Research Paper

Molecular characterization of multidrug-resistant *Klebsiella pneumoniae* isolates

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Abstract

Klebsiella pneumoniae is an important cause of healthcare-associated infections worldwide. Selective pressure, the extensive use of antibiotics, and the conjugational transmission of antibiotic resistance genes across bacterial species and genera facilitate the emergence of multidrug-resistant (MDR) *K. pneumoniae*. Here, we examined the occurrence, phenotypes and genetic features of MDR *K. pneumoniae* isolated from patients in intensive care units (ICUs) at the First Affiliated Hospital of Xiamen University in Xiamen, China, from January to December 2011. Thirty-eight MDR *K. pneumoniae* strains were collected. These MDR *K. pneumoniae* isolates possessed at least seven antibiotic resistance determinants, which contribute to the high-level resistance of these bacteria to aminoglycosides, macrolides, quinolones and β-lactams. Among these isolates, 24 strains were extended-spectrum β-lactamase (ESBL) producers, 2 strains were AmpC producers, and 12 strains were both ESBL and AmpC producers. The 38 MDR isolates also contained class I (28/38) and class II integrons (10/38). All 28 class I-positive isolates contained *aacC1*, *aacC4*, *orfX*, *orfX'* and *aadA1* genes. β-lactam resistance was conferred through bla_{SHV} (22/38), bla_{TEM} (10/38), and bla_{CTX-M} (7/38). The highly conserved bla_{KPC-2} (37/38) and bla_{OXA-23} (1/38) alleles were responsible for carbapenem resistance, and a *gyrA* site mutation (27/38) and the plasmid-mediated *qnrB* gene (13/38) were responsible for quinolone resistance. Repetitive-sequence-based PCR (REP-PCR) fingerprinting of these MDR strains revealed the presence of five groups and sixteen patterns. The MDR strains from unrelated groups showed different drug resistance patterns; however, some homologous strains also showed different drug resistance profiles. Therefore, REP-PCR-based analyses can provide information to evaluate the epidemic status of nosocomial infection caused by MDR *K. pneumoniae*; however, this test lacks the power to discriminate some isolates. Thus, we propose that both genotyping and REP-PCR typing should be used to distinguish genetic groups beyond the species level.

Key words: *Klebsiella pneumoniae*, multidrug resistance, molecular characterization.

Introduction

Klebsiella pneumoniae has been medically recognized as one of the most important opportunistic pathogens, causing hospital-acquired and healthcare-associated pulmonary system, urinary tract, circulating system and soft tissue infections worldwide (Podschun and Ullmann, 1998). However, *K. pneumoniae* has become a clinically important micro-organism, particularly in last two decades due to its tendency to develop antibiotic resistance and cause fatal outcomes (Nordmann *et al.*, 2009; Ko *et al.*, 2002).

In recent years, *K. pneumoniae* has been identified as a major cause of community-acquired pneumonia (CAP) and is responsible for approximately 10% of all hospital-acquired infections, ranking second among Gram-negative pathogens (Nordmann *et al.*, 2009). Studies performed in Asia have demonstrated that the fatality rate of *K.*

pneumoniae-induced pneumonia in elderly people was 15% to 40%, equal to or even greater than that of *Haemophilus influenzae* (Molton *et al.*, 2013). The production of extended-spectrum β-lactamases (ESBL) in this organism contributes to the emergence and dissemination of *K. pneumoniae* infections. Effective anti-infective drugs, such as aminoglycosides, fluoroquinolones, and carbapenems, have been used to treat ESBL-producing *K. pneumoniae* infections (Sanchez *et al.*, 2013). In particular, the use of carbapenems against ESBL-producing Gram-negative micro-organisms poses a serious problem in the management of healthcare-associated infections because the abuse of antibiotics might lead to the emergence of carbapenem-resistant organisms. Notably, the selective pressure posed by the extensive use of antibiotics has facilitated the emergence of multidrug-resistant (MDR) *K. pneumoniae*. Furthermore, conjugational transmission of antibiotic resistance genes across bacterial species and genera has aggravated the problem of *K. pneumoniae* antibiotic resistance.

MDR *K. pneumoniae* was first reported in the United States, followed by Europe, South America, and Asia (Ko *et al.*, 2002; Pfaller *et al.*, 2001; Winokur *et al.*, 2001; Yigit *et al.*, 2001). At present, infections caused by MDR *K. pneumoniae* have become a major problem, as few antibiotics are available, resulting in higher morbidity, longer hospitalization, increased mortality rates, and excessive health care costs compared with infections associated with antibiotic-susceptible micro-organisms (Correa *et al.*, 2013; Ma and Wang, 2013). However, the prevalence of antibiotic-resistant bacteria significantly varies according to region, country, and susceptible population, as the seriousness of this problem is significantly associated with the measures applied to control the spread of drug-resistant bacteria (Ko *et al.*, 2002). The aim of this study was to characterize the drug resistance phenotypes and molecular antibiotic resistance mechanisms of MDR *K. pneumoniae* strains isolated from patients in intensive care units (ICUs). The relatedness of the strains was also investigated using the repetitive-sequence-based PCR (REP-PCR)-based DiversiLab system.

Materials and Methods

Bacterial identification and antimicrobial susceptibility testing

A total of 38 MDR *K. pneumoniae* strains were isolated from sputum, bronchial-alveolar perfusate, blood, intraperitoneal drainage, pleural drainage of patients and surfaces of objects (respirators, bedrails and bedclothes) in ICUs from January to December 2011. Thirty nosocomial infection strains were identified according to the *Diagnostic Criteria for Nosocomial Infection* established by the Ministry of Health of China. Five strains (K33, K34, K35, K36 and K37) were isolated from ventilators, two strains

(K19 and K20) were isolated from bedrails, and one (K31) strain was isolated from bedclothes. Bacterial identification and antimicrobial susceptibility testing were performed using the Vitek2 semi-automated system with GN13 and AST13 cards (bioMérieux Inc., Durham, NC), respectively. The minimum inhibitory concentrations (MICs) for 28 antimicrobial agents for MDR isolates were confirmed using the broth microdilution method. In addition, polymyxin B and tigecycline susceptibilities were determined through Etests (bioMérieux Inc., Durham, NC). Multidrug resistance was defined as non-susceptibility to at least one agent in three or more antimicrobial categories according to the breakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI), excluding tigecycline and polymyxin B. For these studies, *K. pneumoniae* ATCC 700603 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls. Disks of cefotaxime (30 μg), cefotaxime/clavulanic acid (30/10 μg), ceftazidime (30 μg), and ceftazidime/clavulanic acid (30/10 μg) were used to confirm ESBL production of these *K. pneumoniae* isolates using the Double Disc Synergy Test (DDST) according to the recommendations of the CLSI. For the phenotypic identification of AmpC-producing isolates, the DDST was applied using disks containing ceftazidime (30 μg) and ceftazidime/3-aminophenylboronic acid (30/300 μg). A ≥ 5-mm enhancement of the zone of inhibition around the ceftazidime disk was considered AmpC-positive. The three-dimensional test was also used to confirm AmpC-producing isolates.

DNA extraction

Genomic DNA from MDR strains was prepared for PCR and genetic analysis using the UltraClean® Microbial DNA Isolation Kit (MO BIO Labs Inc., Solana Beach, CA) according to the manufacturer's instructions. Briefly, MDR strains were grown to the desired optical density and were collected after centrifugation at 10,000 x *g* for 60 s. The bacterial cells were resuspended in bead solution and transferred to a bead-beating tube containing microbeads and proteinase K. Subsequently, the cells were lysed through mechanical disruption using a specially designed MO BIO Vortex Adapter on a standard vortex. The supernatant was loaded onto a Spin Filter mini column and washed twice with Wash buffer, and the genomic DNA was eluted with DNA-free Tris buffer.

Resistance determinants detection

The genes involved in the antibiotic resistance of MDR strains were screened through PCR assays as previously described (Perez-Perez and Hanson, 2002; Wen, 2010; Mak, 2009), except that the PCR conditions used to detect *bla*$_{KPC}$ and *bla*$_{NDM-1}$, included a pre-denaturing step at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, with polishing at 72 °C for 10 min. The PCR products were purified using the

QIAGEN Gel Extraction Kit (Qiagen Inc., Hilden, Germany) and were subjected to sequencing using the same primers used for PCR amplification. The resistance genes and the primers used in the present study are listed in Table 1.

Genetic relatedness determination

The genetic similarities of the MDR *K. pneumoniae* strains were further analyzed using REP-PCR, a semi-automated PCR technique for the amplification of the re-gions between the non-coding repetitive sequences in bacterial genomes. REP-PCR was performed using the DiversiLab Klebsiella Kit (bioMérieux Inc., Durham, NC) according to the manufacturer's instructions. The PCR products were separated on an Agilent B2100 Bioanalyzer using DNA LabChip reagents (Agilent Technologies Inc., Palo Alto, CA). The data were uploaded to the web-based DiversiLab system for further analysis. All results, including dendrograms, scatterplots, electropherograms, and virtual gel images, were illustrated using DiversiLab software

Table 1 - Primer sequences used in this study.

Primer	Sequence	Target gene	Source or reference
KPC-F	ATGTCACTGTATCGCCGTC	bla_{KPC}	This study
KPC-R	TTACTGCCCGTTGACGCC	bla_{KPC}	This study
NDM-1-F	TGCATTGATGCTGAGCGGGTG	bla_{NDM-1}	This study
NDM-1-R	ATCACGATCATGCTGGCCTTG	bla_{NDM-1}	This study
IMP-F	GGAATAGAGTGGCTTAAYTCTC	bla_{IMP}	(Wen and Mi, 2010)
IMP-R	CCAAACYACTASGTTATCT	bla_{IMP}	(Wen and Mi, 2010)
VIM-F	GATGGTGTTTGGTCGCATA	bla_{VIM}	(Wen and Mi, 2010)
VIM-R	CGAATGCGCAGCACCAG	bla_{VIM}	(Wen and Mi, 2010)
EBC-F	TCTATAAGTAAAACCTTCACCG	bla_{EBC}	(Perez-Perez and Hanson, 2002)
EBC-R	CCAGGTATGGTCCAGCTTGAG	bla_{EBC}	(Perez-Perez and Hanson, 2002)
ACC-F	AACAGCCTCAGCAGCCGGTTA	bla_{ACC}	(Perez-Perez and Hanson, 2002)
ACC-R	TTCGCCGCAATCATCCCTAGC	bla_{ACC}	(Perez-Perez and Hanson, 2002)
FOX-F	TTCGAGATTGGCTCGGTCAGC	bla_{FOX}	(Perez-Perez and Hanson, 2002)
FOX-R	CAAAGCGCGTAACCGGATTGG	bla_{FOX}	(Perez-Perez and Hanson, 2002)
DHA-F	AACTTTCACAGGTGTGCTGGGT	bla_{DHA}	(Perez-Perez and Hanson, 2002)
DHA-R	CCGTACGCATACTGGCTTTGC	bla_{DHA}	(Perez-Perez and Hanson, 2002)
CIT-F	TGGCCAGAACTGACAGGCAAA	bla_{CIT}	(Perez-Perez and Hanson, 2002)
CIT-R	TTTCTCCTGAACGTGGCTGGC	bla_{CIT}	(Perez-Perez and Hanson, 2002)
TEM-F	ACAGCGGTAAGATCCTTGAGAG	bla_{TEM}	(Wen and Mi, 2010)
TEM-R	GAAGCTAGAGTAAGTAGTTCG	bla_{TEM}	(Wen and Mi, 2010)
SHV-F	ACCTTTAAAGTAGTGCTCTGC	bla_{SHV}	(Wen and Mi, 2010)
SHV-R	CACCATCCACTGCAGCAGCTG	bla_{SHV}	(Wen and Mi, 2010)
CTX-M-F	ATGGTTAAAAAATCACTGCGYCAGTTC	bla_{CTX-M}	(Wen and Mi, 2010)
CTX-M-R	TCACAAACCGTYGGTGACGATTTTAGCCGC	bla_{CTX-M}	(Wen and Mi, 2010)
OXA-F	CTGTTGTTTGGGTTTCGCAAG	bla_{OXA}	(Wen and Mi, 2010)
OXA-R	CTTGGCTTTTATGCTTGATG	bla_{OXA}	(Wen and Mi, 2010)
PER-F	GCCTGACGATCTGGAACC	bla_{PER}	(Wen and Mi, 2010)
PER-R	GATACTGCACCTGATCATC	bla_{PER}	(Wen and Mi, 2010)
VEB-F	GCGTTATGAAATTTCCGATTG	bla_{VEB}	(Wen and Mi, 2010)
VEB-R	CAACATCATTAGTGGCTGCTG	bla_{VEB}	(Wen and Mi, 2010)
GES-F	ATGCGCTTCATTCACGCAC	bla_{GES}	(Wen and Mi, 2010)
GES-R	CTATTTGTCCGTGCTCAGG	bla_{GES}	(Wen and Mi, 2010)
1Int-F	ATCATCGTCGTAGAGACGTCGG	Class I Integron	(Wen and Mi, 2010)
1Int-R	GTCAAGGTTCTGGACCAGTTGC	Class I Integron	(Wen and Mi, 2010)
2Int-F	GCAAATAAAGTGCAACGC	Class II Integron	(Wen and Mi, 2010)
2Int-R	ACACGCTTGCTAACGATG	Class II Integron	(Wen and Mi, 2010)

Table 1 - Cont.

Primer	Sequence	Target gene	Source or reference
3Int-F	GCAGGGTGTGGACGAATACG	Class III Integron	(Wen and Mi, 2010)
3Int-R	ACAGACCGAGAAGGCTTATG	Class III Integron	(Wen and Mi, 2010)
qnrA-F	ATTTCTCACGCCAGGATTTG	*qnrA*	(Mak JK, 2009)
qnrA-R	GATCGGCAAAGGTTAGGTCA	*qnrA*	(Mak JK, 2009)
qnrB-F	GATCGTGAAAGCCAGAAAGG	*qnrB*	(Mak JK, 2009)
qnrB-R	ACGATGCCTGGTAGTTGTCC	*qnrB*	(Mak JK, 2009)
qnrS-F	ACGACATTCGTCAACTGCAA	*qnrS*	(Mak JK, 2009)
qnrS-R	TAAATTGGCACCCTGTAGGC	*qnrS*	(Mak JK, 2009)
strA-F	ATGATGTCTAACAGCAAACTG	*strA*	(Mak JK, 2009)
strA-R	TCAACCCCAAGTAAGAGG	*strA*	(Mak JK, 2009)
strB-F	ATGGGGTTGATGTTCATGCCGC	*strB*	(Mak JK, 2009)
strB-R	CTAGTATGACGTCTGTCGCAC	*strB*	(Mak JK, 2009)
aphA1-F	ATGAGCCATATTCAACGGG	*aphA1*	(Mak JK, 2009)
aphA1-R	TCAGAAAAACTCATCGAGCATC	*aphA1*	(Mak JK, 2009)
gyrA-F	GCGATGTCGGTCATTGTTGG	*gyrA*	This study
gyrA-R	CCGAACTGGTCACGGATCAG	*gyrA*	This study
gyrB-F	CTCCGTCTCCGTACAGGATGAC	*gyrB*	This study
gyrB-R	TGTGATAGCGCAGTTTATCC	*gyrB*	This study

(v.r.3.3.40). Pearson's correlation coefficient and the un-weighted-pair group method were used to determine the genetic similarity of the tested isolates. Strains with similarities > 98% are indistinguishable and designated as one pattern, and strains with similarities between 95% and 98% are homologous and designated as one group (Tenover, 2009; Marchaim, 2011).

Results

In vitro antimicrobial susceptibility

All 38 MDR *K. pneumoniae* strains showed resistance to aminoglycosides, macrolides, quinolones and β-lactams in the semi-automated systems. All isolates yielded MIC values ≥ 128 mg/L for cefotaxime and ceftazidime; ≥ 8 mg/L for imipenem, ertapenem and meropenem; ≥ 128 mg/L for kanamycin and amikacin; and ≥ 16 mg/L for ciprofloxacin. All MDR isolates were susceptible to colistin (≤ 1 mg/L), tigecycline (≤ 1 mg/L) and fosfomycin (≤ 64 mg/L). Among the 38 MDR *K. pneumoniae* isolates, 89.5% (34/38) were confirmed as ESBL producers, while 36.8% (14/38) were AmpC producers, and 31.5% (12/38) produced both ESBL and AmpC β-lactamases. The susceptibility characteristics of the MDR *K. pneumoniae* are shown in Tables 2 and 3.

Detection and characterization of integrons

PCR was used to assess the presence of integrons in the MDR *K. pneumoniae* strains as previously described (Mak, 2009). Class I and II integrons were detected in

73.7% (28/38) and 26.3% (10/38) of the strains, respectively. No class III integrons were detected. Subsequent PCR amplification and sequencing revealed that all 28 class I integron-positive *K. pneumoniae* isolates contained *aacC1*, which confers a high level of gentamicin resistance, aacC4, which encodes amikacin and tobramycin resistance, *aadA1*, which confers spectinomycin and streptomycin resistance, and two unknown open reading frames, *orfX* and *orfX'*.

Aminoglycoside resistance

All 38 MDR isolates were resistant to streptomycin, while only 28 class I integron-positive isolates contained *aadA1*, *aacC1* and *aacC4*. Therefore, the other two streptomycin resistance genes, *strA* and *strB*, were screened through PCR. Eight class II integron-positive *K. pneumoniae* isolates containing the *strB* gene were detected, whereas no isolates containing the *strA* gene were observed. The control strain *K. pneumoniae* ATCC 700603 was completely sensitive to both spectinomycin and streptomycin; this strain also lacked *aadA1*, *strA* and *strB* genes.

Genetic basis of β-lactam resistance

The PCR analyses demonstrated that ESBL-producing MDR *K. pneumoniae* strains were rich in β-lactamase genes, such as bla_{SHV} (22/38), bla_{TEM} (10/38) and bla_{CTX-M} (7/38) (Table 3). Non-ESBL producers also harbored at least one β-lactamase gene. bla_{SHV-12} and $bla_{CTX-M-9}$ genes were highly prevalent, accounting for 42% and 13%, respectively, of class A β-lactamase genes, while

Table 2 - MICs for the 38 *K. pneumoniae* isolates, determined through agar dilution

Strain No.	MIC (mg/L)[a]														
	AMC	SAM	TZP	CAZ	CRO	CTT	FEP	ATM	ETP	IPM	MEM	AMK	TOB	CIP	LVX
K25, K32	>128	>256	128	128	>256	>256	32	>128	8	16	16	128	128	16	32
K7, K9, K22, K24, K1, K17, K19, K16, K15, K13, K31	>128	>256	>256	>128	128	>256	32	>128	16	32	16	128	>128	32	64
K20, K21, K14	128	128	256	>128	>256	128	128	>128	16	32	32	256	>128	16	64
K33, K18, K8	>128	>256	>256	>128	>256	>256	128	>128	>32	>32	>32	>256	64	32	32
K4, K36, K12, K6	>128	>256	>256	>128	>256	>256	64	>128	8	16	16	128	64	32	32
K5, K4, K34, K11	>128	>256	>256	>128	>256	>256	128	>128	16	32	32	>256	128	32	>64
K35, K23	>128	>256	>256	>128	>256	>256	256	>128	8	16	16	256	128	16	64
K10, K37	>128	>256	>256	>128	>256	>256	64	>128	32	>32	>32	>256	>128	>32	>64
K38, K2	>128	>256	>256	>128	>256	>256	128	>128	16	32	32	256	128	32	16
K28, K29	>128	>256	>256	>128	>256	>256	32	>128	16	>32	32	>256	>128	16	>64
K26, K27, K30	>128	>256	>256	>128	>256	>256	128	>128	8	16	16	>256	>128	32	32

AMC, amoxicillin/clavulanate; SAM, ampicillin/sulbactam; TZP, piperacillin/tazobactam; CAZ, ceftazidime; CRO, ceftriaxone; CTT, cefotetan; FEP, cefepime; AET, aztreonam; ETP, Ertapenem; MEM, meropenem; IPM, imipenem; AMK, amikacin; TOB, tobramycin; KAN, kanamycin; CIP, ciprofloxacin; LVX, levofloxacin.
[a]The MICs of the following antibiotics were identical for all 38 isolates: cefotaxime ≥ 128 mg/L; ampicillin ≥ 512 mg/L; cefazolin ≥ 128 mg/L; piperacillin ≥ 128 mg/L; cefuroxime ≥ 128 mg/L; gentamycin ≥ 128 mg/L; kanamycin ≥ 64 mg/L; chloramphenicol ≥ 32 mg/L; sulfafurazole ≥ 128 mg/L; norfloxacin ≥ 128 mg/L; nitrofurantoin ≥ 128 mg/L.

Table 3 - Resistance determinants detected through the PCR screening of 38 *K. pneumoniae* isolates

Strain No.	Resistance determinants in *K. pneumoniae*																
	ESBL	AmpC	Integron	bla_{SHV}	bla_{CTX-M}	bla_{TEM}	bla_{OXA}	bla_{DHA}	bla_{CIT}	bla_{VIM}	bla_{KPC}	gyrA mutation	qnrB	aacC1	aacC4	aadA1	strB
K25, K32	+	-	Class 2	-	+	-	-	-	+	-	+	+	-	-	-	-	+
K7, K9, K22, K24, K1, K17, K19, K16, K15, K13, K31, K34	+	-	Class 1	+	-	-	-	-	-	-	+	+	-	+	+	-	-
K20, K21, K14, K33, K18, K8, K4, K36, K12, K6	+	-	Class 1	-	-	+	-	-	-	-	+	-	+	+	+	+	-
K35, K23, K10, K5, K4, K11	+	+	Class 2	+	-	-	-	+	-	-	+	+	-	-	-	-	+
K38, K2	+	+	Class 2	+	-	-	-	+	-	-	+	+	+	-	-	-	-
K29	+	+	Class 1	-	-	-	+	-	-	+	-	-	+	+	+	+	-
K26, K27, K38, K28, K37	+	+	Class 1	-	+	-	-	+	-	-	+	+	-	+	+	+	-

+ and - indicate the presence and absence, respectively, of a particular resistance determinant.

$bla_{CTX-M-1}$ was not detected. The bla_{DHA} gene was detected in 11 ESBL-positive strains and 2 ESBL-negative strains, representing the most prevalent AmpC gene. Notably, the bla_{DHA} gene was detected in all *qnr*-positive strains and 5 *qnr*-negative strains (Table 2 and 3). PCR analysis revealed that 37 carbapenem-resistant isolates harbored KPC carbapenemase. Sequencing and alignment analyses revealed the presence of a highly conserved bla_{KPC-2} allele in KPC-positive isolates, and bla_{OXA-23} and bla_{VIM} were only detected in KPC-negative isolates. bla_{CMY}, bla_{ACC}, bla_{FOX}, bla_{PER}, bla_{VEB}, bla_{GES}, bla_{IMP} and bla_{SPM} were not detected in any of the clinical isolates examined.

Quinolone resistance is conferred through a *gyrA* site mutation or the *qnrB* gene

All 38 MDR isolates showed resistance to norfloxacin (MIC $\geq$ 256 mg/L) and ciprofloxacin (MIC $\geq$ 16 mg/L). To identify the quinolone resistance determinants, three subtypes of *qnr* genes (*qnrA*, *qnrB*, and *qnrS*) and two subtypes of *gyr* genes (*gyrA* and *gyrB*) were screened through PCR and sequencing analyses. Among the 38 MDR *K. pneumoniae* isolates, 13 isolates contained the plasmid-mediated quinolone resistance determinant *qnrB*, and 27 isolates contained the DNA gyrase gene *gyrA*, containing a nucleotide mutation in the quinolone-resistance determining region (QRDR). This mutation results in an amino acid change of S83L, which confers quinolone resistance in many bacteria. The gene *qnrB* was present in isolates that did not contain the *gyrA* mutation, and high rates of the *qnrB4* subtype were detected among *qnrB*-positive isolates (10/12). The plasmid-mediated *qnrB* gene was also detected among 27 *gyrA* mutation strains of the two isolates K38 and K2. The *qnrS* gene was not detected in any of the MDR *K. pneumoniae* strains. The control strain *K. pneumoniae* ATCC 700603, which did not carry the *gyrA* mutation or any *qnr* gene, was susceptible to quinolones. These findings suggest that the S83L *gyrA* site mutation conferred quinolone resistance to 27 of 38 isolates, and *qnrB* conferred quinolone resistance to the other 11 isolates. *qnrA*, *qnrS* and mutations in *gyrB* were not detected in any of the 38 MDR *K. pneumoniae* isolates.

REP-PCR and genetic diversity

To characterize the genetic diversity of the 38 MDR *K. pneumoniae* isolates with similar multidrug resistance profiles, strain typing based on REP-PCR was performed using genomic DNA. Five REP-PCR groups and sixteen patterns were observed (Figure 1). K7, K9, K22, K24, K1, K11, K17, K19, K16, K15, K13, and K31 showed high similarity (> 95%) in group II, designating the type 3 REP-PCR pattern. Moreover, the strains in group II pattern III, except for K19 (isolated from a bedrail) and K33 (isolated from bedclothes), were isolated from ICU patients. All group II strains, except K11 and K2, possessed class I integrons and *aphA*. Therefore, REP-PCR lacked the power to discriminate MDR *K. pneumoniae* strains, suggesting that a combination of genotypic data and REP-PCR types should be used to group the 38 isolates.

Discussion

The incidence of MDR *K. pneumoniae* infections has increased during the last decade, reflecting the extensive use of antimicrobial agents (Wu *et al.*, 2012). MDR *K. pneumoniae* is considered as a significant health problem because of limited options for antibiotic treatment. In this study, we conducted the phenotypic characterization and investigated the molecular mechanisms of antibiotic resistance for MDR *K. pneumoniae* strains isolated from ICUs. REP-PCR-based homology analyses provide information concerning the epidemic status of nosocomial infections caused by MDR *K. pneumoniae*.

In this study, 89.5% of the MDR *K. pneumoniae* strains isolated from ICU patients during a 12-month period were ESBL producers. This percentage was much higher than those reported in previous studies concerning other types of bacteria in China (Huang *et al.*, 2007; Wang *et al.*, 2012; Yong-Hong, 2012; Chen *et al.*, 2012). The ESBL-positive MDR *K. pneumoniae* strains possessed at least seven resistance determinants conferring resistance phenotypes to nine classes of antimicrobials. This finding was troubling, as most clinical therapeutic regimens are restricted to a maximum of 4 classes. No significant associations among ESBL-producing strains, antibiotic susceptibilities and integron carriage profiles were observed. However, isolates with similar resistance determinant profiles showed similar antibiotic resistance phenotypes. Notably, class I integrons were predominantly detected among ESBL-positive *K. pneumoniae* isolates (82.4%), consistent with the results of previous Asian studies. However, this percentage is slightly higher than those reported in Australian and American studies and much higher than those reported in European studies (Wu *et al.*, 2012; Rao *et al.*, 2006; Yao *et al.*, 2007; Machado *et al.*, 2007; Jiang *et al.*, 2012). According to the results obtained in the present study, 26.3% of the MDR *K. pneumoniae* isolates and 15.7% of the ESBL-producing isolates harbored class II integrons. Several studies have also reported the distribution of class II integrons among the *Enterobacteriaceae* family, including *Escherichia coli, Salmonella* and *Enterobacter cloacae*, and *Acinetobacter baumannii* in China (Zhou *et al.*, 2013; Gu *et al.*, 2006; Li *et al.*, 2008). The co-existence of class I and II integrons was not observed in any of the MDR *K. pneumoniae* strains. To our knowledge, there has only one report describing the co-existence of class I and II integrons among *Enterobacteriaceae* in China (Li *et al.*, 2008).

The prevalence and wide distribution of plasmid-mediated quinolone resistance determinants has been demonstrated among clinical isolates in China. In the present study, plasmid-mediated quinolone resistance determi-
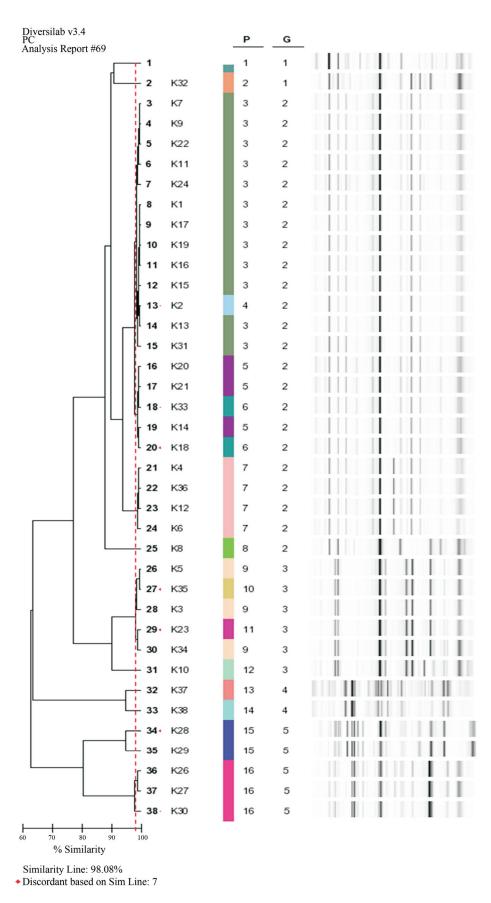
Figure 1 - Dendrograms and virtual gel images of the MDR *K. pneumoniae* isolates identified using web-based DiversiLab software.

nants (*qnr* genes) conferred quinolone resistance in 13 *K. pneumoniae* strains. Furthermore, at least one ESBL subtype (CTX-M, TEM and SHV) or AmpC β-lactamase (DHA) was present in *qnr*-positive *K. pneumoniae* isolates (Table 3). Previous studies have also shown that most ESBL genes are located in transposons and self-transferable plasmids, which typically co-exist with plasmid-mediated quinolone resistance determinants (Jiang *et al.*, 2012; Hassan *et al.*, 2012; Park *et al.*, 2012; Han *et al.*, 2010). This phenotype might reflect the excessive use of cephalosporins and quinolones and the absence of strict antimicrobial policies in medical care facilities. The most frequent ESBL genotype among the *qnr*-positive *K. pneumoniae* strains was SHV-12, a result that was not consistent with results showing that SHV-7 was more common in *qnr*-positive isolates (Jiang *et al.*, 2012).

Carbapenems have been considered as last resort treatments against infections caused by MDR Gram-negative organisms. However, *Klebsiella* has developed an efficient carbapenem resistance mechanism, known as KPC (*Klebsiella pneumoniae* carbapenemase) (Naas *et al.*, 2008). In China, KPC-producing *K. pneumoniae* was first identified in 2007 (Leavitt *et al.*, 2007; Cuzon *et al.*, 2008). Since then, the prevalence of KPC-producing *K. pneumoniae* has increased to epidemic proportions, particularly in large and public city hospitals. An increase in carbapenem-resistant strains has recently been reported in China (Cai *et al.*, 2008), likely reflecting the horizontal and vertical transmission of KPC-encoding genes, overcrowded environments and insufficient infection control measures in these hospitals. In the present study, sequencing and alignment revealed the presence of the bla_{KPC-2} allele in 37 MDR *K. pneumoniae* isolates. The bla_{KPC-2} genes amplified from these isolates were highly conserved, and no nucleotide differences were observed. However, the KPC-negative isolate was also OXA-23-positive. To our knowledge, few studies have revealed the contribution of bla_{OXA-23} to carbapenem resistance in other bacteria, with the exception of *Acinetobacter baumannii*. Because the bla_{OXA-23} gene identified in the KPC-negative *K. pneumoniae* isolate showed 100% identity to *Acinetobacter baumannii* bla_{OXA-23} (data not shown), we speculated that bla_{OXA-23} was transferred from *A. baumannii* through mobile genetic elements, which have been implicated in the dissemination of antimicrobial resistance through horizontal transmission. Previous studies have shown that integrons contribute to the prevalence of transferable extended-spectrum cephalosporin resistance through Enterobacteriaceae (Pisney *et al.*, 2013; Magiorakos *et al.*, 2013). Therefore, an in-depth investigation is required to study the mechanism of reduced susceptibility and to predict the clinical efficiency of carbapenem drugs.

The results of the present study demonstrated that the streptomycin resistance of class I integron-positive *K. pneumoniae* isolates was conferred through the *aadA1* gene cassette. However, the widespread streptomycin resistance gene *strB* was only detected in class II integron-positive isolates. So far, the genes *strA* and *strB* are considered as a gene pair, comprising a mobile genetic element of the conjugative antibiotic resistance plasmid, which confers high-level streptomycin resistance in bacteria (Chiou *et al.*, 1995; Tauch *et al.*, 2003). We did not detect the *strA* gene in any of the isolates examined; thus, it is likely that the *strB* gene is an element of class II integrons. Currently, we are examining the genetic elements of class II integrons and the mechanism of streptomycin resistance in two other class II integron-positive strains, which have been demonstrated as *strB*-negative.

To provide evidence for the prevention and control of nosocomial infections, the genotypic similarities of MDR K. pneumoniae strains were investigated using the REP-PCR-based DiversiLab system. Five different groups and sixteen patterns of MDR K. pneumoniae were isolated from ICU patients. The MDR K. pneumoniae isolated from the ventilators and bed rails shared genetic similarities with the strains isolated from patients. The homology between some strains isolated from ventilators and bedrails suggested that the equipment or object surfaces in the ICU had persistent MDR K. pneumoniae colonization. Although REP-PCR is a powerful tool for strain typing, with resolution beyond the species level, this technique lacked the power to discriminate group II strains, as all of the MDR strains in group II, except K11 and K2, possessed class I integrons and *aphA*. Furthermore, the K. pneumoniae strains in the same clone group could possess different resistance phenotypes and different drug resistance determinants. Therefore, REP-PCR fingerprinting should be combined with other detection methods to distinguish genetic groups beyond the species level.

In conclusion, MDR K. pneumoniae is becoming a serious problem in ICUs, with many strains developing resistance to most available antibiotics. The results of this study provide evidence for appropriate surveillance and outbreak investigations. We propose that infection control measures and strict antimicrobial stewardship policies should be applied to reduce the selective pressure that inevitably favors the emergence and epidemic of MDR strains and to increase the therapeutic usefulness of these antibiotics.

Acknowledgments

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Associate Editor: Roxane Maria Fontes Piazza