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SHORT REPORT

Coexistence Of Plasmid-Mediated *mcr-1* And *bla*$_{NDM-4}$ Genes In A *Klebsiella pneumoniae* Clinical Strain In Vietnam

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Abstract: In this study, we characterized the first clinical *Klebsiella pneumoniae* strain co-harboring *mcr-1* and *bla*$_{NDM-4}$ genes in Vietnam, which was recovered from a patient admitted to hospital in 2015. This strain demonstrated nonsusceptible to all tested antibiotics, including last-line antibiotics such as carbapenems (MICs ≥128 μg/mL) and colistin (MIC =32 μg/mL), except tigecycline (MIC =1 μg/mL). Whole-genome analysis using both MinION and MiSeq data revealed that the strain carried 29 resistance genes. Particularly, *mcr-1* and *bla*$_{NDM-4}$ genes were carried by different self-conjugative plasmids and able to be transferred to a recipient by conjugation. The colistin resistance of this strain was conferred by *mcr-1* and additional chromosomal resistance determinants. Eight amino acid substitutions found in PmrA, PmrB, PmrC, PmrI, and PmrJ, all proteins that are involved in lipopolysaccharide modifications, may be associated with chromosomal colistin resistance. The accumulation of multiple antibiotic resistance mechanisms in this clinical isolate raises alarm on potential spread of extensively drug-resistant *K. pneumoniae* in healthcare settings.

Keywords: coexistence, *mcr-1*, *bla*$_{NDM-4}$, *Klebsiella pneumoniae*, Vietnam

Colistin and carbapenems have been considered as last-line antibiotics against serious infections caused by multidrug-resistant Gram-negative bacteria. However, public health concern has intensified from instances of colistin- and carbapenem-resistant *Enterobacteriaceae* infections increasingly reported worldwide.[1–3] Particularly, the coexistence of transferable New Delhi metallo-β-lactamase (NDM)-encoding gene *bla*$_{NDM}$ and mobile colistin resistance gene *mcr* in *Enterobacteriaceae* poses a serious threat to global health, as its extensive spread could lead to outbreaks of untreatable infections.[2,3] In this study, we characterized a clinical strain of *Klebsiella pneumoniae*, co-harboring *mcr-1* and *bla*$_{NDM-4}$ resistance genes in Vietnam.

K. pneumoniae strain PI15KP27 was recovered from the sputum of a less than one-year-old patient admitted to a pediatric hospital in Ho Chi Minh City, Vietnam in 2015 with a diagnosis of pneumonia on day 21 after hospital admission. The child, still experiencing diarrhea, sepsis and severe pneumonia, was discharged from the hospital at the request of his family after staying in the intensive care unit and respiratory department for 34 days. During his hospitalization, the child was treated with metronidazole, levofloxacin, meropenem, and colistin. PI15KP27 was likely a hospital-acquired strain because it was identified more than two days after hospitalization.

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Antimicrobial susceptibility evaluations of *K. pneumoniae* PI15KP27, recipient *E. coli* J53Az^R and transconjugants were performed by disk diffusion and agar dilution method according to the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2018), except fosfomycin, which was interpreted by the European Union Committee for Antimicrobial Susceptibility Testing (EUCAST, 2019). Minimum inhibitory concentrations (MICs) for colistin and tigecycline were determined by broth microdilution method using guidelines of the EUCAST and the United States Food and Drug Administration (FDA), respectively. The results showed that PI15KP27 was resistant to 23 antibiotics which belonged to 17 classes, including last-resort antibiotics such as carbapenems (MICs ≥128 μg/mL) and colistin (MIC =32 μg/mL). It was intermediate resistance to amikacin (MIC =32 μg/mL) and susceptible to tigecycline (MIC =1 μg/mL) (Table 1).

The whole-genome sequencing was carried out using the MinION nanopore sequencer (Oxford Nanopore Technologies, UK). Genome assembly was performed by Canu 1.6[4] and then corrected using Pilon 1.22[5] with reads obtained from 250-bp paired-end Illumina MiSeq sequencing (Illumina Inc., USA). Genetic analysis of the complete genome sequence indicated that PI15KP27 consisted of a 5,247,824-bp chromosome with an average 50.1% GC content and three plasmids pKP27-MCR1, pKP27-NDM4 and pKP27-MPH with a sequencing depth of 110. Identification of antimicrobial resistance genes using the ResFinder 3.0 database (https://cge.cbs.dtu.dk/services/ResFinder/) revealed the presence of 29 resistance genes, most of them located on three plasmids (Table 2). Particularly, the *mcr-1* gene was found on a 144,138-bp IncA/C2-type plasmid pKP27-MCR1 and *bla*$_{NDM-4}$ was found on a 121,851-bp IncFIIK-type plasmid pKP27-NDM4, which were able to be transferred into the recipient *E. coli* strain J53 by conjugation assays.

Sodium azide-resistant *E. coli* J53Az^R strain was used as the recipient for the conjugation experiments to examine the transferability of plasmids in the clinical strain. A total of 1ml of each clinical donor and recipient culture was mixed together and incubated at 37°C for 6h. Transconjugants were selected on brain-heart infusion agar (BHIA) (Difco) containing sodium azide (100 μg/mL) plus imipenem (1 μg/mL), colistin (4 μg/mL), or azithromycin (20 μg/mL). As a result, transconjugants selected on the imipenem supplemented medium or colistin supplemented medium showed antimicrobial susceptibility profiles compatible with resistant phenotypes encoded by genes located on plasmid pKP27-NDM4 (*aac(3)-Iid, aadA1, aac(6')-Ib, bla*$_{NDM-4}$, *bla*$_{CTX-M-14}$, *bla*$_{OXA-9}$, *aac(6')-Ib-cr, qnrS1*) and pKP27-MCR1 (*mcr-1, floR, sul2, tet*(A)), respectively (Table 2). In particular, transconjugants selected on imipenem supplemented medium showed resistant to gentamicin, tobramycin, kanamycin, ertapenem, imipenem, meropenem, cefuroxime, cefotaxime, ceftazidime, cefepime, cefoxitin, ampicillin, amoxicillin-clavulanic acid; intermediate to ciprofloxacin and susceptible to trimethoprim-sulfamethoxazole, aztreonam, chloramphenicol, tetracycline, azithromycin, sulfonamides, nitrofurantoin. Transconjugants selected on colistin supplemented medium showed resistant to colistin, chloramphenicol, sulfonamides, tetracycline; intermediate to ampicillin and susceptible to other above remain antibiotics (Table 1). There was no strain selected on azithromycin supplemented medium. The presence of *mcr-1* and *bla*$_{NDM4}$ in the transconjugants was confirmed by PCR. These results illustrated that *mcr-1* and *bla*$_{NDM-4}$ genes were carried by different self-conjugative plasmids.

There was a significant difference in minimal inhibitory concentration (MIC) for colistin of the clinical strain (MIC =32 μg/mL) in comparison to its transconjugant carrying *mcr-1* positive plasmid (MIC =4 μg/mL), indicating that colistin resistance of this strain was conferred by *mcr-1* and additional chromosomal resistance determinants. Eight amino acid substitutions were identified by comparison to the genome of *K. pneumoniae* HS11286 (GenBank accession no. CP003200), including E57G in PmrA; G248R in PmrB; C27F and Q330R in PmrC; I260L in PmrI; V53I, I94L and I300V in PmrJ, all proteins that are involved in lipopolysaccharide modifications, may be associated with colistin resistance. All those mutations, except G248R and Q330R, were earlier observed in some colistin-resistant *K. pneumoniae* isolates.[6–9] Further research is required to clarify the significance of these mutations in colistin resistance. The complete sequences of *K. pneumoniae* strain PI15KP27 and three plasmids have been deposited in GenBank under the accession numbers CP041639-CP041642.

The result of multilocus sequence typing (MLST) using MLST1.8 database (https://cge.cbs.dtu.dk/services/MLST/) indicated that PI15KP27 belonged to sequence type 16 (ST16). This clone has disseminated worldwide with different antimicrobial resistance profiles, which was reported as a β-lactamase producer or involved in outbreaks of CTX-M-15-producing *K. pneumoniae*.[10,11] Additionally, the ST16 clone was also found in some carbapenem-resistant *K. pneumoniae* isolates spread in a

Table 1 Antimicrobial Susceptibility Of Clinical Strain PI15KP27, Recipient *E. Coli* J53Az^{R} And Its Transconjugants

Antimicrobial Susceptibility		**PI15KP27**	**Transconjugants**		**J53**
			pKP27-MCR1	**pKP27-NDM4**	
Disk Diffusion assay					
Resistant		GM, TOB, K, ETP, IMP, MEM, CXM, CAZ, FEP, FOX, CIP, SXT, ATM, AMP, AUG, C, TE, AZ, S, F	C, S, TE	GM, TOB, K, ETP, IMP, MEM, CXM, CAZ, FEP, FOX, AMP, AUG	–
Intermediate			AMP	CIP	AMP
Susceptible		–	GM, TOB, K, ETP, IMP, MEM, CXM, CAZ, FEP, FOX, CIP, SXT, ATM, AUG, AZ, F	SXT, ATM, C, TE, AZ, S, F	GM, TOB, K, ETP, IMP, MEM, CXM, CAZ, FEP, FOX, CIP, SXT, ATM, AUG, C, TE, AZ, S, F
Minimum inhibitory concentration (µg/mL)					
Antimicrobial class	Antimicrobial agent				
Aminoglycosides	GM	>256	2	48	1.5
	AK	32	2	32	2
Carbapenems	ETP	>128	0.008	32	0.008
	IMP	128	0.125	16	0.125
	MEM	>128	0.015	8	0.015
Extended-spectrum cephalosporins; 3rd and 4th generation cephalosporins	CTX	>256	0.125	>256	0.125
	CAZ	>256	0.25	>256	0.25
	FEP	>256	<0.016	8	0.016
Cephamycins	FOX	>256	8	>256	8
Fluoroquinolones	CIP	>256	0.03	0.5	0.015
Folate pathway inhibitors	SXT	32	0.05	0.032	0.05
Glycylcyclines	TGC	1	0.06	0.06	0.06
Penicillins	AMP	>256	3	>256	4
Penicillins + β-lactamase inhibitors	AUG	>256	3	48	3
Phenicols	C	>256	256	4	4
Phosphonic acids	FOS	>256	0.5	0.5	0.5
Polymyxins	CS	32	4	0.5	0.5
Tetracyclines	TE	>256	128	2	2

Abbreviations: GM, gentamicin; AK, amikacin; TOB, tobramycin; K, kanamycin; ETP, ertapenem; IMP, imipenem; MEM, meropenem; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; FOX, cefoxitin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; TGC, tigecycline; ATM, aztreonam; AMP, ampicillin; AUG, amoxicillin-clavulanic acid; C, chloramphenicol; FOS, fosfomycine; CS, colistin; TE, tetracycline; AZ, azithromycin; S, sulfonamides; F, nitrofurantoin; NA, not available.

Table 2 Genomic Characteristics Of *K. Pneumoniae* PI15KP27

	Chromosome	Plasmids		
		pKP27-MPH	pKP27-MCR1	pKP27-NDM4
Accession no.	CP041639	CP041640	CP041641	CP041642
Sequence type/Plasmid replicon type	ST-16	IncFIB(K)	IncA/C2	IncFII(K)
Size (bp)	5,247,824	222,330	144,138	121,851
Resistance genes	*bla*$_{SHV-1}$, *oqxB*, *oqxA*, *fosA*	*aac(6')-Ib-cr, aadA2, bla*$_{OXA-1}$, *bla*$_{CTX-M-15}$, *bla*$_{TEM-1B}$, *aac(6')-Ib-cr, mph*(A), *catB4, sul1, tet*(A), *dfrA12*	*aph(6)-Id, aph (3")-Ib, mcr-1, floR, sul2, tet*(A)	*aac(3)-Iid, aadA1, aac(6')-Ib, bla*$_{NDM-4}$, *bla*$_{CTX-M-14}$, *bla*$_{OXA-9}$, *aac(6')-Ib-cr, qnrS1*

clinical setting in Vietnam.[12] ST16 may become a high-risk clone causing multidrug-resistant hospital-acquired infections.[11] The MLST results in this study support this assumption.

The coexistence of *mcr* and *bla*$_{NDM}$ has been commonly identified in *E. coli* but rarely noted in *K. pneumoniae*.[13] The co-harboring of those genes was reported in *K. pneumoniae* isolates from both livestock and human clinical samples.[2,3] To the best of our knowledge, this was the first report in Vietnam of co-production of MCR and NDM in a clinical *K. pneumoniae* strain.

The accumulation of multiple antibiotic resistance mechanisms in this clinical isolate raises alarm on potential spread of extensively drug-resistant *K. pneumoniae* in healthcare settings. The genomic epidemiology surveillance of hospital-acquired infection pathogens, including colistin- and carbapenem-resistant *K. pneumoniae*, is highly necessary to prevent their dissemination.

Ethics Approval

Ethics approval for this study was granted by the Ethics Committee in Biomedical Research – Pasteur Institute in Ho Chi Minh City (Certificate no. 09/CN_HDDD).

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Disclaimer

Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense.

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Author Contributions

VC, JWJ, and TI conceived and designed the study. LL, LKT, BPTT, YNN, HLN, and KQHN collected samples and performed experiments. LL, LKT, TDLH, HNLV, YM, DM, and SN performed data analysis. LL, LKT, TDLH, and YNN wrote the paper. All authors contributed to drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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