiplex AmpC-producing isolates than in the simplex AmpC-producing isolates; however, the differences were not significant (100.00% and 82.50%, respectively, $P = 0.570$; 100.00% and 87.50%, respectively, $P = 1.000$; Table 3).

| Characteristics                  | AmpC $\beta$-lactamase | $P$ value |
|----------------------------------|-------------------------|-----------|
|                                  | simplex AmpC ($n = 40$) | multiplex AmpC ($n = 6$) |
| Fluoroquinolone nonsusceptibility| 35 (87.50%)              | 6 (100.00%) | 1.000 |
| Isolates carrying PMQR           | 33 (82.50%)              | 6 (100.00%) | 0.570 |
| $qnrA$                           | 2 (5.00%)                | 0 (0.00%) | 1.000 |
| $qnrB$                           | 33 (82.50%)              | 6 (100.00%) | 0.570 |
| $qnrS$                           | 2 (5.00%)                | 0 (0.00%) | 1.000 |

Among all isolates, the other $\beta$-lactamase genes $bla_{\text{SHV}}$, $bla_{\text{CTX-M}}$, $bla_{\text{TEM}}$ and $bla_{\text{OXA}}$ were detected in 39, 37, 8 and 26 isolates, respectively. DNA sequence analysis revealed five $bla_{\text{SHV}}$ subtypes: $bla_{\text{SHV}-12}$ ($n = 23$), $bla_{\text{SHV}-11}$ ($n = 9$), $bla_{\text{SHV}-33}$ ($n = 4$), $bla_{\text{SHV}-28}$ ($n = 2$) and $bla_{\text{SHV}-27}$ ($n = 1$). Among the $bla_{\text{CTX-M}}$ gene family, four variants were identified: $bla_{\text{CTX-M-3}}$ ($n = 24$), $bla_{\text{CTX-M-15}}$ ($n = 6$), $bla_{\text{CTX-M-14}}$ ($n = 6$) and
bla_{CTX-M-22} (n = 1). Of the 26 OXA-positive isolates, all harboured \textit{bla}_{OXA-10}, while the 8 TEM-positive isolates harboured \textit{bla}_{TEM-1} (Table 2).

**Resistance Transfer And Molecular Characterization Of Plasmids**

Plasmids carrying ac-AmpC and \textit{qnrB} genes were successfully transferred via conjugation (n = 2) and/or electroporation (n = 4) from the 6 multiple AmpC-producing \textit{K. pneumoniae} isolates. The resistance profiles of the transconjugants/transformants were similar to those of the \textit{K. pneumoniae} donor strains, demonstrating the transfer of antimicrobial resistance, including resistance to β-lactam-based antimicrobial compounds and fluoroquinolones.

S1-PFGE plasmid profiles and PCR replicon typing identified 3 replicons, IncFIA, IncFIB and IncA/C (ranging from ~ 78 to 217 kb), which were found in both donors and transconjugants/transformants and were associated with the transfer of \textit{bla}_{DHA-1}, \textit{bla}_{CMY-2}, \textit{qnrB} and several other β-lactamase genes (Table 4 and Fig. 1).
Table 4
Resistance transfer and plasmid characteristics of K. pneumoniae harbouring multiple acquired AmpC and qnrB genes

| Isolate<sup>a</sup> | Resistance pattern | bla<sub>AmpC</sub> | β-lactamase and PMQR | Replicon type |
|---------------------|--------------------|---------------------|----------------------|---------------|
| Kp1                 | AMP, KZ, CXM, CTX, CAZ, CRO, ATM, AMC, LEV, CIP | bla<sub>DHA−1</sub>, bla<sub>CMY−2</sub> | bla<sub>SHV−28</sub>, bla<sub>OXA−10</sub>, qnrB | A/C, FIA |
| El Kp1              | AMP, KZ, CXM, CTX, CAZ, CRO, ATM, AMC, LEV, CIP | bla<sub>DHA−1</sub> | bla<sub>SHV−28</sub>, qnrB | FIA |
| Kp2                 | AMP, KZ, CXM, CTX, CAZ, CRO, FEP, FOX, ATM, AMC, LEV, CIP | bla<sub>DHA−1</sub>, bla<sub>CMY−2</sub> | bla<sub>SHV−33</sub>, bla<sub>CTX−M−15</sub>, qnrB | A/C |
| Tc Kp2              | AMP, KZ, CXM, CTX, CAZ, CRO, ATM | bla<sub>DHA−1</sub> | bla<sub>CTX−M−15</sub>, qnrB | A/C |
| Kp7                 | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, ATM, AMC, LEV, CIP | bla<sub>DHA−1</sub>, bla<sub>CMY−2</sub> | bla<sub>SHV−11</sub>, bla<sub>CTX−M−3</sub>, qnrB | A/C |
| Tc Kp7              | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, ATM, AMC | bla<sub>DHA−1</sub>, bla<sub>CMY−2</sub> | bla<sub>CTX−M−3</sub> | A/C |
| Kp11                | AMP, KZ, CXM, CTX, CAZ, CRO, FEP, FOX, ATM, LEV, CIP | bla<sub>DHA−1</sub>, bla<sub>CMY−2</sub> | bla<sub>SHV−12</sub>, bla<sub>CTX−M−15</sub>, bla<sub>OXA−10</sub>, qnrB | A/C, FIB |
| El Kp11             | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, ATM, LEV, CIP | bla<sub>DHA−1</sub>, bla<sub>CMY−2</sub> | bla<sub>CTX−M−15</sub>, qnrB | A/C, FIB |
| Kp13                | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, ATM, AMC, LEV, CIP | bla<sub>DHA−1</sub>, bla<sub>CMY−2</sub> | bla<sub>SHV−12</sub>, bla<sub>CTX−M−15</sub>, bla<sub>OXA−10</sub>, qnrB | A/C, FIB |
| El Kp13             | AMP, KZ, CXM, CTX, CAZ, CRO, FOX, ATM, AMC, LEV | bla<sub>DHA−1</sub> | bla<sub>CTX−M−15</sub>, qnrB | FIB |
| Kp20                | AMP, KZ, CXM, CTX, CAZ, CRO, ATM, AMC, LEV, CIP | bla<sub>DHA−1</sub>, bla<sub>CMY−2</sub> | bla<sub>SHV−12</sub>, bla<sub>CTX−M−22</sub>, bla<sub>OXA−10</sub>, qnrB | A/C, FIB |
| El Kp20             | AMP, KZ, CXM, CTX, CAZ, CRO, ATM, LEV | bla<sub>DHA−1</sub>, bla<sub>CMY−2</sub> | bla<sub>CTX−M−22</sub>, qnrB | A/C, FIB |

<sup>a</sup>Tc, transconjugant; El, electroporant. Abbreviations: PMQR, plasmid mediated quinolone resistance. AMP, ampicillin; KZ, cefazolin; CXM, cefazolin; CTX, cefotaxime; CAZ, ceftazidime; CRO, cefazolin; FEP, cefepime; FOX, cefoxitin; ATM, aztreonam; AMC, cefazolin; IPM, imipenem; LEV, levofloxacin; CIP, ciprofloxacin; AMK, amikacin.

**Virulence Factors**
Six *K. pneumonia* strains carrying multiple ac-AmpC and *qnrB* genes showed important virulence profiles, including the *entB* (n = 4), *mrkD* (n = 6) and *ybtS* (n = 6) genes, and had the capsular serotype K5 (n = 4). Two-three virulence factors were found per isolate and the results are presented in Table 5.

**Table 5**

| Isolate | ERIC profile<sup>a</sup> | ST   | Virulence profile | Virulence score | Capsular serotype |
|---------|--------------------------|------|-------------------|-----------------|------------------|
| Kp1     | A21                      | STnew1 | *ybtS, mrkD*      | 2               | K5               |
| Kp2     | A12                      | ST1   | *ybtS, mrkD*      | 2               | K5               |
| Kp7     | A10                      | ST1   | *ybtS, mrkD, entB*| 3               | ND               |
| Kp11    | A10                      | ST1   | *ybtS, mrkD, entB*| 3               | K5               |
| Kp13    | A10                      | ST1   | *ybtS, mrkD, entB*| 3               | K5               |
| Kp20    | A10                      | ST1   | *ybtS, mrkD, entB*| 3               | ND               |

<sup>a</sup>ERIC profile, Enterobacterial repetitive intergenic consensus PCR profile. Abbreviations: ST, sequence type; ND, not determined.

**Genetic Context Of Ac-ampc-encoding Genes**

Analysis of the genetic context of the 6 strains that harboured multiple ac-AmpC genes revealed that the *bla<sub>CMY−2</sub>* gene was associated with IS<sub>Ecp1</sub>, which is responsible for the transfer of the *bla<sub>CMY−2−like</sub>*-*blc*-*sugE* region from the chromosome of *Citrobacter freundii* to plasmids. In our study, six strains contained IS<sub>Ecp1</sub>, the outer membrane lipoprotein gene *blc* and the drug efflux channel *sugE* upstream and downstream of the *bla<sub>CMY−2</sub>* gene. However, truncation at the 5′ end of IS<sub>Ecp1</sub> was observed in the isolate Kp7 (Fig. 2a).

We found a structure composed of the *bla<sub>DHA−1</sub>*-*ampR, qacEΔ1,* and *sul1* genes in 6 *bla<sub>DHA−1</sub>*-carrying isolates. All isolates had the IS<sub>CR1</sub> element upstream of *bla<sub>DHA−1</sub>*, but no IS26 element was found upstream or downstream (Fig. 2b).

**Clonal Relationships**

The clonal relatedness of all isolates was determined by ERIC-PCR. By analysing the ERIC-PCR profiles (Fig. 3), the 46 isolates were categorized into two main phylogenetic groups including 25 ERIC types. In the major phylogenetic subgroup, 95.65% (44/46) of the isolates were clustered. The 25 distinct types were then labelled A01 to A25. We found that 6 isolates harboured multiple ac-AmpC β-lactamase and *qnrB* genes belonging to ST1 or STnew1 (Table 5).
Discussion

The spread of antimicrobial resistance is primarily caused by the dissemination of large plasmids carrying multiple antibiotic resistance genes [27]. In the last decade, the prevalence of plasmid-mediated AmpC-producing *K. pneumoniae* isolates has increased in nosocomial acquired and healthcare-associated infections [28, 29]. Plasmids carrying ampC genes are often co-harboured with different antibiotic resistance markers, such as ESBL and PMQR genes [30]. Co-transfer of these resistance genes may contribute to the emergence of multidrug-resistant strains, increasing the risk for antibiotic treatment failure. Our study focused on the dissemination of *K. pneumoniae* strains carrying multiple AmpC and PMQR genes in patients with nosocomial UTI. To the best of our knowledge, this is the first report on the co-occurrence of various plasmid-borne bla\textsubscript{AmpC} and PMQR genes in *Enterobacteriaceae* causing nosocomial infections.

In the studied isolates, non-susceptibility of both third-generation cephalosporin and fluoroquinolone was very high (CTX, 100%; CAZ, 89.13%; CRO, 97.83%; LEV, 82.61%; and CIP, 89.13%), as non-susceptibility to at least one third-generation cephalosporin was one of the major criteria adopted in this study. However, with the increasing use of fluoroquinolone-class drugs in community- and hospital-acquired UTI patients, the rates of resistance to fluoroquinolones have increased [31]. In this study, the resistance rates of two fluoroquinolones (LEV and CIP) in *K. pneumoniae* isolates were prominently higher than those reported in a prior Asia-Pacific survey (51–55%) of pathogens causing UTI [31]. And recent report mentioned above suggested that fluoroquinolones are not recommended as a UTI therapy empirically unless in vitro data show susceptible results [31].

The most commonly occurring gene encoding an ac-AmpC enzyme in this study was DHA-1, which was found in 89.13% of the *K. pneumoniae* isolates and is the most prevalent plasmid-mediated AmpC in China and other areas of Asia; this study also found a low prevalence of \textit{bla\textsubscript{CMY-2}} (23.91%), which has been mostly reported in Europe. Notably, the simultaneous production of multiple AmpC β-lactamases in a single isolate (as observed in 6 of the 46 isolates), complicates the detection of both enzymes and treatment of infections caused by these isolates. The multiplex AmpC-producing isolates exhibited a higher percentage (100%) of PMQR prevalence than the simplex AmpC-producing isolates (82.5%), but the difference was not statistically significant. Multiplex PCR also revealed that \textit{qnrB} had the highest prevalence (84.78%), followed by \textit{qnrA} (4.35%) and \textit{qnrS} (4.35%). Earlier studies also indicate that \textit{qnrB} is the most prevalent PMQR gene in China and that its distribution is closely related to the presence of acquired AmpC β-lactamases [32, 33].

Co-transfer of PMQR genes along with \textit{bla\textsubscript{AmpC}} in large individual plasmids co-harboured many other resistance genes has been shown in other studies [18]. The successful transfer of \textit{bla\textsubscript{AmpC}} along with \textit{qnrB} genes was studied in the 6 isolates carrying multiple ac-AmpC β-lactamase genes. However, this study showed that not all multiple AmpC genes were co-transferred with the \textit{qnrB} gene, which indicates that these genes are likely located on different plasmids. Previous studies on plasmid replicon types show that the IncI1, IncA/C, IncFI1 and IncX1 plasmids carry \textit{bla\textsubscript{AmpC}} [34, 35]. PMQR genes are associated
with the IncN, IncL/M, IncFII, IncHI1, IncI1, IncR, and colE types [36]. In the present study, we found that in *K. pneumoniae*, plasmids carrying both *bla*<sub>AmpC</sub> and PMQR genes were of the replicon type IncA/C, followed by IncFIB and IncFIA. IncF-group plasmids are highly conjugative and are widely distributed in *Enterobacteriaceae*, and the presence of any gene in this group of plasmids will escalate the spread of the gene to other organisms [36]. Furthermore, we found that *bla*<sub>AmpC</sub> genes were generally located on plasmids of various sizes ranging from ~ 78 to 217 kb. Our results are in agreement with reports of ac-AmpC genes found to be encoded by plasmids of sizes varying from 7 to 180 kb [16].

Subsequently, we analysed the regions surrounding the ac-ampC genes. The genetic organization of *bla*<sub>CMY-2</sub> was highly conserved. All the isolates carried the mobile element IS<sub>Ecp1</sub> (*IS<sub>Ecp1-bla</sub>_<sub>CMY-2</sub>-*blc*-sugE*), as documented in previous reports [26, 37]. A well-conserved structure was also found in all isolates co-harbouring the *bla*<sub>DHA-1</sub> and *bla*<sub>CMY-2</sub> genes. However, although IS26 or ISCR1 elements are commonly related to the transmission of DHA-1 enzymes [38], none of our isolates harboured the IS26 insertion sequence.

Based on the distinct patterns observed with ERIC-PCR typing, the dissemination of AmpC-producing *K. pneumoniae* strains is multifactorial and cannot be fully attributed to a predominant clone, as was reported by Thouraya *et al* [37]. In addition, we found that the six isolates co-harbouring multiple acquired *bla*<sub>AmpC</sub> genes belonged to the ST1/STnew1 clone. This ST has been described recently in a clinical *K. pneumoniae* isolate of swine origin [39]. Moreover, we found that clonal *K. pneumoniae* strains had the capsular serotype K5 and showed important virulence profiles, including the three genes *entB*, *mrkD* and *ybtS*. *mrkD* and *ybtS* are widely distributed in *K. pneumoniae* and encode type 3 fimbriae and phenolate-type siderophores.

**Conclusions**

The present study indicates that third-generation cephalosporin and fluoroquinolone resistance is high in clinical isolates causing nosocomial UTI. PMQR genes were highly prevalent in AmpC-producing *K. pneumoniae* isolates, and *qnrB* was predominant. AmpC genes were co-transferred with *qnr*<sub>B</sub> and several other β-lactamase genes, conferring resistance to most antibiotics tested. All transconjugants/transformants were associated with IncFIA, IncFIB and IncA/C plasmids and a conservative genetic environment. This study has several limitations. First, this study was a retrospective analysis, and only limited UTI patient information was available. Another limitation of the study was the small number of multiple AmpC-producing *K. pneumoniae* isolates obtained from a single medical centre. However, the study focused on the transfer of plasmid-mediated resistance genes among clinical AmpC-producing *K. pneumoniae* strains to better guide the prevention and clinical treatment of nosocomial infections. Further studies are needed to address these limitations.

**Abbreviations**
UTI, urinary tract infection; PMQR, Plasmid-mediated quinolone resistance; ac-AmpC, acquired AmpC; ERIC-PCR, enterobacterial repetitive intergenic consensus-PCR; PBRT, PCR-based replicon typing; PFGE, Pulsed-field gel electrophoresis; ST, sequence type; AMP, ampicillin; KZ, cefazolin; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; FEP, cefepime; FOX, cefoxitin; ATM, aztreonam; AMC, amoxicillin/clavulanic acid; IPM, imipenem; LEV, levofloxacin; CIP, ciprofloxacin; AMK, amikacin.

Declarations

Ethics approval and consent to participate

The study protocol was carefully reviewed and approved by the institutional Ethics Committee of the Dalian Medical University.

Consent for publication

Not Applicable.

Competing interests

The authors declare that they have no competing interest.

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Authors’ contributions

YC conceived and designed the experiments and contributed to the writing of the manuscript. YLX, CZ, YQH and SYJ performed the experiments. ZZN and JW analysed the data. WTG and YM contributed reagents and materials. QQZ coordinated collection of specimens. All authors read and approved the final manuscript.

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