Diagnostic Microbiology and Infectious Disease 93 (2019) 355–361

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Antimicrobial Susceptibility Studies

Characterization of an NDM-19-producing *Klebsiella pneumoniae* strain harboring 2 resistance plasmids from China

Yao Liu [a,1], Hong Zhang [a,b,1], Xinzhuo Zhang [a], Nan Jiang [a], Zhikun Zhang [a], Jinping Zhang [a], Baoli Zhu [c], Guangxi Wang [a,*], Kelei Zhao [d,**], Yingshun Zhou [a,*]

[a] Department of Pathogenic Biology, School of Basic Medicine, Southwest Medical University, Luzhou 646000, Sichuan, China
[b] Nanchong Central Hospital of Sichuan, Nanchong 637000, Sichuan, China
[c] Key Laboratory of Pathogenic Microbiology & Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China
[d] Antibiotics Research and Re-evaluation Key Laboratory of Sichuan Province, Sichuan Industrial Institute of Antibiotics, Chengdu University, Chengdu 610052, Sichuan, China

ARTICLE INFO

Article history:
Received 21 July 2018
Received in revised form 6 November 2018
Accepted 13 November 2018
Available online 20 November 2018

Keywords:
CRKP
Plasmid
bla_{NDM-19}
WGS

ABSTRACT

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has become a major cause of nosocomial infections and posed challenges on clinical treatments. The main objective of this study was to determinate the genetic characteristics of the NDM-19–producing CRKP strain SCM96. From 2015 to 2017, 18 CRKP strains were recovered from sputum samples of patients in respiratory medicine in 6 hospitals from 5 provinces and cities in China. Polymerase chain reaction results for carbapenem resistance genes detection showed strain SCM96 carried bla_{NDM-19}. Three types of transconjugants harboring different plasmids were selected by conjugation experiment. The Whole Genome Sequencing (WGS) was performed using the PacBio RS platform. The genome size of SCM96 was 5,579,775 bp and composed of chromosomal DNA (5,398,745 bp) and 2 plasmids, IncFII type plasmid pSCM96-1 (134,869 bp) and IncX3 type plasmid pSCM96-2 (46,161 bp). SCM96 belonged to ST15 and K28. In addition to the 4 antibiotic resistance genes located in the chromosome, pSCM96-1 carried a complex resistance region containing 17 resistance genes and several mobile genetic elements (MGEs) like △Tn*6029*, In4-like integron, and Tn*3*, and pSCM96-2 had only 1 bla_{NDM-19} gene. As far as we know, this was the first description of bla_{NDM-19} in *K. pneumoniae*. Up to 22 antibiotic resistance genes, several important MGEs, and transferable plasmids might increase the possibility of co-spreading of bla_{NDM-19} with other resistance genes.

1. Introduction

It is reported that the prevalence of carbapenem resistance in bacterial pathogens has become a serious problem which enormously threatens human health worldwide (Fu et al., 2018; Lemos et al., 2014; Zhang et al., 2017). Especially, the New Delhi metallo-β-lactamase (NDM)–mediated carbapenem resistance has been identified in *Enterobacteriaceae* species from many countries (Di et al., 2017; Rahman et al., 2014), and various mobilizable genetic elements are found to play an important role in the spreading of bla_{NDM} (Datta et al., 2017). As of June 2018, a total of 22 variants of bla_{NDM-1} have been deposited in the NCBI database, including the recently identified bla_{NDM-19} (GenBank accession no. MF370080.1), bla_{NDM-20} (GenBank accession no. KY654092.1), bla_{NDM-21} (GneBank accession no. MG183694), and bla_{NDM-22} (GneBank accession no. MH24335).

K. pneumoniae is a common opportunistic pathogen that normally causes a wide range of lethal diseases such as pneumonia, urinary tract infections, and other community-acquired infections in nosocomial patients (Holt et al., 2015). The emergence of multiple-drug–resistant *K. pneumoniae* has been identified as an urgent threat to human health according to the US Centers for Disease Control and Prevention (Holt et al., 2015), and the carbapenem-resistant *K. pneumoniae* has been recognized as critical (Priority 1) antibiotic-resistant bacteria by the World Health Organization (http://www.who.int/medicines/publications/global-priority-list-antibiotic-resistant-bacteria/en/). Here, as far as we know, is the first description about the genetic characterization of a new metallo-β-lactamases (MBLs)–producing *K. pneumoniae* strain which harbors 2 different plasmids and carries a total of 22 resistance genes including bla_{NDM-19}.

2. Materials and methods

2.1. Strains, antimicrobial susceptibility testing, and resistance genes detection

From 2015 to 2017, a total of 18 carbapenem-resistant *K. pneumoniae* strains were collected from sputum samples of patients in respiratory medicine in 6 hospitals in Sichuan, Henan, Hunan, and Fujian provinces

* Corresponding authors. Tel.: +86-0830-3160073.
** Corresponding author. Tel.: +86-028-84216035.
E-mail addresses: guangxiwang@swmu.edu.cn (G. Wang), zkl5228@163.com (K. Zhao), yingshunzhou@swmu.edu.cn (Y. Zhou).
[1] Yao Liu and Hong Zhang contributed equally to this work.

https://doi.org/10.1016/j.diagmicrobio.2018.11.007
0732-8893/

and the city of Shanghai in China. Routine species identification and antimicrobial susceptibility testing were carried out by the Vitek2 system (BioMérieux, France). The minimal inhibitory concentrations (MICs) were determined by broth microdilution method according to the recommendations of the Clinical and Laboratory Standards Institute (Institute, 2018). *Escherichia coli* strain ATCC 25922 was used as quality control. Polymerase chain reaction (PCR) amplification and DNA sequencing were performed to identify the key carbapenemase encoding genes (bla_{NDM}, bla_{KPC}, bla_{VIM}, bla_{IMP}, and bla_{OXA-48}) and the key extended-spectrum beta-lactamase (ESBLs) gene (bla_{CTX-M}, bla_{SHV}, and bla_{TEM}) as previously described (Poirel et al., 2011; Tian et al., 2009).

2.2. Conjugation experiment

SCM96 was cultured in Mueller–Hinton (MH) broth as the donor, and azide-resistant *E. coli* strain J53 was used as the recipient. Transconjugants were selected on MacConkey agar containing 2 mg/L of ceftazidime and 100 mg/L of sodium azide or containing 1 mg/L of imipenem and 100 mg/L of sodium azide. PCR amplification was performed to confirm the transconjugants thorough detecting the antibiotics resistance genes.

2.3. WGS and analysis

The total genomic DNA of SCM96 was extracted by using DNA extraction kit (QIAGEN, Shanghai, Co Ltd). Fragment libraries were constructed using library prep kit (SMRTbell™ Template Prep Kit 1.0-SPv3) and sequenced using the Illumina Miseq and Pac Bio RS platforms (Majorbio Co., Ltd. Shanghai, China). The clean reads were assembled using SOAP*denovo* software package (Luo et al., 2012). Prediction and annotation of the open reading frames (ORFs) were carried out using Glimmer (Delcher et al., 2007). Multilocus sequence typing (MLST) was determined by the online software (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). Capsular serotyping based on the nucleotide sequence of *wzc* gene was performed using the BIGSdb-Kp database (http://bigsdb.web.pasteur.fr). The resistome of SCM96 was identified using ResFinder 3.0 (https://cge.cbs.dtu.dk/services/ResFinder/). Plasmid classification was performed using PlasmidFinder 1.3 (https://cge.cbs.dtu.dk/services/PlasmidFinder/) and BIGSdb (http://pubmlst.org/plasmid/) (Jolley and Maiden, 2010). Pairwise alignment was performed by BLASTN and BLASTP homology search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Zhang et al., 2017). The phylogenetic relationship between amino acid sequences of NDM variants was aligned with ClustalW method, and the phylogenetic tree was constructed by using the MEGA7 program (Kumar et al., 2016).

3. Results

3.1. Resistance phenotypes and distribution of antimicrobial resistance genes

All the 18 identified *K. pneumoniae* strains in the current study were resistant to imipenem, meropenem, and broad-spectrum cephalosporins. The MICs of SCM96 strain were listed in Table 2. Notably, all the strains carried 1 carbapenemase gene; 14 strains were found to carry the bla_{KPC-2} gene, 3 carry the bla_{NDM-1}, and 1 carries the bla_{NDM-19} which was named SCM96. More details were listed in Table 1.

3.2. Selection of 3 types of transconjugants

Three types of transconjugants were obtained. And then they were all confirmed by PCR amplification for resistance genes that exist in strain SCM96 such as bla_{NDM-19} and $bla_{CTX-M-3}$. One only carrying the bla_{NDM-19} (J53-SCM96-NDM) was selected on plates containing 1 mg/L of imipenem and 100 mg/L of sodium azide; meanwhile, one carrying the $bla_{CTX-M-3}$ but no bla_{NDM-19} (J53-SCM96-CTX-M) and one co-carrying the bla_{NDM-19} and $bla_{CTX-M-3}$ (J53-SCM96-NDM-CTX-M) were screened from the plates containing 2 mg/L of ceftazidime and 100 mg/L of sodium azide. By the way, the MICs of J53 and transconjugants were listed in Table 2.

3.3. WGS, MLST, serotype and resistance genes distribution of SCM96

The result of sequence assembly showed that the genome of *K. pneumoniae* SCM96 consisted of a circular chromosome (GenBank accession no. CP028716) and 2 plasmids: pSCM96-1 (GenBank accession no. CP028717) and pSCM96-2 (GenBank accession no. CP028718). Strain SCM96 was classified as sequence type ST15 and serotype K28. The result of resistome analysis of SCM96 confirmed that there were 4 drug-resistance genes (bla_{SHV-28}, *oqxA, oqxB*, and *fosA*) in the chromosomal DNA, 17 resistance genes (aminoglycosides resistance genes, *strA*, *strB*, *aphA1a*, *aac(3)-IId*, *aac(6')Ib-cr*, *aadA16*; β-Lactams resistance genes, bla_{TEM-1}, $bla_{CTX-M-3}$; fluoroquinolone resistance genes, *qnrS1*; rifampicin resistance genes, *arr-3*; tetracycline resistance genes *tet(A)*; phenicol, *floR*; sulphonamides, *qacEΔ1*, *sul1*, *sul2*; macrolides, *mph(A)*; trimethoprim, *dfrA27*) in the plasmid pSCM96-1, and only 1 resistant gene (bla_{NDM-19}) in the plasmid pSCM96-2 (Table 3).

3.4. Two plasmids' analysis

The plasmid pSCM96-1 was 134,869 bp in length and contained 174 annotated ORFs, and the average GC content was 51.58%. The plasmid pSCM96-1 belonged to the IncFII incompatibility plasmid type as determined by the software PlasmidFinder 1.3 (Carattoli et al., 2014). The backbone composition of pSCM96-1 was the most similar to that of the plasmid pK1HV (GenBank accession no. HF545434) with 99% nucleotide identity and 81% sequence coverage (Fig. 1a). Compared to pK1HV, a complex resistance region (44,920 bp) containing 17 resistance genes was identified in pSCM96-1. This region showed no significant relation with the 49-kb unique region of pK1HV referred to by Ying et al. (2015). However, its origin might relate to plasmid pKF3-94 (GenBank accession no. NC_013950.1). pFK3-94 and pSCM96-1 carried 2 replicons: *repA* (858 bp, encoding RepA) and *repA2* (252 bp, encoding the replication regulatory protein RepA2), with FIIK2 coexisting in all of them. And a complete *phd1* gene (561 bp, encoding phospholipase D) is downstream of *repA2* in pFK3-94, while the *phd1* was incomplete (480 bp, designated *△pld1*) in pSCM96-1 (Fig. 1b). Sequence analysis showed about 28 kb sequence encoding some resistance genes, transposase genes, and integrase gene inserted between *△pld1* and Tn*3*-*tnpA* in pSCM96-1 and replaced the location between *pld1* and Tn*3*-*tnpA* in pFK3-94 (Fig. 1b). In this region, a few of the mobile genetic elements (MGEs) were noticed. Six copies of intact or truncated IS*26* were located at different sites, and a complete IS*Ecp1* (1656 bp) was divided into 2 parts (403 bp and 1091 bp) by an intact IS*1*. Additionally, an IS*Kpn19* was truncated by a recombinase site (data not shown) and composed an IS*Kpn19*-*qnrS1* composite transposon with the other intact IS*Kpn19*. Moreover, a Tn*6029*-like element (designed △Tn*6029*) which lost IS*26*-△Tn*2* fragment was found. Also, an In4-like integron from class 1 integron, which contained a variable region [VR; *aac(6′)-Ib-cr5, arr-3*, *dfrA-27*, *aadA16*], a 5′-conserved sequence (5′-CS) possessing *intI* gene, and a 3′-conserved sequence (3′-CS) possessing the *qacEΔ1* and *sul1* genes, was also identified in the resistance region of pSCM96-1. Further analysis revealed a fragment including a partial copy of IS*6100* and IRt flanking a complete IS*6100* was missing in pSCM96-1. A Tn*3* transposon element bearing bla_{TEM-1B} and $bla_{CTX-M-3}$ genes with linear structure of Tn*3*-bla_{TEM-1B}-$bla_{CTX-M-3}$-ΔIS*Ecp1*-IS*1*-ΔIS*Ecp1*, which mediated the resistance of SCM96 to cephalosporins and penicillins, was located downstream of the In4.

In addition, the second plasmid pSCM96-2 was 46,161 bp in length and had 60 annotated ORFs with an average GC content of 46.65%. The result of plasmid typing showed that pSCM96-2 belonged to the IncX3 incompatibility plasmid type. Subsequent nucleotide sequence analysis revealed that the resistance gene bla_{NDM-19} was flanked in the upstream region by IS*3000*-*tnpA*-ΔIS*Aba125*-*tnpA*-IS*5*-*tnpA* and downstream by

Table 1
Resistance phenotype and distribution of carbapenemase and ESBLs encoding genes.

Strain	Samples	Underlying disease	Resistance phenotype	Carbapenemase and AmpC encoding genes
SCM1	Sputum	Diffuse alveolar damage	IMP/MEP/CAZ/CTX/FEP/TZP/CRO/CIP/SMZ-TMP/GM/AMK	bla_{KPC-2}, $bla_{CTX-M-65}$, bla_{SHV11}, bla_{TEM-1}
SCM2	Sputum	Pulmonary contusion	IMP/MEP/CAZ/CTX/FEP/TZP/CRO/CIP/SMZ-TMP/CIP/GM/AMK	bla_{KPC-2}, bla_{SHV12}, $bla_{CTX-M-65}$
SCM7	Sputum	Chronic obstructive pulmonary disease	IMP/MEP/CAZ/CTX/FEP/TZP/CRO/CIP/SMZ-TMP/CIP/GM/AMK	bla_{KPC-2}, $bla_{CTX-M-28}$, bla_{SHV12}
SCM9	Sputum	Lung cancer	IMP/MEP/CAZ/CTX/FEP/TZP/CRO/CIP/SMZ-TMP/CIP/GM/AMK	bla_{NDM-1}, bla_{SHV12}, $bla_{CTX-M-3}$
SCM17	Sputum	Chronic obstructive pulmonary disease	IMP/MEP/CAZ/CTX/FEP/TZP/CRO/CIP/SMZ-TMP	bla_{KPC-2}, $bla_{CTX-M-28}$, $bla_{SHV12,}$
SCM32	Sputum	Diabetics	IMP/MEP/CAZ/CTX/FEP/TZP/CRO/CIP/SMZ-TMP	bla_{NDM-1}, bla_{SHV12}, $bla_{CTX-M-3}$
SCM45	Sputum	Chronic obstructive pulmonary disease	IMP/MEP/CAZ/CTX/FEP/TZP/CRO/CIP/SMZ-TMP/CIP/AMK	bla_{KPC-2}, $bla_{CTX-M-14}$, bla_{TEM-1}
SCM78	Sputum	Multiple injuries	IMP/MEP/CAZ/CTX/FEP/TZP/CRO/CIP/SMZ-TMP/AMK	bla_{KPC-2}, $bla_{CTX-M-14}$, bla_{TEM-1}
SCM79	Sputum	Diffuse alveolar damage	IMP/MEP/CAZ/CTX/FEP/TZP/CRO/CIP/SMZ-TMP/AMK	bla_{KPC-2}, $bla_{CTX-M-14}$, bla_{TEM-1}
SCM80	Sputum	Diffuse alveolar damage	IMP/MEP/CAZ/CTX/FEP/TZP/CRO/CIP/SMZ-TMP	bla_{KPC-2}, $bla_{CTX-M-14}$, bla_{TEM-1}
SCM81	Sputum	Pulmonary infection	IMP/MEP/CAZ/CTX/FEP/TZP/CRO/CIP/SMZ-TMP/CIP	bla_{KPC-2}, $bla_{CTX-M-14}$, bla_{TEM-1}
SCM83	Sputum	Pulmonary infection	IMP/MEP/CAZ/CTX/FEP/TZP/CRO/CIP/SMZ-TMP/AMK/GM	bla_{NDM-1}, bla_{SHV12}, bla_{TEM-1}, $bla_{CTX-M-15}$
SCM85	Sputum	Pulmonary infection	IMP/MEP/CAZ/CTX/FEP/TZP/CRO/CIP/SMZ-TMP	bla_{KPC-2}, $bla_{CTX-M-28}$, bla_{SHV12}, bla_{TEM-1}
SCM88	Sputum	Pulmonary infection	IMP/MEP/CAZ/CTX/FEP/TZP/CRO/CIP/SMZ-TMP	bla_{KPC-2}, $bla_{CTX-M-22}$, bla_{SHV11}, bla_{TEM-1}
SCM89	Sputum	Pulmonary infection	IMP/MEP/CAZ/CTX/FEP/TZP/CRO/CIP/SMZ-TMP/ AMK/GM	bla_{KPC-2}, $bla_{CTX-M-15}$, bla_{SHV11}, bla_{TEM-1}
SCM91	Sputum	Pulmonary infection	IMP/MEP/CAZ/CTX/FEP/TZP/CRO/CIP/SMZ-TMP	bla_{KPC-2}, $bla_{CTX-M-15}$
SCM96	Sputum	Chronic obstructive pulmonary disease	IMP/MEP/CAZ/CTX/FEP/TZP/CRO/CIP/SMZ-TMP	bla_{NDM-19}, bla_{SHV12}, $bla_{CTX-M-3}$
SCM103	Sputum	Chronic obstructive pulmonary disease	IMP/MEP/CAZ/CTX/FEP/TZP/CRO/CIP/SMZ-TMP	bla_{KPC-2}, $bla_{CTX-M-15}$

Abbreviations: IMP = imipenem; MEP = meropenem; CAZ = ceftazidime; CTX = cefotaxime; FEP = cefepime; TZP = piperacillin–tazobactam; CRO = ceftriaxone; CIP: ciprofloxacin; SMZ-TMP = trimethoprim/sulfamethoxazole; GM = gentamicin; AMK = amikacin.

ble_{MBL}-*trpF*-*dsbC*-IS*26*-*umuD*. The genetic structure of this plasmid was almost equal to other bla_{NDM} bearing IncX3 plasmids, such as pEC50-NDM7 (bla_{NDM-7}; GenBank accession no. KX470735), pEc1929 (bla_{NDM-5}; GenBank accession no. KT824791), and pNDM-20 (bla_{NDM-20}, GenBank accession no. MF458176) (Fig. 2a). The only difference is the bla_{NDM} variants have some point mutations (Fig. 2b). The result of amino acid sequences based homologous and phylogenetic analysis base on the 22 registered bla_{NDM} variants showed that bla_{NDM-19} shared the closest evolutionary relationship with bla_{NDM-7} with only 1 mutation at site A233V (Fig. 2b).

4. Discussion

Carbapenem-resistant *K. pneumoniae* is an enteric bacterium that has been responsible for an increasing number of deadly outbreaks and hospital acquired infections and become a serious public health threat (Cprek and Gallagher, 2016; Diago-Navarro et al., 2018; Nordmann et al., 2011). Since bla_{NDM-1} has been first reported in India, NDM-positive isolates emerged and disseminated all over the world (Johnson and Woodford, 2013; Rahman et al., 2014; Yong et al., 2009). While *K. pneumoniae* strains producing bla_{NDM} have already achieved international spread, a characteristic of NDM-producing *K. pneumoniae* has so far been their rapid dissemination (Tzouvelekis et al., 2012). In this study, 18 carbapenem-resistant *K. pneumoniae* isolates were selected to detect drug-resistant phenotype and -resistant genotype. We found the antimicrobial resistance gene bla_{KPC} identified more frequently than other carbapenem-resistant genes (bla_{MBLs} and bla_{OXA}) in the 18 CRKP isolates, which was consistent with some studies in other areas (Del Franco et al., 2015; Rimoldi et al., 2017; Vanegas et al., 2016). More importantly, 1 bla_{NDM-19} gene was found in *K. pneumoniae strian* SCM96 and was also the first time identified in a

Table 2
MICs of *K. pneumoniae* (SCM96), transconjugants (J53-SCM96-NDM, J53-SCM96-CTX-M, J53-SCM96-NDM-CTX-M), and recipient strain (J53).

	SCM96	J53-SCM96-NDM	J53-SCM96-CTX-M	J53-SCM96-NDM-CTX-M	J53
Imipenem	>128	>128	1	>128	1
Meropenem	>128	>128	<0.25	>128	<0.25
Ceftazidime	>512	>512	32	>512	4
Cefotaxime	>128	>128	64	>128	1
Cefoxitin	>256	>256	>256	>256	4
Aztreonam	>256	0.25	>256	>256	0.125
Ampicillin	>256	>256	>256	>256	2
Ciprofloxacin	>128	32	4	64	1
Gentamicin	>512	8	256	128	4

Table 3
Antibiotic resistance genes of *Klebsiella pneumoniae* SCM96.

Antibiotic class	Resistance gene	Genetic context	Position	%identity[a]	Accession no.
Aminoglycosides	*strA*	pSCM96-1	101959-102762	100.00	AF321551
	strB	pSCM96-1	102762-103598	100.00	M28829
	aphA1a	pSCM96-1	104514-105329	100.00	KY270849
	aac(3)-IId	pSCM96-1	106456-107316	100.00	MG299130
	aac(6')-Ib-cr	pSCM96-1	110538-111137	100.00	DQ303918
	aadA16	pSCM96-1	112473-113318	100.00	CP025966
β-Lactams	bla_{TEM-1}	pSCM96-1	129833-130693	100.00	JF910132
	bla_{NDM-19}	pSCM96-2	41427-42239	100.00	NG_055498
	$bla_{CTX-M-3}$	pSCM96-1	131475-132350	100.00	EF437434
	bla_{SHV-28}	chromosome	3196079-3196939	100.00	HM751101
Fluoroquinolone	*qnrS1*	pSCM96-1	1446-2102	100.00	AB187515
	oqxA	chromosome	4748214-4749389	99.23	EU370913
	oqxB	chromosome	4749413-4752565	98.86	EU370913
Rifampicin	*arr-3*	pSCM96-1	111234-111686	100.00	JF806499
Tetracycline	*tet(A)*	pSCM96-1	121889-123163	100.00	AF534183
Phenicol	*floR*	pSCM96-1	123764-124977	100.00	MG860488
Sulphonamides	*sul1*	pSCM96-1	113689-114615	100.00	CP002151
	qacEΔ1	pSCM96-1	113435-113782	100.00	CP000891
	sul2	pSCM96-1	101083-101898	100.00	HQ840942
Macrolides	*mph(A)*	pSCM96-1	118826-119731	100.00	D16251
Trimethoprim	*dfrA27*	pSCM96-1	111819-112292	100.00	FJ459817
Fosfomycin	*fosA*	chromosome	1274841-1275252	100.00	ACWO01000079

[a] Percent nucleotide identity and corresponding GenBank accession number for reference sequence.

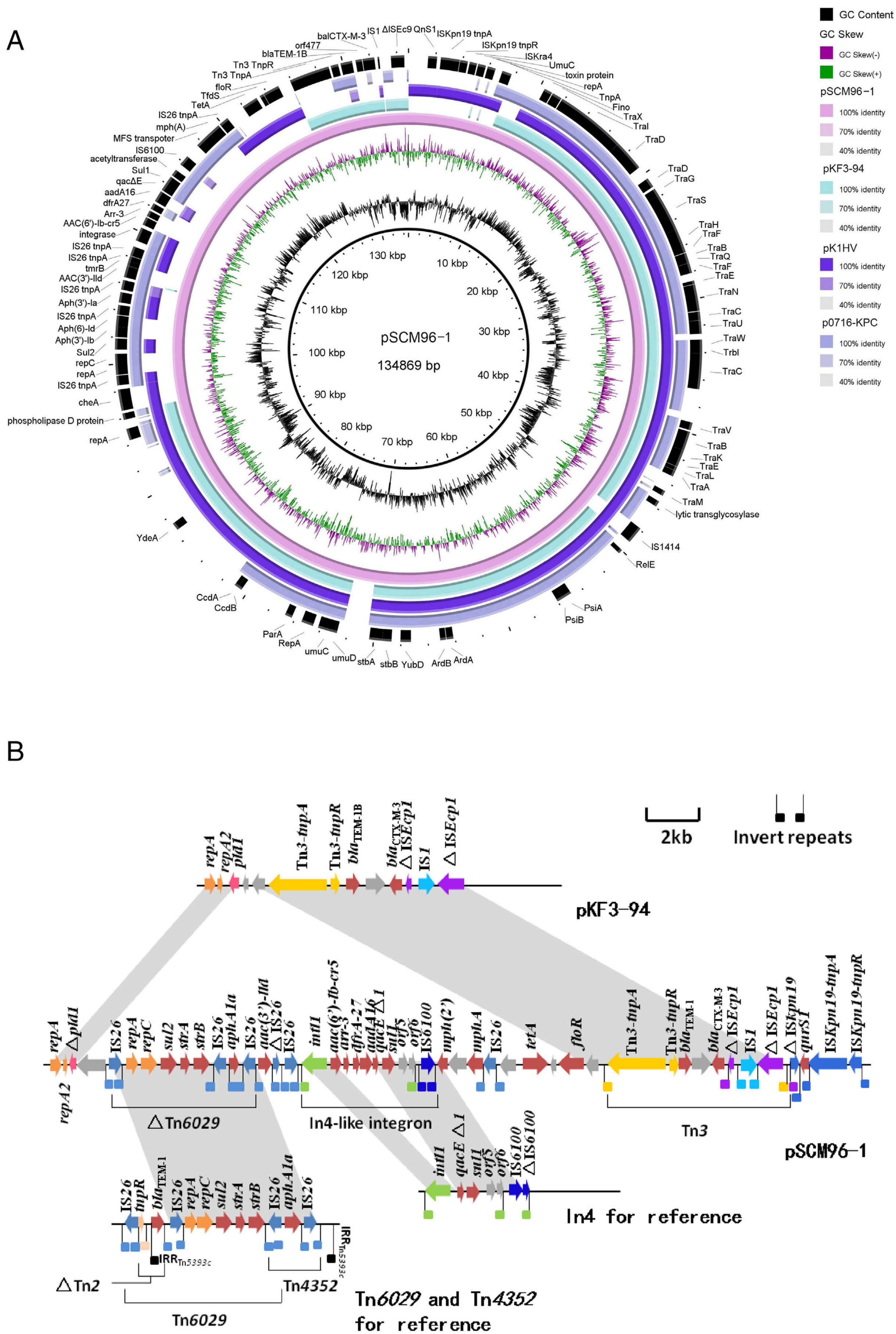
K. pneumoniae species. By the way, although the bla_{NDM-19} was detected in an *Escherichia coli* strain N17-00233 (Boyd, D.A. et al., unpublished results) before, unfortunately, the genetic characteristic of this gene has not yet been studied.

The results of WGS revealed SCM96 harbored 2 different plasmids, pSCM96-1 and pSCM96-2, which indicated the high transmission possibility of resistance genes and virulence genes. Plasmid pSCM96-1 was a large plasmid carrying a complex resistance region harboring 17 antimicrobial resistance genes, a gene encoding virulence factor (*phd1*), and several significant the MGEs. These 17 antimicrobial resistance genes covered most types of antibiotics, which led to SCM96 being not susceptible to almost all the common antibiotics. Insertion of the 28-kb sequence between Δ*phd1* and Tn*3*-*tnpA* indicated the recombination of this region in pSCM96-1. The recombination of Δ*phd1* flanking sequences also occurred in pK1HV. It seemed that the region downstream of *phd1* was a hot spot of recombination (Ying et al., 2015). Notably, the sequence of the In4 adjacent to an IS*26* in the 5' end included additional resistance gene *aac (3′)-lld*. IS*26* is implicated in the dissemination of resistance genes and also seems to facilitate the mobilization of chromosomal sequences containing resistance with evolution of the multiresistance plasmid pSCM96-1. IS*6100* was considered to be a characteristic of integrons belonging to In4-like groups. Its deletion at the direct duplication, right-hand end, or left-hand end was usually a way of In4-like integrons originating from In4 (Partridge et al., 2001). In addition, majority mobile elements are bounded by invert repeats (IRs) (Mahillon and Chandler, 1998). On this basis, the boundaries of the Tn*3* were those 2 IRs (Fig. 1b), which mean that the Tn*3* was able to transpose the bla_{TEM-1B} and $bla_{CTX-M-3}$. Moreover, the IS*Ecp1* element is associated with genes that encode CTX-M-type β-lactamase and appears to play an important role in the mobilization and expression of genes that encode these enzymes (Eckert et al., 2006; Poirel et al., 2005; Tian et al., 2011). However, sequence analysis showed that it was divided into 2 segments by IS*1*, and a sequence of 163 bp was deleted in the middle of IS*Ecp1*, which might cause the defective function of IS*Ecp1* transposeae. So did pKF3-94(Ying et al., 2015). But IS*Ecp1* in pKF3-94 was divided into 2 portions of 1057 and 309 bp which was shorter than pSCM96-1. This suggested the region in pSCM96-1 was probably an origin of the counterpart in pKF-94 (Ying et al., 2015).

Plasmid pSCM96-2, belonging to IncX3 type plasmid, harbored only 1 antimicrobial resistance gene, bla_{NDM-19}, which showed almost 100% nucleotide identity with pNDM20 except point mutations on bla_{NDM} (Liu et al., 2018b). To our knowledge, IncX3-type plasmids were the most frequently reported to mediate the dissemination of bla_{NDM} including bla_{NDM-1}, bla_{NDM-5}, bla_{NDM-7}, bla_{NDM-13}, bla_{NDM-20}, and bla_{NDM-21} in China (An et al., 2016; Liu et al., 2018a, 2018b; Lv et al., 2016; Yang et al., 2014; Zhang et al., 2016). Interestingly, those IncX3-type plasmids carrying bla_{NDM} showed high similarity in nucleotide sequence. These findings here further suggested that the bla_{NDM} bearing IncX3-type plasmids might have evolved from the same ancestral plasmid through a series of mutations. Easy spread of IncX3 and point mutations of bla_{NDM-1} were responsible for the IncX3 plasmids disseminating multiple variants of NDM. In addition, connection between plasmid replicons and sequence types is usually significant to study the spreading of resistance genes. Until now, *K. pneumoniae* ST14 and ST147 have been found in IncX3-positive NDM producers in China (Mei et al., 2017; Wang et al., 2014). As far as we know, *K. pneumoniae* ST15 was for the first time identified in IncX3-positive NDM producers in China.

In conclusion, here we characterize a *K. pneumoniae* clinical stain SCM96 harboring 2 plasmids and carrying up to 22 antimicrobial resistance genes, including the bla_{NDM-19} gene which was the first time to be reported in *K. pneumoniae* specie, and these might confer the resistance of SCM96 against almost all of the commonly used clinical antibiotics. Moreover, the identified important MGEs in the 2 plasmids might enable the global dissemination of resistance genes. Further mechanistic studies concerning the co-spreading of multiple resistance genes may significantly contribute to the control of prevalent antibiotic resistance and the development of novel antimicrobial drugs.

Fig. 1. Sequence analysis of the plasmid pSCM96-1. (a) Comparative analysis of pSCM96-1 with 3 closely related plasmids (pKF3-94, pK1HV, and p0716-KPC) in circle structure. Concentric rings represent the similarity between the reference sequence in the inner ring and the other sequences in the outer rings. Color levels indicate the result of BLAST with a matched degree in the shared regions. (b) Structure feature of the complex resistance region in pSCM96-1 compared with the reference sequences. Grey shading indicates the shared regions with a high degree of nucleotide identity (>99%). ORFs are indicated as arrows and are colored according to their putative functions. Orange arrows indicate replication-associated genes. Red arrows indicate antimicrobial resistance genes. Mobile elements are indicated by other different colors, whose invert repeats are defined with corresponding color. Gray arrows indicated other function genes.


A
GC Content
GC Skew
GC Skew(-)
GC Skew(+)
pSCM96−1
100% identity
70% identity
40% identity
pKF3-94
100% identity
70% identity
40% identity
pK1HV
100% identity
70% identity
40% identity
p0716-KPC
100% identity
70% identity
40% identity
pSCM96−1
134869 bp
10 kbp
20 kbp
30 kbp
40 kbp
50 kbp
60 kbp
70 kbp
80 kbp
90 kbp
100 kbp
110 kbp
120 kbp
130 kbp
balCTX-M-3
IS1
ΔISEc9
QnS1
ISKpn19 tnpA
ISKpn19 tnpR
ISKra4
UmuC
toxin protein
repA
TnpA
Fino
TraX
TraI
TraD
TraD
TraG
TraS
TraH
TraF
TraB
TraQ
TraF
TraE
TraN
TraC
TraU
TraW
TrbI
TraC
TraV
TraB
TraK
TraE
TraL
TraA
TraM
lytic transglycosylase
IS1414
RelE
PsiA
PsiB
ArdA
ArdB
YubD
stbB
stbA
umuD
umuC
RepA
ParA
CcdB
CcdA
YdeA
repA
phospholipase D protein
cheA
IS26 tnpA
repA
repC
Sul2
Aph(3')-Ib
Aph(6)-Id
IS26 tnpA
Aph(3')-Ia
IS26 tnpA
AAC(3')-IId
tmrB
IS26 tnpA
IS26 tnpA
integrase
AAC(6')-Ib-cr5
Arr-3
dfrA27
aadA16
qacΔE
Sul1
acetyltransferase
IS6100
MFS transporter
mph(A)
IS26 tnpA
TetA
TfdS
floR
Tn3 TnpA
Tn3 TnpR
blaTEM-1B
orf477
B
2kb
Invert repeats
pKF3−94
repA
repA2
pld1
Tn3-tnpA
Tn3-tnpR
blaTEM-1B
blaCTX-M-3
ΔISEcp1
IS1
ΔISEcp1
pSCM96−1
repA
repA2
Δpld1
IS26
repA
repC
sul2
strA
strB
IS26
aphA1a
IS26
aac(3')-IId
ΔIS26
IS26
intI1
aac(6')-Ib-cr5
arr-3
dfrA-27
aadA16
qacEΔ1
sul1
orf5
orf6
IS6100
mph(2')
mphA
IS26
tetA
floR
Tn3-tnpA
Tn3-tnpR
blaTEM-1
blaCTX-M-3
ΔISEcp1
IS1
ΔISEcp1
ΔISKpn19
qnrS1
ISKpn19-tnpA
ISKpn19-tnpR
ΔTn6029
In4-like integron
Tn3
intI1
qacEΔ1
sul1
orf5
orf6
IS6100
ΔIS6100
In4 for reference
IS26
tnpR
blaTEM-1
IS26
repA
repC
sul2
strA
strB
IS26
aphA1a
IS26
IRRTn5393c
IRRTn5393c
ΔTn2
Tn4352
Tn6029
Tn6029 and Tn4352
for reference

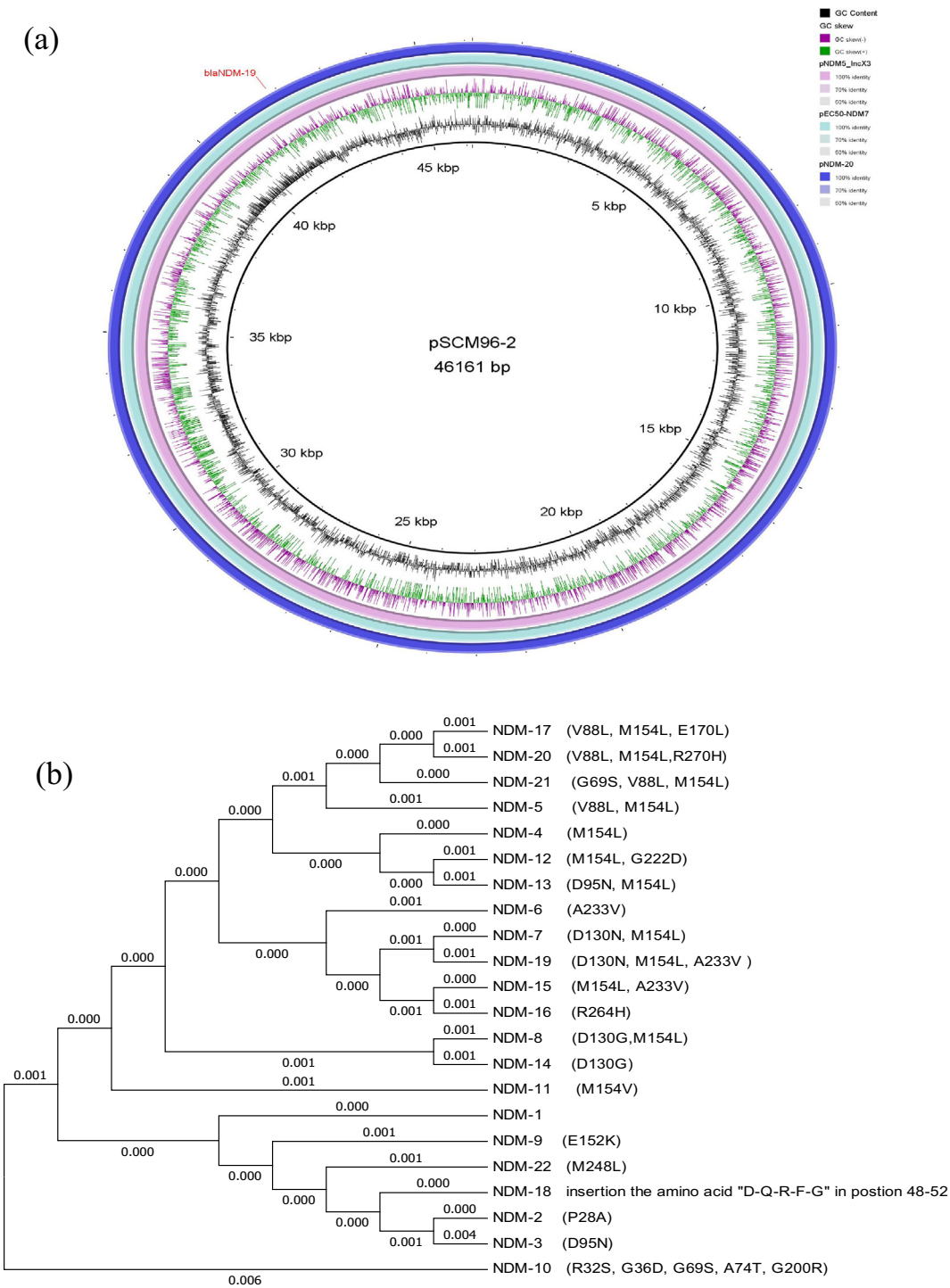


Fig. 2. Sequence analysis of the plasmid pSCM96-2. (a) Comparative analysis of pSCM96-2 with 3 closely related plasmids (pNDM-20, pNDM5_IncX3, pEC50-NDM7) in circle structure. Concentric rings represent the similarity between the reference sequence in the inner ring and the other sequences in the outer rings. Color levels indicate the result of BLAST with a matched degree in the shared regions. The map was drawn using BRIG (http:// sourceforge.net/projects/brig/). (b) The phylogenetic relationship between amino acid sequences of NDM variants is shown. All the sequences were aligned with ClustalW method. The phylogenetic tree was constructed by using the neighbor-joining method with 1000 bootstrap replicates and the amino acid mutations shown in the brackets.

Availability of data and material

All data generated or analyzed during this study are included in this published article. Sequence data that support the findings of this study have been deposited in GenBank with the accession codes CP028716 (https://www.ncbi.nlm.nih.gov/nuccore/CP028716), CP028717 (https://www.ncbi.nlm.nih.gov/nuccore/CP028717), and CP028718 (https://www.ncbi.nlm.nih.gov/nuccore/CP028718).

Funding information

This research was funded by the National Natural Science Foundation of China [31500114 and 31700111], the Sichuan Province Science and Technology project [2016JY0223], the Health and Family Planning Commission of Sichuan Province Grant [16PJ538] and the Southwest Medical University Natural Science Foundation [2015LZCYD-S09 (7/8)].

Competing interests

The authors have nothing to declare.

Ethical approval

Not required.

References

An J, Guo L, Zhou L, Ma Y, Luo Y, Tao C, et al. NDM-producing Enterobacteriaceae in a Chinese hospital, 2014-2015: identification of NDM-producing Citrobacter werkmanii and acquisition of blaNDM-1-carrying plasmid in vivo in a clinical Escherichia coli isolate. J Med Microbiol 2016;65(11):1253–9.

Carattoli A, Zankari E, Garcìa-Fernandez A, Larsen MV, Lund O, Villa L, et al. PlasmidFinder and pMLST: in silico detection and typing of plasmids. Antimicrob Agents Chemother 2014;7:3895–903.

Cprek JB, Gallagher JC. Ertapenem-containing double-carbapenem therapy for treatment of infections caused by carbapenem-resistant Klebsiella pneumoniae. Antimicrob Agents Chemother 2016;60(1):669–73.

Datta S, Mitra S, Chattopadhyay P, Som T, Mukherjee S, Basu S. Spread and exchange of Bla NDM-1 in hospitalized neonates: role of mobilizable genetic elements. Eur J Clin Microbiol Infect Dis 2017;36(2):255–65.

Del Franco M, Paone L, Novati R, Giacomazzi CG, Bagattini M, Galotto C, et al. Molecular epidemiology of carbapenem resistant Enterobacteriaceae in Valle d'Aosta region, Italy, shows the emergence of KPC-2 producing Klebsiella pneumoniae clonal complex 101 (ST101 and ST1789). BMC Microbiol 2015;15(1):260.

Delcher AL, Bratke KA, Powers EC, Salzberg SL. Identifying bacterial genes and endosymbiont DNA with Glimmer. Bioinformatics 2007;23(6):673–9.

Di DY, Jang J, Unno T, Hur HG. Emergence of Klebsiella variicola positive for NDM-9, a variant of New Delhi metallo-beta-lactamase, in an urban river in South Korea. J Antimicrob Chemother 2017;72(4):1063–7.

Diago-Navarro E, Motley MP, Ruiz-Peréz G, Yu W, Austin J, Seco BMS, et al. Erratum for Diago-Navarro et al., "Novel, broadly reactive anticapsular antibodies against carbapenem-resistant *Klebsiella pneumoniae* protect from infection". mBio 2018;9(3).

Eckert C, Gautier V, Arlet G. DNA sequence analysis of the genetic environment of various blaCTX-M genes. J Antimicrob Chemother 2006;57(1):14–23.

Fu L, Huang M, Zhang X, Yang X, Liu Y, Zhang L, et al. Frequency of virulence factors in high biofilm formation blaKPC-2 producing Klebsiella pneumoniae strains from hospitals. Microb Pathog 2018;4:168–72.

Holt KE, Wertheim H, Zadoks RN, Baker S, Whitehouse CA, Dance D, et al. Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in Klebsiella pneumoniae, an urgent threat to public health. Proc Natl Acad Sci U S A 2015;112(27):3574–81.

Institute CaLS. Performance standards for antimicrobial susceptibility testing: twenty-eight informational supplement M100-S28; 2018.

Johnson AP, Woodford N. Global spread of antibiotic resistance: the example of New Delhi metallo-beta-lactamase (NDM)-mediated carbapenem resistance. J Med Microbiol 2013;62(Pt 4):499–513.

Jolley KA, Maiden MC. BIGSdb: scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics 2010;11(1):595.

Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets. Mol Biol Evol 2016;33(7):1870–4.

Lemos EV, de la Hoz FP, Alvis N, Einarson TR, Quevedo E, Castaneda C, et al. Impact of carbapenem resistance on clinical and economic outcomes among patients with Acinetobacter baumannii infection in Colombia. Clin Microbiol Infect 2014;20(2):174–80.

Liu L, Feng Y, McNally A, Zong Z. blaNDM-21, a new variant of blaNDM in an Escherichia coli clinical isolate carrying blaCTX-M-55 and rmtB. J Antimicrob Chemother 2018a;73(9):2336–9.

Liu Z, Li J, Wang X, Liu D, Ke Y, Wang Y, et al. Novel variant of New Delhi metallo-beta-lactamase, NDM-20, in Escherichia coli. Front Microbiol 2018b;9:248.

Luo R, Liu B, Xie Y, Li Z, Huang W, Yuan J, et al. SOAPdenovo2: an empirically improved memory-efficient short-read de novo assembler. Gigascience 2012;1(1):18.

Lv J, Qi X, Zhang D, Zheng Z, Chen Y, Guo Y, et al. First report of complete sequence of a blaNDM-13-harboring plasmid from an Escherichia coli ST5138 clinical isolate. Front Cell Infect Microbiol 2016;6:130.

Mahillon J, Chandler M. Insertion Sequences. Microbiol Mol Biol Rev 1998;62(3):725–74.

Mei YF, Liu PP, Wan LG, Liu Y, Wang LH, Wei DD, et al. Virulence and genomic feature of a virulent Klebsiella pneumoniae sequence type 14 strain of serotype K2 harboring blaNDM-5 in China. Front Microbiol 2017;8:335.

Nordmann P, Naas T, Poirel L. Global spread of carbapenemase-producing Enterobacteriaceae. Emerg Infect Dis 2011;17(10):1791–8.

Partridge SR, Brown HJ, Stokes HW, Hall RM. Transposons Tn1696 and Tn21and their Integrons In4 and In2 have independent origins. Antimicrob Agents Chemother 2001;45(4):1263–70.

Poirel L, Lartigue MF, Decousser JW, Nordmann P. ISEcp1B-mediated transposition of blaCTX-M in Escherichia coli. Antimicrob Agents Chemother 2005;49(1):447–50.

Poirel L, Walsh TR, Cuvillier V, Nordmann P. Multiplex PCR for detection of acquired carbapenemase genes. Diagn Microbiol Infect Dis 2011;70(1):119–23.

Rahman M, Shukla SK, Prasad KN, Ovejero CM, Pati BK, Tripathi A, et al. Prevalence and molecular characterisation of New Delhi metallo-beta-lactamases NDM-1, NDM-5, NDM-6 and NDM-7 in multidrug-resistant Enterobacteriaceae from India. Int J Antimicrob Agents 2014;44(1):30–7.

Rimoldi SG, Gentile B, Pagani C, Di Gregorio A, Anselmo A, Palozzi AM, et al. Whole genome sequencing for the molecular characterization of carbapenem-resistant Klebsiella pneumoniae strains isolated at the Italian ASST Fatebenefratelli Sacco Hospital, 2012–2014. BMC Infect Dis 2017;17(1):666.

Tian GB, Wang HN, Zou LK, Tang JN, Zhao YW, Ye MY, et al. Detection of CTX-M-15, CTX-M-22, and SHV-2 extended-spectrum β-lactamases (ESBLs) in Escherichia coli fecal-sample isolates from pig farms in China. Foodborne Pathog Dis 2009;6(3):297–304.

Tian SF, Chu YZ, Chen BY, Nian H, Shang H. ISEcp1 element in association with bla(CTX-M) genes of E. coli that produce extended-spectrum beta-lactamase among the elderly in community settings. Enferm Infecc Microbiol Clin 2011;29(10):731–4.

Tzouvelekis LS, Markogiannakis A, Psichogiou M, Tassios PT, Daikos GL. Carbapenemases in Klebsiella pneumoniae and other Enterobacteriaceae: an evolving crisis of global dimensions. Clin Microbiol Rev 2012;25(4):682–707.

Vanegas JM, Parra OL, Jiménez JN. Molecular epidemiology of carbapenem resistant gram-negative bacilli from infected pediatric population in tertiary-care hospitals in Medellín, Colombia: an increasing problem. BMC Infect Dis 2016;16(1):463.

Wang X, Xu X, Li Z, Chen H, Wang Q, Yang P, et al. An outbreak of a nosocomial NDM-1-producing Klebsiella pneumoniae ST147 at a teaching hospital in mainland China. Microb Drug Resist 2014;20(2):144–9.

Yang P, Xie Y, Feng P, Zong Z. blaNDM-5 carried by an IncX3 plasmid in Escherichia coli sequence type 167. Antimicrob Agents Chemother 2014;58(12):7548–52.

Ying J, Wu S, Zhang K, Wang Z, Zhu W, Zhu M, et al. Comparative genomics analysis of pKF3-94 in Klebsiella pneumoniae reveals plasmid compatibility and horizontal gene transfer. Front Microbiol 2015;6:831.

Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, et al. Characterization of a new metallo-β-lactamase gene, blaNDM-1, and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India. Antimicrob Agents Chemother 2009;53(12):5046–54.

Zhang F, Xie L, Wang X, Han L, Guo X, Ni Y, et al. Further spread of Bla NDM-5 in Enterobacteriaceae via IncX3 plasmids in Shanghai, China. Front Microbiol 2016;7:424.

Zhang L, Li Y, Shen W, Wang SM, Wang G, Zhou Y. Whole-genome sequence of a carbapenem-resistant hypermucoviscous Klebsiella pneumoniae isolate SWU01 with capsular serotype K47 belonging to ST11 from a patient in China. J Glob Antimicrob Resist 2017;11:87–9.