Characteristics of carbapenemase-producing *Klebsiella pneumoniae* as a cause of neonatal infection in Shandong, China

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Received May 8, 2015; Accepted September 1, 2016

DOI: 10.3892/etm.2017.4070

Abstract. The goal of the present study was to examine the characteristics of carbapenem-resistant *Klebsiella pneumoniae* as a cause of neonatal infection. A total of 37 carbapenem-resistant *Klebsiella pneumoniae*-positive newborns hospitalized in Shandong Provincial Hospital, China between April 2011 and October 2013 were examined. Antibiotic susceptibility testing was performed using the agar dilution method and the Etest. Resistance genes were assessed by polymerase chain reaction (PCR) and sequencing. Pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) were used to determine the genotypes and homology of these isolates. Plasmids were analyzed by PFGE and conjugation experiments. The outer membrane proteins were examined by PCR and SDS-PAGE. All of the isolates were revealed to be resistant to the third and fourth generation cephalosporins and piperacillin-tazobactam. Tigecycline, colistin, levofloxacin and amikacin were successful against all of the isolates. The antibiotic resistance rates of aztreonam, gentamicin, trimethoprim-sulfamethoxazole and fosfomycin were 13.51, 48.64, 78.38 and 86.49%, respectively. Of the 37 cases, 25 isolates (67.57%) were bla_{NDM-1} positive, 13 isolates (35.14%) were bla_{IMP-4} positive and 1 isolate (2.70%) was bla_{IMP-8} positive. Two isolates carried both bla_{NDM-1} and bla_{IMP-4}. The isolate carrying 2-4 plasmids and bla_{NDM-1} and bla_{IMP-4} was transferable between strains. SDS-PAGE data indicated that outer membrane proteins remained present. PFGE revealed 7 distinct clusters, and MLST reported the presence of ST20, ST17, ST54, ST705 and ST290 sequences, which indicated that there was clone and plasmid spread between newborns. The main resistance mechanism of carbapenem-resistant *Klebsiella pneumoniae* was that the isolates expressed the carbapenemase resistance of bla_{NDM-1} and bla_{IMP-4} genes. The current study indicates that early detection of these genes may be helpful in infection prevention and control.

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Key words: *Klebsiella pneumoniae*, carbapenem-resistance, infection of newborns, NDM-1, IMP-4

Introduction

Klebsiella pneumoniae is an important pathogen of community-acquired and nosocomial neonatal infections, with mortality rates varying between 18 and 68% (1,2). Carbapenem is the most effective drug used to treat infection with gram-negative bacteria, due to its marked antibacterial activity and stability in response to β-lactamases (3). However, the inappropriate use of carbapenem has accelerated the emergence of resistant strains in recent years (4). Infections associated with carbapenem-resistant *K. pneumoniae* are a public health problem globally due to their transmission mechanism and the limited therapeutic options available (5).

The most prevalent carbapenemase genes expressed by Enterobacteriaceae are in Ambler classes A and B, particularly bla_{KPC}, bla_{IMP} and bla_{NDM} (6). Steinmann *et al* (7) described a hospital outbreak caused by *K. pneumoniae* carbapenemase (KPC)-2-producing *K. pneumoniae* in Germany between July 2010 and January 2011 that resulted in the death of four people. Similarly, in March 2011, a New Delhi Metallo-β-lactamase (NDM)-1-producing *Escherichia coli* strain was isolated in Hong Kong, China (8).

The prevalence of carbapenem-resistant Enterobacteriaceae is increasing in mainland China, and has caused a number of nosocomial outbreaks. In 2012, Hu *et al* (9) reported an outbreak of 77 KPC-2-producing Enterobacteriaceae in Shanghai Huashan Hospital, China. Finally, in 2015, Chen *et al* (10) reported Enterobacteriaceae co-expressing bla_{NDM-1} and bla_{IMP-4} in China. The prevalence of carbapenem-resistant Enterobacteriaceae is a current challenge during treatment and infection control in hospitals; fortunately, neonatal cases are typically rare.

Table I. Primers used in the present study.

Primer	Forward (5'-3')	Reverse (5'-3')
KPC	ATTGGCTAAAGGGAAACACGACC	GTAGACGGCCAACACAAT
SME	AGATAGTAAATTTTATAG	CTCTAACGCTAATAG
IMIF	ATAGCCATCCTTGTTTAGCTC	TCTGCGATTACTTTATCCTC
NMC	GCATTGATATACCTTTAGCAGAGA	CGGTGATAAAATCACACTGAGCATA
GES	GTTTTGCAATGTGCTCAACG	TGCCATAGCAATAGGCGTAG
IMP-1	TGAGCAAGTTATCTGTATTC	TTAGTTGCTTGGTTTTGATG
VIM-1	TTATGGAGCAGCAACCGATGT	CAAAAGTCCCGCTCCAACGA
GIM-1	AGAACCTTGACCGAACGCAG	ACTCATGACTCCTCACGAGG
SIM-1	TACAAGGGATTCGGCATCG	TAATGGCCTGTTCCCATGTG
SPM-1	CCTACAATCTAACGGCGACC	TCGCCGTGTCCAGGTATAAC
NDM-1	ATTAGCCGCTGCATTGAT	GGCATGTCGAGATAGGAAGT
OXA	ACACAATACATATCAACTTCGC	AGTGTGTTTAGAATGGTGATC
CTX-M-cons	TTTGCGATGTGCAGTACCAGTAA	CGATATCGTTGGTGGTGCCATA
CTX-M-1	GGTTAAAAAATCACTGCGTC	TTACAAACCGTYGGTGACGA
CTX-M-2	ATGATGACTCAGAGCATTCGCCGC	TCAGAAACCGTGGGTTACGATTTT
CTX-M-9	GTGACAAAGAGAGTGCAACGG	ATGATTCTCGCCGCTGAAGCC
CTX-M-15	CACACGTGGAATTTAGGGACT	GCCGTCTAAGGCGATAAACA
TEM	ACATGGGGGATCATGTAACT	GACAGTTACAATGCTTACT
SHV	ATGCGTTATATTCGCCTGTG	AGCGTTGCCAGTGCTCGATG
MOX	GCTGCTCAAGGAGCACAGGAT	CACATTGACATAGGTGTGGTGC
FOX	AACATGGGGTATCAGGGAGATG	CAAAGCGCGTAACCGGATTGG
DHA	AACTTTCACAGGTGTGCTGGGT	CCGTACGCATACTGGCTTTGC
CTT	TGGCCAGAACTGACAGGCAAA	TTTCTCCTGAACGTGGCTGGC
EBC	TCGGTAAAGCCGATGTTGCGG	CTTCCACTGCGGCTGCCAGTT
OMPK35	ATGATGAAGCGCAATATTCTGGCAGTGG	TGGGCTTTGTCGCCATTGCCGTCA
OMPK36	ATGAAAGTTAAAGTACTGTCCCTC	GCCGGTATCTCTACCGACGAC

In the present study, patients who were carbapenem-resistant *Klebsiella pneumoniae*-positive in the neonatal, pediatric intensive care and cardiac intensive care units (ICUs) were enrolled, between April 2011 and October 2013. In order to better understand infection control, the antibiotic resistance of the strains, and homogeneity and transmission mechanisms of the resistance genes, were investigated.

Materials and methods

Collection of isolates and susceptibility testing. In the current study, 37 carbapenem-resistant *K. pneumoniae* samples were collected in Shandong Provincial Hospital, China, between April 2011 and October 2013. Of these, 31 isolates were from the neonatal unit, 5 were from the pediatric ICU and 1 was from the cardiac ICU. All isolates were identified using a VITEK-2 compact 60 system (bioMérieux, Marcy l'Etoile, France). It is of note that 21 of these *K. pneumoniae* isolates carried with NDM-1 have been described in a previous article (11). The novel isolates were Kpn1, 3-8, 12, 14-18, 21, 24 and 39. Antibiotic susceptibility testing was performed by the agar dilution method (12). All antibiotics, except tigecycline and colisin, were administered according to the approved standard of the Clinical and Laboratory Standards Institute 2014 guidelines (13). The minimum inhibitory concentrations (MICs) of meropenem, imipenem, ertapenem, tigecycline, colisin and fosfomycin were determined by an Etest (bioMérieux). The 2014 European Committee on Antimicrobial Susceptibility Testing breakpoint (www.eucast.org/clinical_breakpoint) was used to determine resistance in colistin and tigectcline. All isolates were screened for carbapenemase production using the modified Hodge test (MHT) and imipenem-ethylenediaminetetraacetic acid (EDTA) double-disc synergistic test (14). *E. coli* ATCC25922 (American Type Culture Collection Center, Manassas, VA, USA) acted as the control.

Polymerase chain reaction (PCR) and DNA sequence analysis of drug resistance genes. Samples were screened for the presence of carbapenem resistance genes (*bla*KPC, *bla*SME, *bla*IMI/*bla*NMC, *bla*GES, *bla*IMP, *bla*VIM, *bla*GIM, *bla*SIM-1, *bla*SPM, *bla*NDM-1 and *bla*OXA), common extended-spectrum β-lactamase (ESBL) genes (*bla*CTX-M, *bla*TEM and *bla*SHV) and AmpC β-lactamase (AMPC) genes (*bla*MOX, *bla*FOX, *bla*DHA, *bla*CIT and *bla*EBC) in all 37 strains using PCR and previously described primers (15-18). The primers used are listed in Table I. The genome used as a template was extracted using a Rapid Bacterial Genomic DNA Isolation Kit (Sangon Biotech Co., Ltd., Shanghai, Chin). PCR was performed according to the manufacturer's instruction of the PCR (Taq) kit (B532501-0100; Sangon Biotech Co., Ltd.). The reaction was performed using the following thermal cycling conditions: 1 Cycle at 94˚C for 5 min; 30 cycles at 94˚C for 1 min, 55-58˚C for 45 sec and 72˚C for 1 min; and 1 cycle at 72˚C for 10 min. The results of PCR were screened

Table II. Characteristics of 37 carbapenem-resistant *K. pneumoniae*.

Isolate no.	Patient age	Gender	Ward	Isolate date	Specimen	ST	PFGE	MHT	EDTA	Resistance genes			
										carbapenemase	ESBL	AMPC	
Kpn1	9 m	F	pICU	2011/4/3	sputum	705	F	-	+	IMP-4	TEM-1	CTX-M-14	-
Kpn3	2 m	F	pICU	2011/9/2	sputum	20	B	-	+	IMP-4	TEM-1	CTX-M-14	DHA-1
Kpn4	7 d	M	neonatal unit	2012/3/16	blood	290	A	-	+	IMP-8	TEM-1	CTX-M-15	-
Kpn5	7 d	F	neonatal unit	2012/5/17	sputum	54	E	-	+	NDM-1	TEM-1	CTX-M-14	DHA-1
Kpn6	1 m	F	neonatal unit	2012/6/5	sputum	54	E	-	+	NDM-1	TEM-1	CTX-M-14	DHA-1
Kpn7	17 d	M	neonatal unit	2012/7/27	sputum	54	E	-	+	NDM-1	TEM-1	CTX-M-14	DHA-1
Kpn8	1 m	M	neonatal unit	2012/7/26	sputum	54	E	-	+	NDM-1, IMP-4	TEM-1	CTX-M-14	DHA-1
Kpn9	2 m	M	neonatal unit	2012/8/1	sputum	20	B	-	+	NDM-1	TEM-1	CTX-M-15	DHA-1
Kpn10	21 d	M	neonatal unit	2012/8/23	sputum	20	B	-	+	NDM-1	TEM-1	CTX-M-15	DHA-1
Kpn11	1 d	M	neonatal unit	2012/8/29	sputum	20	B	±	+	NDM-1	TEM-1	CTX-M-15	DHA-1
Kpn12	5 d	M	pICU	2012/8/28	sputum	705	F	-	+	IMP-4	TEM-1	CTX-M-14	-
Kpn13	13 d	M	neonatal unit	2012/8/30	sputum	20	B	-	+	NDM-1	TEM-1	CTX-M-15	DHA-1
Kpn14	1 m	M	pICU	2012/8/30	sputum	705	F	-	+	IMP-4	TEM-1	CTX-M-14	-
Kpn15	14 d	F	neonatal unit	2012/10/4	sputum	54	E	-	+	IMP-4	TEM-1	CTX-M-14	DHA-1
Kpn16	4 d	F	neonatal unit	2012/10/16	sputum	54	E	-	+	IMP-4	TEM-1	CTX-M-14	DHA-1
Kpn17	23 d	M	neonatal unit	2012/11/5	umbilicus	54	E	-	+	IMP-4	TEM-1	CTX-M-15	-
Kpn18	11 d	F	neonatal unit	2012/11/9	sputum	54	E	-	+	IMP-4	TEM-1	CTX-M-15	DHA-1
Kpn19	2 m	M	neonatal unit	2013/1/1	sputum	20	B	-	+	NDM-1	TEM-1	CTX-M-15	DHA-1
Kpn20	22 d	F	neonatal unit	2013/1/29	sputum	20	B	-	+	NDM-1	TEM-1	CTX-M-15	DHA-1
Kpn21	9 d	M	neonatal unit	2013/1/30	sputum	54	D	-	+	IMP-4	TEM-1	CTX-M-15	-
Kpn23	1 m	M	neonatal unit	2013/2/25	sputum	20	B	-	+	NDM-1	TEM-1	CTX-M-15	DHA-1
Kpn24	12 d	M	neonatal unit	2013/2/27	umbilicus	54	D	-	+	IMP-4	TEM-1	CTX-M-15	DHA-1
Kpn25	2 m	M	neonatal unit	2013/3/5	sputum	20	G	±	+	NDM-1	-	CTX-M-15	DHA-1
Kpn26	1 m	M	neonatal unit	2013/3/6	sputum	20	B	-	+	NDM-1	TEM-1	CTX-M-15	DHA-1
Kpn27	25 d	F	neonatal unit	2013/3/14	sputum	20	B	-	+	NDM-1	TEM-1	CTX-M-15	DHA-1
Kpn28	1 m	M	neonatal unit	2013/3/19	sputum	20	B	-	+	NDM-1	-	CTX-M-15	DHA-1
Kpn29	1 m	M	neonatal unit	2013/4/19	sputum	20	B	-	+	NDM-1	TEM-1	CTX-M-15	DHA-1
Kpn30	13 d	M	neonatal unit	2013/4/23	umbilicus	54	D	+	+	NDM-1	-	CTX-M-15	DHA-1
Kpn31	5 d	F	neonatal unit	2013/5/2	sputum	20	B	-	+	NDM-1	TEM-1	CTX-M-15	DHA-1
Kpn32	9 d	F	neonatal unit	2013/7/13	sputum	20	B	-	+	NDM-1	TEM-1	CTX-M-15	DHA-1
Kpn33	1 m	M	neonatal unit	2013/7/21	sputum	20	B	+	+	NDM-1	TEM-1	CTX-M-15	DHA-1
Kpn34	2 m	M	pICU	2013/7/24	sputum	20	B	-	+	NDM-1	TEM-1	CTX-M-15	DHA-1

Table II. Continued.

Isolate no.	Patient age	Gender	Ward	Isolate date	Specimen	ST	PFGE	MHT	EDTA	Resistance genes		
										Carbapenemase	ESBL	AmpC
Kpn35	1 d	M	neonatal unit	2013/7/27	sputum	17	C	+	+	NDM-1	TEM-1	CTX-M-14	-
Kpn36	1 m	F	neonatal unit	2013/7/29	blood	20	B	±	+	NDM-1	TEM-1	CTX-M-15	DHA-1
Kpn37	2 d	F	neonatal unit	2013/8/19	sputum	17	C	-	+	NDM-1	TEM-1	CTX-M-14	-
Kpn38	1 y	F	cICU	2013/9/10	sputum	17	C	±	+	NDM-1, IMP-4	TEM-1	CTX-M-14	-
Kpn39	16 d	M	neonatal unit	2013/10/17	sputum	54	D	-	+	IMP-4	TEM-1	CTX-M-15	DHA-1

Kpn, *Klebsiella pneumonia*; d, days; m, months; y, years; M, male; F, female; pICU, pediatric intensive care unit; cICU, cardiac intensive care unit; ST, strain; PFGE, pulse-field gel electrophoresis analysis result; MHT, modified Hodge test; EDTA, EDTA synergistic test; NDM-1, New Delhi Metallo-β-lactamase-1; ESBL, extended-spectrum-β-lactamase.

by electrophoresis on a 1% agarose gel and the results were sequenced by Sangon Biotech Co., Ltd.

Resistance gene transfer experiments. Transfer experiments were performed using azide-resistant *E. coli* J53 (donated by Peking Union Medical College Hospital, Beijing, China) as the recipient strain. Overnight single colonies of donor and acceptor strains on MacConkey agar plates were inoculated into 5 ml nutrient broth containing imipenem (0.5 μg/ml) or sodium azide (200 μg/ml). These were incubated at 37˚C for 8 h. Cultures of the donor strain (10 μl) and recipient strain (20 μl) were mixed with 10 ml fresh Mueller-Hinton broth and incubated for 24 h at 35˚C. The mixture was then inoculated onto MacConkey agar plates containing sodium azide (200 μg/ml) and imipenem (0.5 μg/ml) for 24 h at 35˚C. Conjugation was confirmed with a VITEK-2 compact system for drug sensitivity testing. The presence of carbapenemase was confirmed by MHT, imipenem-EDTA double-disc synergistic test and PCR analysis.

Pulse-field gel electrophoresis (PFGE) and plasmid analysis. An overnight bacterial culture was suspended in cell suspension buffer [100 mM EDTA, 100 mM Tris-HCl (pH 8.0)] and adjusted to an optical density of 4.0 at a wavelength of 600 nm. The suspension was mixed with equal volumes of low-melting agarose in Tris-EDTA (TE) buffer in a 2% solution of low-melting-temperature agarose in TE buffer [1 mM EDTA, 10 mM Tris-HCl (pH 8.0)]. Following cooling, the agarose sections were incubated for 4 h at 54˚C in cell lysis buffer [50 mM Tris-HCl, 50 mM EDTA (pH 8.0), 0.01 g/ml N-lauroyl-sarcosine, sodium salt, 0.1 mg/ml proteinase K]. These were then washed thoroughly with TE buffer and digested overnight with *XbaI* restriction endonuclease (Takara Bio, Inc., Otsu, Japan). DNA separation was performed in 0.5X Tris/borate/EDTA (TBE) buffer in a PFGE system (CHEF Mapper; Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 14˚C, using a voltage of 6 V/cm, a switch angle of 120˚ and a switch ramp of 4-40 sec for 21 h. The *Salmonella enterica* serotype *Braenderup* H9812 (Peking Union Medical College Hospital) was used as a marker for PFGE. The restriction patterns were analyzed and interpreted in accordance with the protocol implemented by Tenover *et al* (19).

The plasmid number and size of the 37 isolates, and their transconjugants, were analyzed by S1-PFGE. In summary, the gels embedded with bacterial DNA were digested with proteinase K and Sl nuclease. DNA separation was performed in 0.5X TBE buffer in the above PFGE system at 14˚C with a voltage of 6 V/cm, a switch angle of 120˚ and a switch ramp of 2.16-63.8 sec for 20 h. *S. enterica* H9812 fragment, digested by *XbaI* enzyme, was used as a molecular weight standard. Subsequent to PFGE, the DNA products of the positive transconjugants were recovered and used as templates to amplify the carbapenem-resistance genes. The PCR reaction conditions and primers used are the same as described above. The PCR products were sequenced and searched on GenBank (www.ncbi.nlm.nih.gov/genbank).

Multilocus sequence typing (MLST). MLST was performed on *K. pneumoniae* using 7 conserved housekeeping genes

Table III. Antibiotic susceptibility of 37 carbapenem-resistant *K. pneumoniae* isolates.

	MICs (μg/ml)															
Isolate no.	IMP	MEM	ETP	CN	AK	TZP	CRO	CAZ	FEP	FOX	LEV	SXT	TGC	CO	FOS	ATM
Kpn1	6	4	32	≥16	≤2	8	≥64	≥64	32	>256	1	≥16	0.75	0.125	1024	≤1
Kpn3	2	0.8	3	≥16	≤2	64	≥64	≥64	32	>256	1	≤1	0.5	0.125	24	≥64
Kpn4	3	2	1	≥16	≤2	64	≥64	≥64	32	>256	1	≤1	0.75	0.125	64	≥64
Kpn5	>32	>32	3	≥16	≤2	64	≥64	≥64	16	>256	1	≤1	0.5	0.125	16	≥64
Kpn6	>32	>32	4	≥16	≤2	64	≥64	≥64	16	>256	0.5	≤1	0.5	0.125	16	≥64
Kpn7	>32	>32	>32	≥16	≤2	64	≥64	≥64	2	>256	0.5	≤1	0.75	0.125	24	4
Kpn8	0.5	0.75	3	≥16	≤2	64	≥64	≥64	16	>256	1	≤1	0.5	0.125	16	≥64
Kpn9	8	8	>32	0.5	2	>256	≥64	≥64	>64	>256	0.5	>32	0.5	0.125	32	>256
Kpn10	24	8	>32	0.5	4	>256	≥64	≥64	>64	>256	0.5	0.125	0.5	0.125	48	>256
Kpn11	>32	>32	>32	0.5	2	>256	≥64	≥64	>64	>256	1	0.125	0.5	0.125	48	>256
Kpn12	>32	>32	8	≥16	≤2	64	≥64	≥64	32	>256	1	≥16	1.5	0.125	1024	2
Kpn13	>32	>32	>32	1	4	>256	≥64	≥64	>64	>256	1	>256	0.5	0.125	>256	1
Kpn14	4	>32	>32	≥16	≤2	64	≥64	≥64	32	>256	1	≥16	1.5	0.125	1024	2
Kpn15	4	32	>32	≥16	≤2	128	≥64	≥64	32	>256	0.5	≤1	0.75	0.25	12	≥64
Kpn16	1	0.75	4	≥16	≤2	64	≥64	≥64	32	>256	0.5	≤1	0.5	0.125	8	≥64
Kpn17	2	32	32	≥16	≤2	64	≥64	≥64	>64	>256	≤0.2	≤1	0.75	0.125	12	≥64
Kpn18	32	32	32	≥16	≤2	64	≥64	≥64	>64	>256	≤0.2	≤1	0.5	0.125	16	≥64
Kpn19	>32	>32	>32	1	4	>256	≥64	≥64	>64	>256	0.5	0.25	0.5	0.125	16	>256
Kpn20	>32	>32	>32	1	2	>256	≥64	≥64	>64	>256	1	0.125	0.5	0.125	32	>256
Kpn21	0.5	1	4	≥16	≤2	64	≥64	≥64	32	>256	≤0.2	≤1	0.5	0.125	24	≥64
Kpn23	>32	>32	>32	0.5	2	>256	≥64	≥64	>64	>256	1	0.25	0.5	0.125	16	>256
Kpn24	>32	>32	>32	≥16	≤2	64	≥64	≥64	>64	>256	≤0.2	≤1	0.5	0.125	24	≥64
Kpn25	>32	>32	>32	0.5	2	>256	≥64	≥64	>64	>256	1	0.25	0.25	0.125	48	>256
Kpn26	32	32	32	0.5	4	>256	≥64	≥64	>64	>256	1	0.25	1	0.125	24	>256
Kpn27	>32	>32	>32	0.5	2	>256	≥64	≥64	>64	>256	1	0.19	0.5	0.032	48	>256
Kpn28	>32	>33	>32	0.5	2	>256	≥64	≥64	>64	>256	1	0.19	0.5	0.032	32	>256
Kpn29	>32	>32	>32	1	2	>256	≥64	≥64	>64	>256	1	0.125	0.5	0.125	48	>256
Kpn30	4	32	32	0.5	4	>256	≥64	≥64	>64	>256	1	0.125	1	0.125	32	>256
Kpn31	32	32	>32	0.5	2	>256	≥64	≥64	>64	>256	1	0.125	1	0.25	48	>256
Kpn32	>32	>32	>32	0.5	4	>256	≥64	≥64	>64	>256	1	0.125	0.5	0.25	128	>256
Kpn33	>32	>32	>32	0.5	4	>256	≥64	≥64	>64	>256	0.5	0.5	1	0.25	16	>256
Kpn34	>32	>32	>32	0.5	4	>256	≥64	≥64	>64	>256	0.5	0.5	0.5	0.25	32	>256

Table III. Continued.

	MICs (μg/ml)												
Isolate no.	IMP	MEM	ETP	CN	AK	TZP	CRO	CAZ	FEP	FOX	LEV	SXT	TGC	CO	FOS	ATM
Kpn35	>32	8	>32	32	2	>256	≥64	≥64	>64	>256	0.5	>32	1	0.25	16	>256
Kpn36	>32	>32	>32	0.5	4	>256	≥64	≥64	>64	>256	0.5	0.5	1	0.25	48	>256
Kpn37	>32	4	32	32	2	>256	≥64	≥64	>64	>256	1	>32	0.5	0.125	16	>256
Kpn38	>32	>32	>32	16	2	>256	≥64	≥64	>64	>256	0.5	>32	1	0.125	16	>256
Kpn39	>32	3	4	≥16	≤2	64	≥64	≥64	16	>256	≤0.2	≤1	0.5	0.125	32	≥64

MIC, Minimum inhibitory concentration; Kpn, *Klebsiella pneumonia*; IMP, imipenem; MEM, meropenem; ETP, ertapenem; CN, gentamicin; AK, amikacin; TZP, piperacillin-tazobactam; CRO, cefatriaxone; CAZ, ceftazidime; FEP, cefepime; FOX, cefoxitin; LEV: levofloxacin; SXT, trimethoprim-sulfamethoxazole; TGC, tigecycline; CO, colisin; FOS, fosfomycin; ATM, aztreonam.

(*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB* and *tonB*) according to protocols available on the MLST Pasteur website (bigsdb.pasteur.fr/klebsiella/primers_used.html).

Analysis of outer membrane proteins (OMPs). The OMP genes were screened in all clinical isolates with previously described primers, using PCR (20). The primers used are listed in Table I. The reaction used the following thermal cycling conditions: 1 Cycle at 94˚C for 5 min; 30 cycles at 94˚C for 45 sec, 55˚C for 45 sec and 72˚C for 1 min; and 1 cycle at 72˚C for 10 min. The results of PCR were screened by electrophoresis on a 1% agarose gel. The PCR negative isolates were investigated for alterations in the OMPs by SDS-PAGE, as previously described (21).

Results

Clinical and epidemiological characteristics of 37 isolates. Between April 2011 and October 2013, 37 carbapenem-resistant *K. pneumoniae* samples were isolated from neonatal inpatients in Shandong Provincial Hospital in Jinan, China. Of these, 32 isolates were from sputum specimens, 3 were from umbilical secretions and 2 were from blood (Table II). As indicated, 21 of these *K. pneumoniae* isolates carried with NDM-1 have been described in a previous article (11). Of these samples, 31 were isolated from patients in the neonatal department, 5 were from the pediatric ICU and 1 was from a patient in the cardiac ICU. These patients included 23 males and 14 females. The 31 patients from the neonatal ward were all premature, with a low birth weight and neonatal pneumonia. A total of 5 infant patients had an intrauterine infection at birth. All patients were initially treated with various antibiotics, including cloxacillin, cefuroxime, and cefotetan. A total of 8 patients were treated with imipenem or meropenem. The cardiac ICU patient had a respiratory tract infection and fever 5 days after their cardiac operation. The prognosis of this cohort was poor; 2 patients succumbed to bloodstream infection.

Susceptibility results. Drug sensitivity was tested with the standard agar dilution method (Table III). These features in 21 previously described *K. pneumoniae* isolates are included in this analysis (11). All 37 isolates were resistant to penicillin, piperacillin-tazobactam, cephalosporins and their composite agents including the enzyme inhibitors cefoxitin and carbapenem. The percentage of the number of bacteria sensitive to antibiotics divided by total strains, the sensitivity rate, to tigecycline, levofloxacin, amikacin and polymyxin were all 100%. The sensitivity rates of aztreonam, gentamicin, trimethoprim-sulfamethoxazole and fosfomycin were 13.51, 48.64, 78.38 and 86.49%, respectively.

The 37 *K. pneumoniae* samples displayed different degrees of resistance to carbapenems. Kpn4 was sensitive to ertapenem, with intermediate sensitivity to imipenem and meropenem. Of the 7 strains with mild sensitivity to ertapenem, 4 strains (Kpn16, 21, 3 and 8) were sensitive to imipenem and meropenem, 2 strains (Kpn5 and 6) were markedly resistant to imipenem and meropenem and Kpn39 was resistant to imipenem, with intermediate resistance to meropenem. The 19 isolates all had high resistance to the three carbapenem antibiotics; the MICs of the three carbapenems were ≥32 μg/ml.

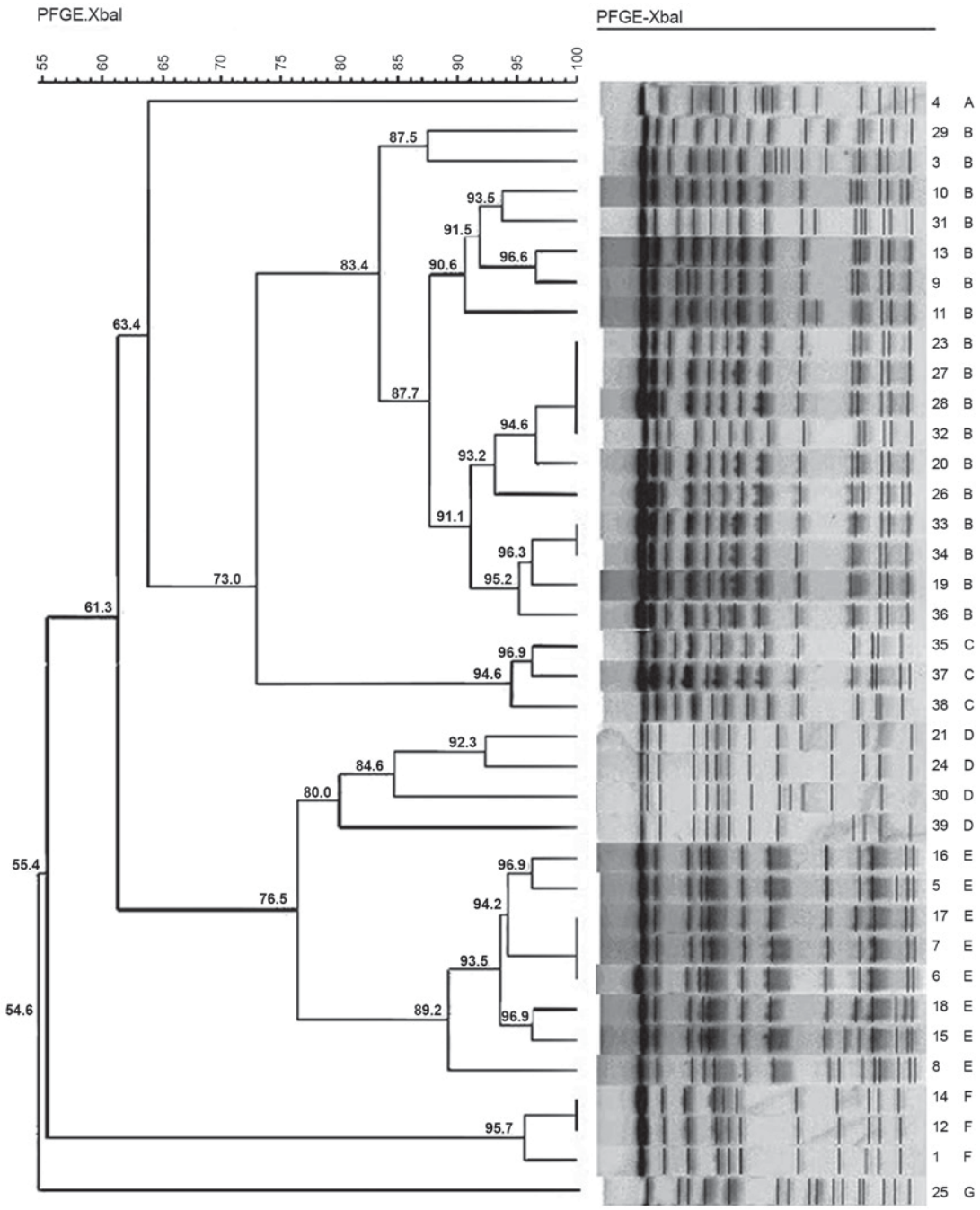

Figure 1. Dendrogram analysis. Dendrogram generated with the Fingerprinting II Informatix software package, demonstrating the relatedness of fingerprints (XbaI-PFGE) for 37 carbapenem-resistant *K. pneumoniae* strains. The phylogenetic tree was constructed using the Dice coefficient and UPGMA clustering. A genetic similarity index scale is shown in the left of the dendrogram. PFGE types and strain number are included along each PFGE lane. PFGE, pulse-field gel electrophoresis.

Resistance characterization and resistance genes. The 37 carbapenem-resistant *K. pneumoniae*, including those from our previous study, all showed positive results in the EDTA synergistic test, and 7 attained positive or weakly positive results in the MHT, at a rate of 18.92% (11). Metallo-β-lactamases (MBL) were confirmed to be present in all isolates through sequencing of the PCR products. Of these, 23 isolates expressed only $bla_{\text{NDM-1}}$, and 11 isolates expressed only $bla_{\text{IMP-4}}$. Kpn4, isolated from blood, expressed only $bla_{\text{IMP-8}}$, and 2 isolates simultaneously expressed $bla_{\text{NDM-1}}$ and $bla_{\text{IMP-4}}$ (11). In addition to MBL, other types of β-lactamase were examined, including ESBLs and AMPCs. The distribution of the resistance genes in these strains is reported in Table II. The ESBL genes were determined to be $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-14}}$ and $bla_{\text{TEM-1}}$ in 24 (64.87%), 13 (35.14%) and 34 (91.89%) isolates, respectively. Genotyping of the AMPC genes confirmed the presence of $bla_{\text{DHA-1}}$ in 28 (75.68%) isolates.

Characterization of OMPs. In our previous study, the outer membrane protein genes OmpK35 and OmpK36 were detected in 21 *K. pneumoniae* isolates expressing NDM-1 (Kpn9, Kpn10, Kpn11, Kpn13, Kpn19, Kpn20, Kpn23 and Kpn25-38) (11). The remaining 16 strains revealed no loss of OmpK35 and OmpK36 at the gene or the protein level.

Transfer of carbapenem resistance. A total of 28 transconjugants were obtained from 37 isolates by transfer experiments, with a success rate of 75.68%. Of these, 20 transconjugants were obtained from 23 isolates encoding bla_{NDM-1} and 8 transconjugants were obtained from 11 isolates encoding bla_{IMP-4} (11). The transfer experiment failed in 2 isolates simultaneously encoding bla_{NDM-1} and bla_{IMP-4}. All transconjugants were confirmed to have the same biochemical spectrum as *E. coli* strain J53, using the VITEK-2 compact system. All transconjugants demonstrated similar carbapenem susceptibility to donor strains Table IV. Compared with *E. coli* strain J53, the MIC of imipenem, meropenem and etarpenem increased 8- to 64-fold, 32- to 512-fold and 256- to 2,048-fold, respectively. The MIC of piperacillin tazobactam, ceftazidime, Cefatriaxone, cefoxitin and cefepime increased 8- to 2,048-fold.

Homology analyzed by PFGE and MLST. MLST revealed that the 37 isolates had 5 different sequence types: ST17, ST20, ST54, ST705 and ST290 (Table III) (11). The ST20 and ST54 sequences represented the majority of the clones. PFGE revealed 7 distinct clusters amongst the 37 isolates (Fig. 1). The 3 strains from the umbilical cord all had ST54 sequences, while two strains (Kpn4 and Kpn9) isolated from blood had ST54 and ST290 sequences. Cluster B included 17 isolates, all of which had ST20 sequences. The 12 isolates with ST54 sequences were divided into clusters D and E. A cluster was defined as strains with homology >80%.There were 5 strains isolated from the pediatric ICU, 3 of which had ST705 sequences and were allocated into cluster F. The Kpn35, Kpn37 and Kpn38 samples belonged to ST17 and the same cluster E in PFGE (11).

Plasmid profiling. The plasmid number and size of all isolates were analyzed by S1-PFGE, which revealed that these isolates contained 2-4 plasmids. As the primary sequences, ST20- and ST54-encoding isolates contained 2 or 3 plasmids, but their transconjugants only had 1 plasmid. The sizes of the plasmid from ST20- and ST54-encoding bacteria were 336 kb and 55 kb, respectively. The bla_{NDM-1} and bla_{IMP-4} were located in the 336 kb plasmid and 55 kb plasmid, respectively, as determined by PCR and sequencing.

Discussion

The standard method of treatment for serious infections caused by ESBL-producing Enterobacteriaceae is carbapenem antibiotics, but drug resistance is a critical problem (22). The present study demonstrated that carbapenem-resistant *K. pneumoniae* primarily manifested in infants within the neonatal unit. Premature birth, low birth weight, low immunity and intrauterine infections are the primary risk factors of nosocomial infection (23). The widespread inappropriate use of carbapenem and cephalosporins is an additional important risk factor (4).

In the current study, all patients were treated with cephalosporins within 2 weeks of admission, and 8 infants were prescribed carbapenem. The use of a ventilator may increase the risk of infection by carbapenem-resistant *K. pneumoniae*, however 10 patients used a ventilator in the present study. Antibiotic sensitivity testing revealed that carbapenem-resistant *K. pneumoniae* was resistant to most antibiotics. Only a few antibiotics, including aminoglycosides, fluoroquinolones, colistin, tigecycline and fosfomycin, were effective *in vitro*.

There is currently no effective drug available to treat carbapenem-resistant Enterobacteriaceae in neonates due to the limited range of drugs available. This it because some antibiotics, such as aminoglycosides, quinolones and tetracyclines, can not be used in pediatric patients because of their side effects. Therefore, infection by carbapenem-resistant Enterobacteriaceae has a very high mortality rate of ~70% in patients with bacteremia (24). In the current study, the prognosis of all neonates was poor; 2 children succumbed to bloodstream infections.

The outbreak of 21 *K. pneumoniae* isolates expressing NDM-1 were reported in a previous article (11). In addition, the present study described an outbreak of the same clone (ST705) was identified in the pediatric ICU. This strain was resistant to all beta-lactam antibiotics except for azteronam. The current results confirmed that this resistance was induced by the metalloenzyme bla_{IMP-4}, belonging to the Ambler class B of carbapenemases. This strain is able to hydrolyze penicillins, cephalosporins and carbapenems, but not aztreonam. In the neonatal ward, there were 2 strains of carbapenem-resistant *K. pneumoniae*: ST20 and ST54. The former carried bla_{NDM-1} and was responsible for an outbreak between August 2012 and July 2013. In comparing the housekeeping genes of ST20 and ST17, only the infB allele was reported to differ, with a base variant at 279 (T-C). This suggested that these 2 sequences are highly homologous. *K. pneumoniae* of the ST54 strain was identified with 5 isolates expressing only bla_{NDM-1}, 7 isolates expressing only bla_{IMP-4}, and Kpn8 expressing bla_{NDM-1} and bla_{IMP-4}. The ST54 strains expressing bla_{NDM-1} were present in samples between May 2012 and July 2012, and the ST54 strains expressing bla_{IMP-4} were prevalent in samples between 2011 and 2013. The outbreak described in the present study was due to the ST54 strain, expressing bla_{NDM-1}. Critically, 2 isolates expressed both bla_{NDM-1} and bla_{IMP-4}.

The production of carbapenemase was the primary mechanism by which Enterobacteriaceae bacteria attained resistance to carbapenem antibiotics. Carbapenemase-producing Enterobacteriaceae induce high mortality and are easily spread by acquiring carbapenemases from different species (25,26). In the present studied population, the most common genes expressed were bla_{NDM-1} and bla_{IMP-4}. Plasmid analysis proved that NDM-1 and IMP-4 were located in 336 kb and 55 kb plasmids, respectively. A transformation test revealed that these are easily transferred between different strains, and may cause spread between bacteria. Previous studies have reported that infection in neonates is caused by $bla_{CTX-M-15}$-expressing *K. pneumoniae* in the strains ST20 and ST17 in Spain and Canada (27,28). The outbreak of carbapenem-resistant *K. pneumoniae* in causing neonatal infection therefore requires additional study.

Previous studies have suggested that the overexpression of ESBLs and AMPC combined with missing outer membrane proteins have an important role in the drug resistance of *K. pneumoniae* (29,30). Currently, the main known outer membrane proteins in *K. pneumoniae* include OmpK37, OmpK35 and OmpK36. Of these, OmpK35 and OmpK36 have crucial roles in regulating drug penetration (11). While previous

Table IV. Results of antibiotic susceptibility testing of *E. coli* J53 transconjugant strains derived from blaNDM-producing *K. pneumonia* (μg/ml).

Transconjugant	IMP	MEM	ETP	CN	AK	TZP	CTX	CAZ	FEP	FOX	LEV	SXT	TGC	CO	FOS	ATM
kpn3-J53	2	1	2	≥16	≤2	32	≥64	≥64	32	256	1	≤1	0.5	0.125	24	32
kpn5-J53	16	16	4	≥16	≤2	16	≥64	≥64	8	64	1	≤1	0.5	0.125	16	≥64
kpn6-J53	8	8	2	≥16	≤2	16	≥64	≥64	16	128	0.5	≤1	0.5	0.125	16	32
kpn7-J53	16	32	32	≥16	≤2	8	≥64	≥64	2	64	0.5	≤1	0.75	0.125	24	4
kpn9-J53	2	2	>32	<1	<2	64	>256	>256	8	>256	<0.25	<0.5	0.25	0.5	2	32
kpn10-J53	2	2	>32	<1	<2	64	>256	>256	>256	>256	<0.25	<0.5	0.25	0.5	2	>256
kpn11-J53	2	4	4	<1	<2	64	>256	>256	8	>256	<0.25	<0.5	0.25	0.5	2	>256
kpn13-J53	>32	>32	>32	1	4	128	≥64	≥64	>64	>256	1	>256	0.5	0.125	>256	1
kpn15-J53	4	16	16	≥16	≤2	128	≥64	≥64	32	128	0.5	≤1	0.75	0.25	12	32
kpn16-J53	<1	<1	4	≥16	≤2	32	≥64	≥64	16	64	0.5	≤1	0.5	0.125	8	16
kpn17-J53	2	8	4	≥16	≤2	32	≥64	≥64	64	128	≤0.2	≤1	0.75	0.125	12	32
kpn18-J53	8	8	16	≥16	≤2	32	≥64	≥64	32	128	≤0.2	≤1	0.5	0.125	16	64
kpn19-J53	>32	>32	>32	<1	<2	>256	>256	>256	>256	>256	<0.25	<0.5	0.25	0.5	2	>256
kpn20-J53	2	2	16	<1	<2	>256	>256	>256	8	>256	<0.25	<0.5	0.25	0.5	2	>256
kpn21-J53	<1	<1	4	≥16	≤2	16	≥64	≥64	8	128	≤0.2	≤1	0.5	0.125	24	32
kpn23-J53	2	2	4	<1	<2	>256	>256	>256	>256	>256	<0.25	<0.5	0.25	0.5	2	>256
kpn24-J53	8	4	8	≥16	≤2	32	≥64	≥64	32	128	≤0.2	≤1	0.5	0.125	24	64
kpn25-J53	4	4	4	<1	<2	>256	>256	>256	>256	>256	<0.25	<0.5	0.25	0.5	2	>256
kpn26-J53	32	32	32	<1	<2	>256	>256	>256	>256	>256	<0.25	<0.5	0.25	0.5	2	>256
kpn27-J53	2	4	>32	<1	<2	>256	>256	>256	8	>256	<0.25	<0.5	0.25	0.5	2	>256
kpn28-J53	>32	>33	>33	<1	<2	>256	>256	>256	>256	>256	<0.25	<0.5	0.25	0.5	2	>256
kpn29-J53	>32	>32	>32	<1	<2	>256	>256	>256	>256	>256	<0.25	<0.5	0.25	0.5	2	>256
kpn30-J53	4	32	32	<1	<2	>256	>256	>256	>256	>256	<0.25	<0.5	0.25	0.5	2	1
kpn31-J53	32	32	>32	<1	<2	>256	>256	>256	>256	>256	<0.25	<0.5	0.25	0.5	2	>256
kpn32-J53	>32	>32	>32	<1	<2	>256	>256	>256	>256	>256	<0.25	<0.5	0.25	0.5	2	>256
kpn33-J53	>32	>32	>32	<1	<2	>256	>256	>256	>256	>256	<0.25	<0.5	0.25	0.5	2	>256
kpn36-J53	>32	8	>32	<1	<2	>256	>256	>256	>256	>256	<0.25	<0.5	0.25	0.5	2	>256
kpn39-J53	16	2	2	≥16	≤2	16	32	32	16	128	≤0.2	≤1	0.5	0.125	32	32
EC J53	<1	<1	<0.5	<1	<2	<0.5	<1	<1	<1	<1	<0.25	<0.5	0.25	0.5	2	<1

IMP, imipenem; MEM, meropenem; ETP, ertapenem; CN, gentamicin; AK, amikacin; TZP, piperacillin-tazobactam; CRO, cefatriaxone; CAZ, ceftazidime; FEP, cefepime; FOX, cefoxitin; LEV: levofloxacin; SXT, trimethoprim-sulfamethoxazole; TGC, tigecycline; CO, colisin; FOS, fosfomycin; ATM, aztreonam.

PCR amplification reported gene deletion of OmpK35 in kpn15 and kpn38, SDS-PAGE did not confirm the loss of OmpK35. This suggested that detection of the membrane protein required a combination of PCR and SDS-PAGE methods. Furthermore, it indicated that the resistance to carbapenems by *K. pneumoniae* was not associated with the outer membrane protein. Carbapenem-resistant *K. pneumoniae* expressed the carbapenemases gene, and ESBLs and AMPC, also permitting resistance to beta-lactam drugs, suggesting the presence of multi-drug resistance genes.

In conclusion, carbapenem-resistant *K. pneumoniae* causes neonatal infection, which may be due to simultaneously expressed multiple drug resistance genes. In the present study, bla_{NDM-1} and bla_{IMP-4} were the predominating resistance genes. Minimizing spread is a critical clinical challenge. carbapenem-resistant *Klebsiella pneumoniae* is a severe threat to the healthcare system, and calls for stringent standard infection control practices in healthcare settings, in addition to the reasonable use of antibiotics.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant no. 81401696) and the Shandong Province Science and Technology Development Program of China (grant no. 2012G0021844).

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