Infection and Drug Resistance

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ORIGINAL RESEARCH

Emergence and molecular characterization of multidrug-resistant *Klebsiella pneumoniae* isolates harboring $bla_{CTX\text{-}M\text{-}15}$ extended-spectrum β-lactamases causing ventilator-associated pneumonia in China

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Background: Ventilator-associated pneumonia (VAP) is a common nosocomial infection associated with high morbidity due to multidrug-resistant (MDR) pathogens. The purpose of this study was to determine the occurrence of extended-spectrum β-lactamase (ESBL) genes, especially $bla_{CTX\text{-}M\text{-}15}$, in *Klebsiella pneumoniae* (*K. pneumoniae*)-associated VAP and to investigate the antimicrobial resistance patterns and molecular epidemiological characteristics of *K. pneumoniae* strains.

Materials and methods: From January 2013 to December 2015, we retrospectively collected 89 VAP-causing *K. pneumoniae* isolates from tertiary-care hospitals in China, among which ESBL-producing strains were assessed for antimicrobial susceptibility. Several antibiotic resistance genes of clinical relevance in *K. pneumonia* isolates producing ESBL were investigated. Polymerase chain reaction (PCR) and DNA sequencing were employed to characterize the genetic contexts of $bla_{CTX\text{-}M\text{-}15}$. Conjugative plasmids carrying $bla_{CTX\text{-}M\text{-}15}$ were obtained by mating and further subjected to replicon typing. The genetic relatedness of isolates was assessed by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing.

Results: All of the 30 ESBL-producing isolates identified displayed MDR phenotype, with bla_{SHV}, $bla_{CTX\text{-}M}$, bla_{OXA}, and bla_{TEM} detected in 21, 21, 1, and 20 isolates, respectively. $bla_{CTX\text{-}M\text{-}15}$ was the most prevalent ESBL gene (19/30, 63.33%), and IS*Ecp1* was detected 48 bp upstream of 15 $bla_{CTX\text{-}M\text{-}15}$ genes. Based on S1-PFGE analyses, 25 isolates exhibited different plasmid profiles, ranging from ~70 to 320 kb. The $bla_{CTX\text{-}M\text{-}15}$ with bla_{TEM} and *qnr* genes and the IS*Ecp1* element from eight isolates were co-transferrable to recipients via conjugation, with IncFIB, IncFIC, and IncFII being the most prevalent replicons. Twenty different PFGE patterns and 11 sequence types were identified, with ST304 being dominant.

Conclusion: This work reports the emergence of $bla_{CTX\text{-}M\text{-}15}$ in *K. pneumoniae*-induced VAP in China. We showed that IncFIB, IncFIC, and/or IncFII plasmids carrying $bla_{CTX\text{-}M\text{-}15}$ with bla_{TEM}, *qnr* resistance genes, and the IS*Ecp1* element mediate the local prevalence in *K. pneumoniae*-associated VAP.

Keywords: *Enterobacteriaceae*, CTX-M-15, antibiotic resistance, horizontal gene transfer, conjugation

Introduction

Ventilator-associated pneumonia (VAP) is one of the most frequent hospital-acquired infections occurring in intubated and mechanically ventilated patients. The rate of VAP

occurrence is reportedly 9%–27%, with mortality reaching 20%–50%.[1,2] Common causative pathogens of VAP include Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli* and Gram-positive bacteria such as *Staphylococcus aureus*.[3–6] Extended-spectrum β-lactamase (ESBL)-producing *K. pneumoniae* is a common organism of nosocomial infections that play an important role in VAP.[7,8]

Some clinical isolates of pathogens produce ESBLs that are able to hydrolyze expanded-spectrum cephalosporins (eg, ceftriaxone, cefotaxime, and ceftazidime), aztreonam, and related oxyimino-β-lactams, but not carbapenems, and are inhibited by clavulanic acid and tazobactam.[9] Genes encoding ESBLs, such as bla_{SHV}, bla_{CTX-M}, bla_{TEM}, bla_{OXA}, bla_{PER}, and bla_{VEB}, are usually located on large plasmids (>30 kb in size) that are highly mobile and often harbor resistance determinants for several unrelated classes of antimicrobials, such as aminoglycosides, trimethoprim/sulfonamides, tetracyclines, and chloramphenicol.[10] Therefore, antibiotic therapy for treating such multidrug-resistant (MDR) pathogen infections is limited to a small number of expensive drugs.

CTX-M enzymes are among the most important ESBLs worldwide, with a clearly higher prevalence compared to other ESBLs. Indeed, emergence and outbreaks of the CTX-M enzyme have been described in bacteria from Africa, Europe, South America, and Asia.[11] At present, more than 90 CTX-M variants have been designated (http://www.lahey.org/Studies/other.asp), of which CTX-M-15 is the most prevalent variant around the world.[12] The global spread of $bla_{CTX-M-15}$ is largely due to *E. coli* of sequence type (ST) 131, IncFII plasmids, and the genetic platforms of the bla_{CTX-M} gene, such as IS*CR1*, IS*Ecp1,* and IS*26*, which act as promoters for expression of various resistance genes and influence the mobilization of bla_{CTX-M} genes.[13,14]

Many recent reports have indicated that VAP may be associated with multiresistant pathogens, such as *P. aeruginosa* and Gram-negative bacilli, characterized by the production of ESBLs.[15,16] However, limited data are available regarding the emergence and prevalence of $bla_{CTX-M-15}$-producing *K. pneumoniae* isolates from VAP cases in China. To better guide prevention efforts and clinical treatment of infections, this cross-sectional study was performed to investigate the antimicrobial resistance and molecular epidemiology of VAP caused by *K. pneumoniae* isolates producing ESBLs, especially $bla_{CTX-M-15}$, over a 3-year period.

Materials and methods

Clinical isolates and ESBL phenotype confirmation

A retrospective cross-sectional study was conducted at the intensive care unit (ICU) of the First Affiliated Hospital of Dalian Medical University, a 3,700-bed tertiary-care hospital with five ICU wards, from January 2013 to December 2015. *K. pneumoniae* strains were collected via endotracheal aspiration from mechanically ventilated patients with suspected pneumonia and stored at −80°C before use. VAP was defined as pneumonia occurring 48 hours or more after endotracheal intubation with at least two of the following criteria: fever greater than 38.3°C, leukocytosis or leucopenia, and purulent tracheal secretions (greater than 25 neutrophils observed per high-power field).[17] In addition, one or more of the following criteria had to be met: new or persistent infiltrates on chest radiographs, the same microorganism isolated from pleural fluid and tracheal secretions, radiographic cavitation or histopathological demonstration of pneumonia, and positive cultures obtained from bronchoalveolar lavage (greater than 10^4 colony forming units per mL).[18–20] A MicroScan WalkAway 96 Plus instrument (Siemens AG, Munich, Germany) was used for bacterial identification. Polymicrobial infections were excluded from analysis. All of the *K. pneumoniae* isolates were screened and confirmed using a double-disk synergy test for ESBL production.

Antimicrobial susceptibility testing

The antimicrobial susceptibility of ESBL-producing *K. pneumoniae* (EPKP) isolates and recipient (J53Az^R) and transconjugant strains was determined using the standard broth microdilution method according to the recommendations of the Clinical and Laboratory Standards Institute.[21] The following antimicrobial compounds were assessed: cefuroxime, cefotaxime, ceftazidime, cefepime, imipenem, aztreonam, amikacin, ciprofloxacin, levofloxacin, and tigecycline. *E. coli* ATCC 25922 was used as a reference strain. MDR *K. pneumoniae* strains were defined as acquired nonsusceptibility to at least one agent in three or more antimicrobial categories.

Molecular detection of resistance genes

To identify ESBL genes carried by the *K. pneumoniae* isolates, cell lysates were subjected to polymerase chain reaction (PCR) detection of bla_{CTX-M}, bla_{SHV}, bla_{TEM}, and bla_{OXA} genes. All PCR products amplified from β-lactamase genes were commercially sequenced, and subsequent searches

in PubMed using the BLAST program (https://blast.ncbi.nlm.nih.gov/) were performed. Specific PCR assays were conducted as previously described to identify the possible association of $bla_{CTX-M-15}$ with IS*Ecp1* or with the IS*26* insertion element.[13,22] Furthermore, plasmid-mediated quinolone resistance (PMQR) genes (*qnrA*, *qnrB*, *qnrS*, and *aac(6′)-Ib-cr*) were confirmed by multiplex PCR using a previously described protocol.[23] Additional genes of antibiotic resistance, such as 16S rRNA methylase-encoding genes (*armA, rmtB*, and *rmtC*), were assessed to further characterize the identified strains.[24]

Multidrug efflux pump gene expression

The expression of genes encoding the multidrug efflux pumps AcrB, OqxB, and KpgB and their global transcriptional regulators RarA and RamA in clinical EPKP isolates was assessed by quantitative reverse-transcription PCR using previously described oligonucleotide primers.[25] Total bacterial RNA was extracted using an E.Z.N.A.™ bacterial RNA Kit (Omega Bio-Tek, Norcross, GA, USA) and was reverse transcribed to complementary cDNA using a PrimeScript RT Reagent Kit (Takara, Dalian, China) according to the manufacturer's instructions. The cDNA was amplified using a SYBR® Premix Ex TaqTM II Kit (Takara) and a Stratagene Mx3005P qPCR System (Stratagene Agilent, Santa Clara, CA, USA) with 40 cycles of 5 seconds at 95°C and 34 seconds at 60°C. Each strain was amplified in triplicate. The expression levels of each target gene were normalized to a housekeeping gene (*rrsE*). Data were analyzed using Agilent MxPro software based on the $2^{-\Delta\Delta Ct}$ method.

Plasmid analysis

S1-nuclease (Takara) digestion followed by pulsed-field gel electrophoresis (S1-PFGE) analysis was performed for all the EPKP isolates and transconjugants. For plasmid size estimation, comparison with the molecular weight marker *Salmonella braenderup* H9812 was performed. Plasmid replicons were determined using the PCR-based replicon typing scheme (PBRT) 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.[26]

Resistance transfer determination

To determine whether plasmids coding for antibiotic resistance enzymes can be transferred, conjugation experiments were performed with all isolates carrying $bla_{CTX-M-15}$ using a broth mating protocol. *K. pneumoniae* isolates were mated with the sodium azide-resistant *E. coli* strain J53Az^R. Transconjugants were selected on LB agar plates containing sodium azide (100 μg/mL) and cefotaxime (10 μg/mL). PCR amplification, antimicrobial susceptibility testing, and plasmid replicon typing were performed for all transconjugants to determine the presence of resistance determinants, antibiotic phenotypes, and incompatibility groups, respectively.

PFGE and multilocus sequence typing (MLST)

The genetic relatedness of the identified *K. pneumoniae* isolates was examined by PFGE and MLST. DNA was extracted and digested with 45 U *Xba*I (Takara) for 2 hours at 37°C. PFGE was performed for the EPKP isolates using a CHEF-DRIII apparatus (Bio-Rad Laboratories, Hercules, CA, USA) as previously described.[27] MLST analysis was conducted by sequencing fragments of seven housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*), and STs were assigned using the *K. pneumoniae* MLST website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae).

Ethical clearance

The collection of *K. pneumoniae* in this study was part of routine hospital laboratory procedure. This retrospective study was performed using samples for secondary use, free of the need for informed consent and ethics committee approval.

Results

Antimicrobial susceptibility testing

From January 2013 to December 2015, 89 mechanically ventilated patients were identified with *K. pneumoniae*-induced VAP in our tertiary-care hospital. The patients, 60 (67.4%) of whom were male and 29 (32.6%) were female, had a mean ± SD age of 56.0±14.0 years. Among the patients, 30 clinical *K. pneumoniae* isolates were screened and confirmed for ESBL production (30/89, 33.71%). High-level resistance to cephalosporins (cefuroxime [28/30, 93.33%; MIC range: 8 to >512 μg/mL], ceftazidime [29/30, 96.67%; MIC range: 8–512 μg/mL], cefotaxime [28/30, 93.33%; MIC range: 2–512 μg/mL], and cefepime [24/30, 80.00%; MIC range: 4–128 μg/mL]) as well as noteworthy resistance to fluoroquinolones (ciprofloxacin [26/30, 86.67%; MIC range: 1–256 μg/mL], levofloxacin [23/30, 76.67%; MIC range: 2–256 μg/mL]), and aminoglycosides (amikacin [16/30, 53.33%; MIC range: 4 to >512 μg/mL]) were found. All 30 EPKP isolates exhibited a MDR phenotype and were examined in subsequent experiments. Their susceptibility profiles for ten antimicrobial agents are shown in Table 1.

Table 1 Resistance features of the ESBL-producing *K. pneumoniae* isolates, transconjugants, and recipients

Strain[a]	Resistance genes and mobile genetic elements	MIC (μg/mL)									
		CFX	CAZ	CTX	FEP	IPM	ATM	AMK	LEV	CIP	TGC
K. pneumoniae											
EPKP1	bla_{SHV-11}, *qnrS*, *rmtB*	512	32	64	128	2	512	32	128	64	2
EPKP2	bla_{TEM-1}, $bla_{CTX-M-15}$, bla_{SHV-11}, *qnrB*, *qnrS*, *armA*, IS*Ecp1*	>512	256	256	64	1	256	512	64	64	1
EPKP3	bla_{TEM-1}, bla_{SHV-33}, *qnrS*	>512	16	64	32	2	512	32	4	2	1
EPKP4	bla_{TEM-1}, $bla_{CTX-M-15}$, bla_{OXA-10}, *qnrB*, *qnrS*, *armA*	>512	512	64	128	2	512	>512	64	64	1
EPKP5	bla_{TEM-1}, $bla_{CTX-M-15}$, bla_{SHV-11}, *qnrB*, *qnrS*, *armA*, IS*Ecp1*	>512	512	128	64	2	512	256	128	128	2
EPKP6	$bla_{CTX-M-15}$, bla_{SHV-11}, *qnrB*, *qnrS*, *armA*, IS*Ecp1*	128	256	64	32	1	256	>512	64	128	8
EPKP7	*qnrA*, *qnrB*, *qnrS*, *armA*	>512	256	256	64	2	256	128	128	64	4
EPKP8	$bla_{CTX-M-15}$, *qnrB*, *armA*, IS*Ecp1*	>512	256	64	16	1	128	>512	128	128	2
EPKP9	bla_{TEM-1}, bla_{SHV-27}, *qnrS*	512	8	32	64	1	8	16	4	1	1
EPKP10	bla_{SHV-11}	8	32	2	8	2	16	8	2	2	8
EPKP11	bla_{TEM-1}, $bla_{CTX-M-15}$, bla_{SHV-11}, *qnrB*, *qnrS*, *aac(6')-Ib-cr*, *armA*, IS*Ecp1*	>512	>512	512	8	2	4	>512	4	8	0.5
EPKP12	$bla_{CTX-M-15}$, bla_{SHV-11}	16	32	2	16	2	512	8	4	2	1
EPKP13	*qnrB*, *armA*	>512	64	256	4	2	256	128	32	64	1
EPKP14	bla_{TEM-1}, $bla_{CTX-M-15}$, bla_{SHV-11}, *qnrB*, *qnrS*, *armA*, IS*Ecp1*	>512	256	64	64	2	256	128	64	128	1
EPKP15	bla_{TEM-53}, $bla_{CTX-M-15}$, *qnrS*, *armA*, IS*Ecp1*	32	256	32	32	2	16	32	128	64	1
EPKP16	$bla_{CTX-M-15}$, bla_{SHV-11}, *qnrB*, *qnrS*, *armA*	>512	256	128	64	2	64	>512	32	64	2
EPKP17	bla_{TEM-1}, $bla_{CTX-M-15}$, bla_{SHV-11}, *qnrB*, *qnrS*, *armA*, IS*Ecp1*	>512	512	128	128	1	512	>512	32	256	1
EPKP18	bla_{SHV-11}, *qnrB*, *armA*	>512	64	256	64	2	256	>512	128	64	2
EPKP19	bla_{TEM-1}, $bla_{CTX-M-15}$, bla_{SHV-11}, *rmtB*, IS*Ecp1*	>512	>512	128	32	1	128	>512	256	128	4
EPKP20	bla_{TEM-1}, $bla_{CTX-M-15}$, *qnrS*, IS*Ecp1*	>512	256	64	128	1	4	32	32	256	16
EPKP21	bla_{TEM-1b}, $bla_{CTX-M-15}$, *qnrS*, IS*Ecp1*	>512	256	256	64	2	256	32	4	64	1
EPKP22	bla_{TEM-1}, $bla_{CTX-M-22}$, bla_{SHV-33}, *qnrS*	512	128	64	32	2	8	8	32	128	8
EPKP23	bla_{TEM-1}, $bla_{CTX-M-15}$, bla_{SHV-11}, *qnrB*, *qnrS*, *armA*, IS*Ecp1*	>512	512	64	64	2	512	>512	64	64	1
EPKP24	bla_{TEM-1}, $bla_{CTX-M-15}$, *qnrB*, *qnrS*, *armA*, IS*Ecp1*	>512	32	128	16	1	256	128	128	128	1
EPKP25	bla_{TEM-1}, $bla_{CTX-M-15}$, bla_{SHV-11}, *qnrB*, *qnrS*, *armA*, IS*Ecp1*	>512	>512	64	64	1	512	128	128	128	1
EPKP26	bla_{TEM-53}, $bla_{CTX-M-15}$, bla_{SHV-11}, *qnrS*, *armA*, IS*Ecp1*	>512	>512	256	32	1	512	16	128	64	16
EPKP27	bla_{TEM-1b}, $bla_{CTX-M-15}$, *qnrS*	>512	256	32	128	2	512	32	128	128	0.5
EPKP28	bla_{SHV-28}, *qnrB*	>512	256	32	64	1	256	8	64	256	1
EPKP29	bla_{TEM-1}, $bla_{CTX-M-14}$, bla_{SHV-11}, *qnrB*	64	128	128	32	1	256	8	4	8	32
EPKP30	bla_{TEM-1}, bla_{SHV-28}, *qnrB*	>512	512	32	32	2	16	4	64	128	2
Transconjugants											
EPKP6C	$bla_{CTX-M-15}$, *qnrB*, IS*Ecp1*	128	256	64	16	1	128	128	128	64	0.5
EPKP8C	$bla_{CTX-M-15}$, *qnrB*, IS*Ecp1*	512	256	256	128	0.5	128	16	128	64	0.5
EPKP19C	bla_{TEM-1}, $bla_{CTX-M-15}$, IS*Ecp1*	512	>512	128	16	1	128	512	128	128	1
EPKP20C	$bla_{CTX-M-15}$, *qnrS*, IS*Ecp1*	>512	256	64	64	2	4	64	64	128	1
EPKP22C	bla_{TEM-1}, *qnrS*	>512	128	128	64	2	8	16	32	64	0.5
EPKP24C	$bla_{CTX-M-15}$, *qnrB*, *qnrS*, IS*Ecp1*	>512	64	128	32	1	128	128	64	128	1
EPKP26C	$bla_{CTX-M-15}$, *qnrS*, IS*Ecp1*	512	512	256	16	1	256	16	128	32	0.5
EPKP30C	bla_{TEM-1}, *qnrB*	>512	512	64	64	4	16	4	64	64	0.5
Recipients											
J53	–	2	0.5	0.5	1	<0.5	4	8	0.5	<0.5	<0.5

Notes: [a]Isolates 6C, 8C, 19C, 20C, 22C, 24C, 26C, and 30C are transconjugants.

Abbreviations: ESBL, extended-spectrum β-lactamase; *K. pneumoniae*, *Klebsiella pneumoniae*; MIC, minimum inhibitory concentration; CFX, cefuroxime; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; IPM, imipenem; ATM, aztreonam; AMK, amikacin; LEV, levofloxacinb; CIP, ciprofloxacin; TGC, tigecycline; "–", negative.

Identification of antibiotic resistance genes and the $bla_{CTX-M-15}$ genetic context

Of the 30 EPKP isolates, bla_{SHV}, bla_{CTX-M}, bla_{OXA}, and bla_{TEM} were identified in 21, 21, 1, and 20 isolates, respectively. Among the ESBL genes detected in this study, the $bla_{CTX-M-15}$ allele was the most prevalent (19/30, 63.33%), followed by bla_{SHV-28} (2/30, 6.67%) and bla_{TEM-53} (2/30, 6.67%). DNA sequence analysis revealed the insertion of IS*Ecp1* 48 bp upstream of the start codon of the 15 CTX-M-15 genes (except EPKP4, EPKP12, EPKP16, and EPKP27). However, PCR amplification with primers specific for IS*26* was negative for all *K. pneumoniae* isolates carrying $bla_{CTX-M-15}$,

Regarding β-lactamases other than ESBLs, bla_{SHV-11} (16/30, 53.33%) and bla_{TEM-1} (16/30, 53.33%) alleles predominated; other minor β-lactamases, including $bla_{CTX-M-14}$, $bla_{CTX-M-22}$, bla_{OXA-10}, bla_{TEM-1b}, bla_{SHV-33}, and bla_{SHV-27}, were also detected. For PMQR genes, 17 isolates were found to carry *qnrB*, 21 isolates *qnrS*, and two other variants such as *qnrA* or *aac-(6′)-Ib-cr*. Resistance genes for other antibiotics included *armA* in 17 isolates and *rmtB* in two (Table 1).

Differential expression of efflux pump genes

The levels of differential gene expression of the assayed multidrug efflux pumps and their global regulators among the 30 EPKP isolates are presented in Figure 1. Real-time PCR analysis showed uniformly high expression levels of *acrB* (1.80- to 4.00-fold) and *ramA* (2.12- to 17.68-fold) of the RamA/AcrB pathway in seven isolates (EPKP3, EPKP4, EPKP13, EPKP18, EPKP20, EPKP23, and EPKP24). Twelve isolates (EPKP3, EPKP4, EPKP6, EPKP9, EPKP10, EPKP17, EPKP18, EPKP20, EPKP22, EPKP25, EPKP28, and EPKP29) exhibited simultaneously high expression levels of *oqxB* (1.08- to 16.77-fold) and *rarA* (1.18- to 84.50-fold) genes. In addition, 13 isolates (EPKP1, EPKP3, EPKP6, EPKP11, EPKP13, EPKP14, EPKP17, EPKP18, EPKP20, EPKP21, EPKP22, EPKP26, and EPKP27) showed upregulation of the *kpgB* gene (2.07- to 18.53-fold).

Plasmid analysis

We examined the plasmid profiles of 30 EPKP isolates by S1-PFGE analysis. As shown in Figure 2, 25 different plasmid profiles were observed among the 30 EPKP isolates (ranging from ~70 to 320 kb). Fourteen strains (EPKP2, EPKP4, EPKP6, EPKP7, EPKP10, EPKP12, EPKP13, EPKP16, EPKP17, EPKP21, EPKP24, EPKP27, EPKP28, and EPKP30) harbored single plasmids of different sizes, and eleven strains (EPKP1, EPKP3, EPKP5, EPKP8, EPKP9, EPKP11, EPKP19, EPKP20, EPKP22, EPKP26, and EPKP29) harbored two to four plasmids. Conversely, five isolates (EPKP14, EPKP15, EPKP18, EPKP23, and EPKP25) contained no detectable plasmid elements.

Resistance transfer and PBRT

The resistance profiles of the eight transconjugants were similar to those of the bla_{CTX-15}-producing *K. pneumoniae* donor strains, demonstrating the transfer of antimicrobial resistance, including the ESBL phenotype. In addition, resistance to several non-β-lactam-based antimicrobial compounds, such as fluoroquinolones, was also cotransferred along with β-lactam resistance; in contrast, resistance to tigecycline was not transferred. For the transconjugants, the most commonly detected resistance genes included $bla_{CTX-M-15}$ (n=6), bla_{TEM-1} (n=3), *qnrS* (n=4), and *qnrB* (n=4), while the IS*Ecp1* element was detected in six isolates (Table 1).

Plasmid replicon typing showed that in the *K. pneumoniae* isolates carrying $bla_{CTX-M-15}$, the plasmids had different replicons, including IncFIC (n=11), IncFIB (n=8), IncFIA (n=2), IncF (n=1), IncFII (n=1), IncK (n=7), and IncL/M (n=1) (Figure 3). However, PCR replicon typing of the transconjugants identified only three replicons, IncFIB, IncFIC, and IncFII, which were present in both donors and transconjugants and were associated with the transfer of the ESBL phenotype (Table 2).

PFGE and MLST of isolates

The 30 EPKP isolates were assigned to 20 distinct PFGE clusters sharing ≥80% band similarity as well as eleven ST types (ST11, ST15, ST37, ST65, ST268, ST304, ST716, ST828, and ST1049), including two new STs (ST2321 and ST2322) (EPKP3 [02-01-02-01-03-01-25] and EPKP19 [03-20-01-01-01-01-04]). The most prevalent ST was ST304 (n=15, 50%), followed by ST716 (n=3, 10%) and ST37 (n=3, 10%). No clear relationship between replicon and sequence type was observed among the isolates identified in this study (Figure 3).

Discussion

This is one of only a few studies performed to date investigating the antimicrobial resistance and molecular epidemiology of *K. pneumoniae* carrying $bla_{CTX-M-15}$-caused VAP in China. The prevalence of ESBL reported herein is a matter of concern because MDR pathogens causing infectious diseases are common in this area, limiting therapeutic options for treating severe infections often associated with a poor outcome. Indeed, the incidence of EPKP is increasing among patients receiving mechanical ventilation in the ICU of our tertiary-care hospital. The prevalence rate of ESBL among *K. pneumonia* isolates causing VAP was 33.71%. Despite the high prevalence of ESBLs reported in this study, it is lower compared with the prevalence of those causing device-associated infections among children in a pediatric ICU of other medical centers.[28]

MIC determinations showed all EPKP isolates to be highly resistant to cephalosporins, with noteworthy resistance to fluoroquinolones and aminoglycosides also observed. As shown in Table 1, although the MDR phenotype reported in our studied isolates is frequently associated with ESBL

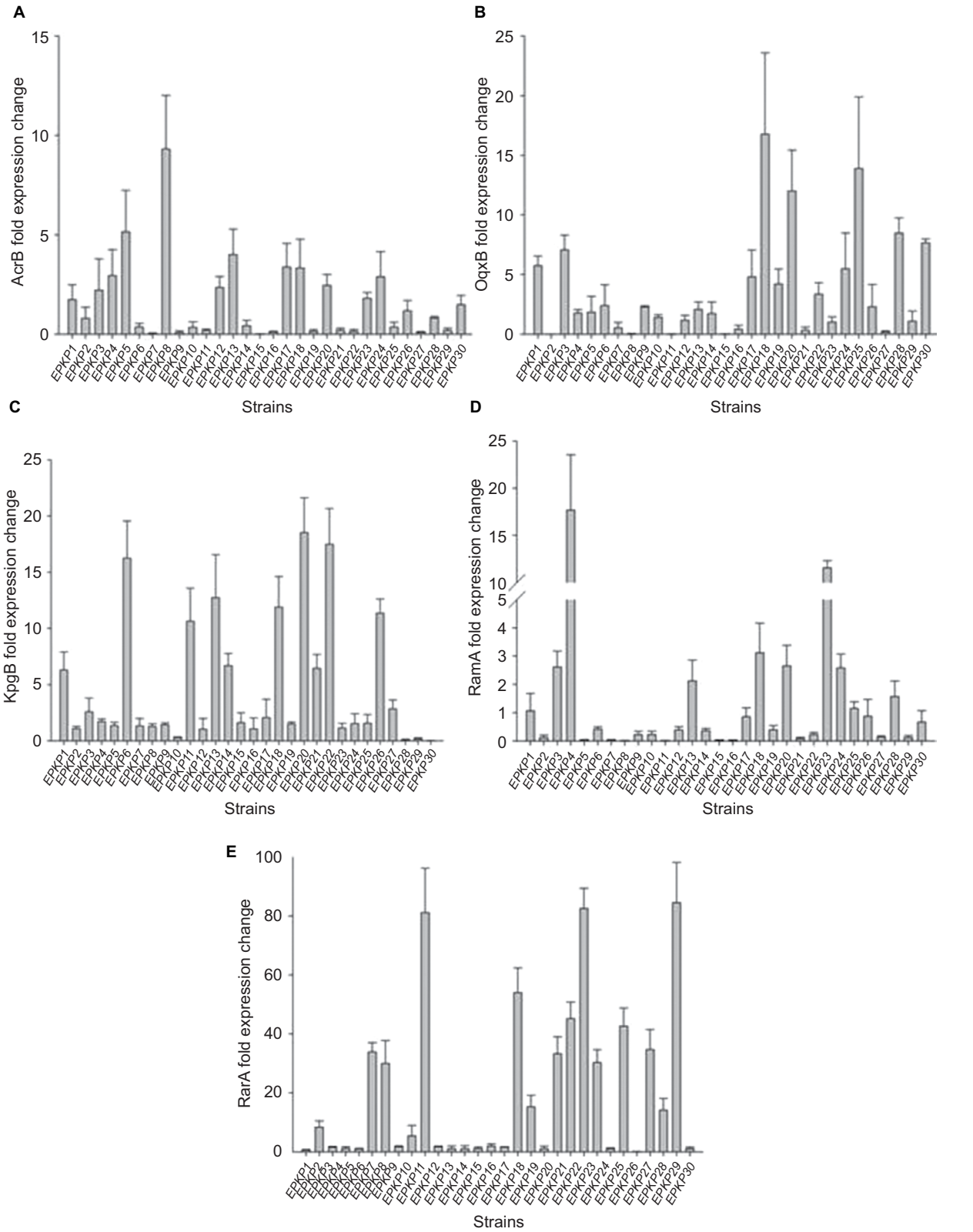


Figure 1 Differential gene expression of multidrug efflux pumps and global regulators among the 30 EPKP isolates.
Notes: Fold change in the expression of genes encoding (**A**) the multidrug efflux pump AcrB; (**B**) the multidrug efflux pump OqxB; (**C**) the multidrug efflux pump KpgB; (**D**) the global regulator RamA; and (**E**) the global regulator RarA. Fold changes in gene expression were determined after normalizing to that of the 16S rRNA gene (*rrsE*) in each strain and then comparing the expression of each gene with corresponding genes in the tigecycline-susceptible *Klebsiella pneumoniae* strain ATCC 13883.
Abbreviations: EPKP, ESBL-producing *K. pneumoniae*; ESBL, extended-spectrum β-lactamase.

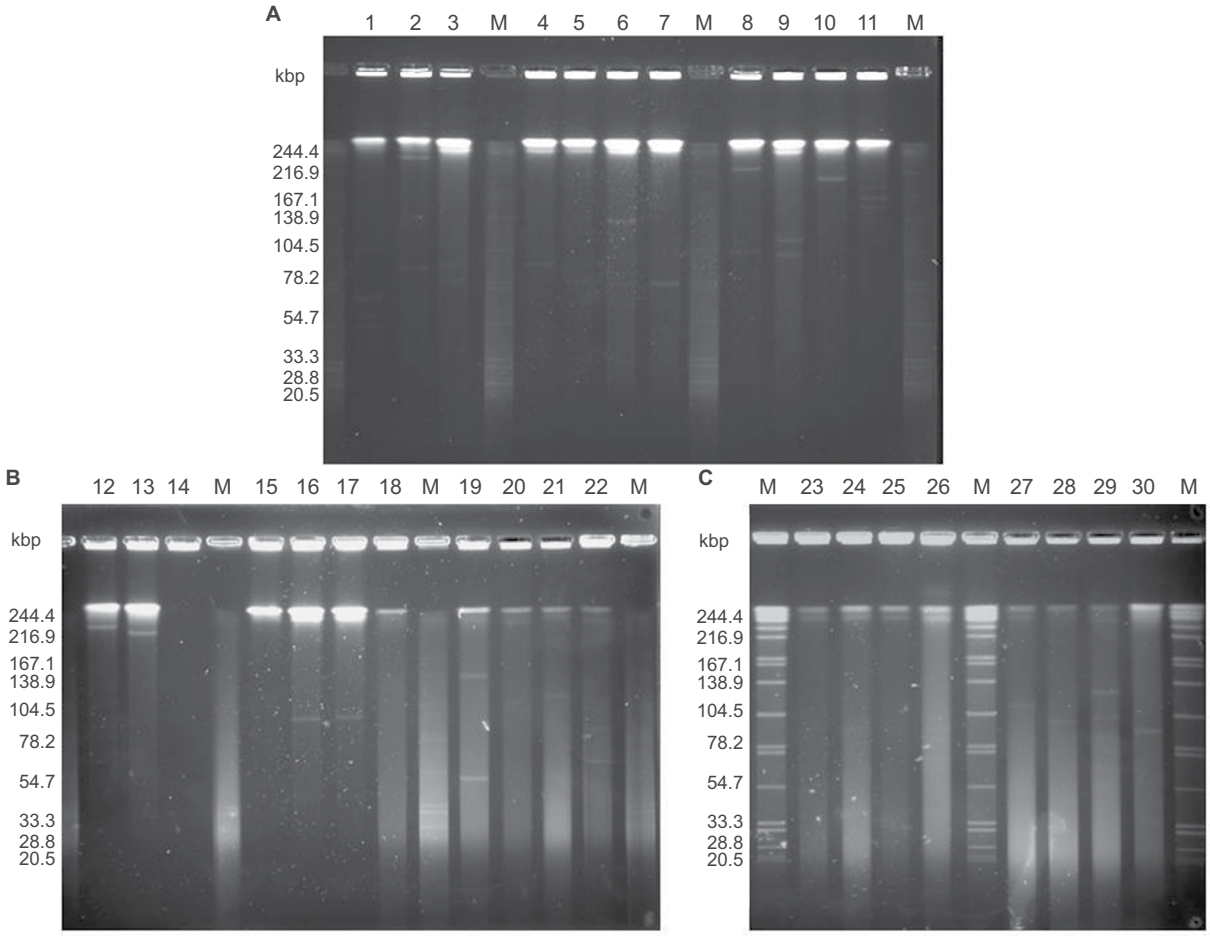
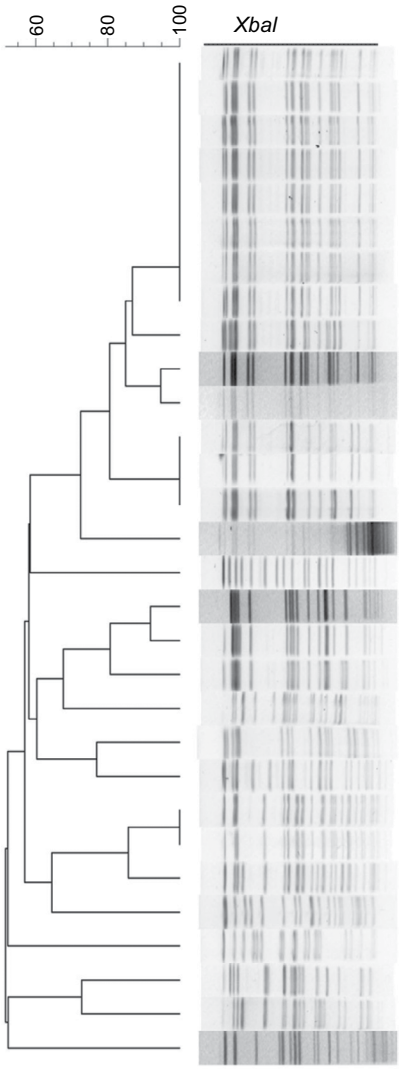


Figure 2 S1 endonuclease pulsed-field gel electrophoresis analysis of plasmids from the 30 VAP-inducing EPKP isolates in China.
Notes: (**A**) "M" indicates the molecular weight marker; Lanes 1–11 show the plasmid profiles of isolates EPKP1–EPKP11, respectively; (**B**) Lanes 12–22 show the plasmid profiles of isolates EPKP12–EPKP22, respectively; and (**C**) Lanes 23–30 show the plasmid profiles of isolates EPKP23–EPKP30, respectively.
Abbreviations: VAP, ventilator-associated pneumonia; EPKP, ESBL-producing *Klebsiella pneumoniae;* ESBL, extended-spectrum β-lactamase.

producers, the concurrent combination of different mechanisms, such as PMQR gene expression, 16S rRNA methylase production, and differential expression of multidrug efflux pump genes may lead to this phenotype. The co-presence of ESBL genes and other resistance determinants on the same plasmid is reported regularly.[29] In agreement with our findings, PMQR has been associated with bla_{CTX-M} genes, with genes conferring resistance to aminoglycosides and tetracycline and other *bla* genes being found on the same plasmids as carrying bla_{CTX-M}.[30]

Over the past decade, predominant CTX-M-type ESBLs have been described globally, including China, South Korea, and many other countries.[11,31,32] In fact, together with $bla_{CTX-M-14}$, $bla_{CTX-M-15}$ is currently the most common variant detected worldwide in clinically important Gram-negative bacteria.[33,34] In this study, we identified the CTX-M-15 enzyme as the most prevalent ESBL in VAP patients. The insertion sequence IS*Ecp1* has previously been shown to play an important role in the mobilization and expression of genes encoding bla_{CTX-M},[13,35] therefore, linkage of $bla_{CTX-M-15}$ with IS*Ecp1* was assessed and shown to be present in all but four of the *K. pneumoniae* isolates carrying the $bla_{CTX-M-15}$ gene. The presence of internal sequences such as IS*26*, which is related to the transmission of β-lactamase genes, such as DHA-1, CFE-1, ACC-1, and SHV-2a, is typically found for the IncFII plasmid.[33,36] Regardless, PCR amplification of the IS*26* gene was negative for all 19 CTX-M-15 genes.

In our study, PBRT and conjugation experiments showed that among the clinical *K. pneumoniae* isolates from mechanically ventilated patients, IncFIB, IncFIC, and IncFII replicons were present in the transconjugants and the $bla_{CTX-M-15}$ gene was co-transferred to the recipient strain with bla_{TEM} and *qnr* genes and the IS*Ecp1* element. These results indicate that IncF-related plasmids carrying $bla_{CTX-M-15}$ are a major vehicle mediating the local prevalence of resistance determinants in *K. pneumoniae* isolates. Nonetheless, it was previously



StrainID	Date of isolation	MLST	Replicon types	Plasmid sizes (kb)
EPKP23	09/06/2013	ST 304	–	–
EPKP2	01/07/2013	ST 304	K	118
EPKP5	20/03/2013	ST 304	FTC	107, 124
EPKP17	16/04/2013	ST 304	FTC	124
EPKP16	17/04/2013	ST 304	FTC	122
EPKP24	24/04/2013	ST 304	FTC	123
EPKP25	24/04/2013	ST 304	–	–
EPKP14	23/05/2013	ST 304	–	–
EPKP6	31/07/2013	ST 304	FIB	169
EPKP4	03/06/2013	ST 304	FIC	122
EPKP26	21/06/2013	ST 304	FIB, FIC	96, 111, 120
EPKP27	22/07/2014	ST 304	K	147
EPKP21	04/08/2014	ST 304	K	147
EPKP7	02/02/2015	ST 304	FIC	104
EPKP15	19/08/2013	ST 304	–	–
EPKP12	10/04/2013	ST 65	Unable	308
EPKP18	03/06/2013	ST 37	–	–
EPKP13	15/07/2013	ST 37	K	290
EPKP8	03/04/2013	ST 37	FIB, K	138, 319
EPKP29	02/07/2015	ST 1049	FIB	134, 160
EPKP1	11/03/2013	ST 11	FIA, F, LM	77, 85, 94, 139
EPKP19	10/06/2014	ST 2322	FIB	74, 172
EPKP20	15/04/2013	ST 716	FIB, FIC	136, 143
EPKP22	18/04/2013	ST 716	FIA, FII	85, 110
EPKP9	28/01/2013	ST 716	FIB, FIC	134, 149
EPKP3	08/03/2013	ST 2321	FIC	107, 120
EPKP10	01/07/2013	ST 828	K	296
EPKP30	30/03/2013	ST 15	FIC	121
EPKP28	20/11/2014	ST 15	K	130
EPKP11	06/03/2013	ST 268	FIB	197, 225

Figure 3 Genetic relatedness, plasmid size, and replicon type of the 30 VAP-inducing EPKP isolates in China.
Note: Dendrogram of patterns generated by PFGE.
Abbreviations: MLST, multilocus sequence typing; ST, sequence type; –, not detected; VAP, ventilator-associated pneumonia; EPKP, ESBL-producing *K. pneumoniae*; ESBL, extended-spectrum β-lactamase; PFGE, pulsed-field gel electrophoresis.

reported that the $bla_{\text{CTX-M-15}}$ gene can also be found in IncN, IncR, IncFIIk, or IncL/M types.[11,37–39]

Regarding our S1-PFGE analysis, the existence of five EPKP isolates (EPKP14, EPKP15, EPKP18, EPKP23, and EPKP25) containing no plasmid suggests that these "plasmid-encoded" resistance genes have been integrated into the chromosome. In addition, smaller plasmids may not have been detected by S1-PFGE analysis.[40] Further studies are needed to investigate other resistance genes possibly carried on smaller plasmids.

A previous retrospective study of 49 mechanically ventilated patients in tertiary hospitals in China showed that ST23 was dominant among hypervirulent *K. pneumoniae* strains,[41] yet ST304 was the most prevalent in our collection of "classic" *K. pneumoniae* isolates (n=15, 50.00%). To the best of our knowledge, this is the first report of ST304 in *K. pneumoniae*, and no clear relationship between replicon and sequence type was observed among the current isolates. This result suggests that this is not a result of the dissemination of particular clones but rather is due to the spread of multiple specific clones and/or mobile genetic elements.

The emergence of MDR pathogens as causative agents of VAP has resulted in a greater administration of inappropriate initial antimicrobial therapies, defined as an antimicrobial regimen that lacks in vitro activity against the isolated organism(s) responsible for the infection.[42] Our data highlights the importance of continuous surveillance of both resistant isolates and genetic elements of resistance to

Table 2 Antibiotic resistance genes and plasmid replicon types in transferable bla_{CTX-15}-producing *Klebsiella pneumoniae* donors and their transconjugants

Isolates[a]	ESBL/PMQR/16S rRNA genes and IS*Ecp1*	Replicon type present
EPKP6	$bla_{CTX-M-15}$, bla_{SHV-11}, *qnrB*, *qnrS*, *armA*, IS*Ecp1*	FIB
EPKP8	$bla_{CTX-M-15}$, *qnrB*, *armA*, IS*Ecp1*	FIB, K
EPKP19	bla_{TEM-1}, $bla_{CTX-M-15}$, bla_{SHV-11}, *rmtB*, IS*Ecp1*	FIB
EPKP20	bla_{TEM-1}, $bla_{CTX-M-15}$, *qnrS*, IS*Ecp1*	FIB, FIC
EPKP22	bla_{TEM-1}, $bla_{CTX-M-22}$, bla_{SHV-33}, *qnrS*	FIA, FII
EPKP24	bla_{TEM-1}, $bla_{CTX-M-15}$, *qnrB*, *qnrS*, *armA*, IS*Ecp1*	FIC
EPKP26	bla_{TEM-53}, $bla_{CTX-M-15}$, bla_{SHV-11}, *qnrS*, *armA*, IS*Ecp1*	FIB, FIC
EPKP30	bla_{TEM-1}, bla_{SHV-28}, *qnrB*	FIC
EPKP6C	$bla_{CTX-M-15}$, *qnrB*, IS*Ecp1*	FIB
EPKP8C	$bla_{CTX-M-15}$, *qnrB*, IS*Ecp1*	FIB
EPKP19C	bla_{TEM-1}, $bla_{CTX-M-15}$, IS*Ecp1*	FIB
EPKP20C	$bla_{CTX-M-15}$, *qnrS*, IS*Ecp1*	FIB, FIC
EPKP22C	bla_{TEM-1}, *qnrS*	FII
EPKP24C	$bla_{CTX-M-15}$, *qnrB*, *qnrS*, IS*Ecp1*	FIC
EPKP26C	$bla_{CTX-M-15}$, *qnrS*, IS*Ecp1*	FIB, FIC
EPKP30C	bla_{TEM-1}, *qnrB*	FIC

Notes: [a]Isolates EPKP6, 8, 19, 20, 22, 24, 26, and 30 are original isolates; EPKP6C, 8C, 19C, 20C, 22C, 24C, 26C, and 30C are transconjugants.
Abbreviations: ESBL, extended-spectrum β-lactamase; PMQR, plasmid-mediated quinolone resistance.

monitor the emergence and trends of ESBL-producing isolates to promote adequate therapeutic strategies for managing MDR bacterial infections.

The present study has several limitations. First, because this study was a retrospective analysis and only limited VAP patient information was available, the study focused on the molecular characterization of the prevalence of genes among clinical EPKP. Another limitation of the study included a lack of analysis of other resistance-related determinants, such as the outer-membrane permeability of EPKP isolates. Further studies are needed to address these limitations.

Conclusion

Although ESBL-producing members of *Enterobacteriaceae* have been reported in China, very limited data are available regarding the susceptibility and molecular characterization of *K. pneumoniae* isolates from mechanically ventilated patients. This study highlights the emergence of ESBLs, particularly the CTX-M-15 type, in *K. pneumoniae*-induced VAP in Chinese hospitals. We showed that the $bla_{CTX-M-15}$ gene was cotransferred with the bla_{TEM} and *qnr* genes and the IS*Ecp1* element, conferring a high level of resistance to most antibiotics tested. All transconjugants were associated with IncFIB, IncFIC, and IncFII plasmids.

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Disclosure

The authors report no conflicts of interest in this work.

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