

Genetic Characterization of an ST5571 Hypervirulent *Klebsiella pneumoniae* Strain Co-Producing NDM-1, MCR-1, and OXA-10 Causing Bacteremia

Xiaolong Ma^{1,*}, Xiaodong Lv^{1,*}, Sihan Feng¹, Ruishan Liu^{2,3}, Hao Fu², Feng Gao¹, Hao Xu²

¹Department of Respiratory Medicine, the First Hospital of Jiaxing (Affiliated Hospital of Jiaxing University), Jiaxing, People's Republic of China;

²Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, the First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, People's Republic of China; ³Department of Laboratory Medicine, the First Affiliated Hospital of Zhengzhou University, Zhengzhou, People's Republic of China

*These authors have contributed equally to this work

Correspondence: Feng Gao, Department of Respiratory Medicine, the First Hospital of Jiaxing (Affiliated Hospital of Jiaxing University), No. 1882 South Zhonghuan Road, Jiaxing, 314000, People's Republic of China, Tel +86 573 825 198 70, Fax +86 573 820 830 06, Email guti-gf@163.com; Hao Xu, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, the First Affiliated Hospital, College of Medicine, Zhejiang University, No. 79 Qing chun Road, Hangzhou, 310003, People's Republic of China, Tel +86 571 872 364 23, Fax +86 571 872 364 21, Email xuhao0523@zju.edu.cn

Purpose: To investigate the phenotypic and genomic characteristics of the multi-drug resistant and hypervirulent *Klebsiella pneumoniae* strain recovered from bacteremia.

Methods: Antimicrobial susceptibility testing (AST) was performed by the microdilution method. Antimicrobial resistance genes, virulence-associated genes, multilocus sequence typing (MLST), and plasmid replicon were characterized by next-generation sequencing (NGS) and nanopore sequencing. S1 nuclease-pulsed field gel electrophoresis (S1-PFGE) and Southern blotting were performed to characterize the plasmid profile.

Results: The hypervirulent colistin- and carbapenem-resistant *K. pneumoniae* strain DY2009 was identified as ST5571, co-carrying *mcr-1*, *bla*_{NDM-1}, and *bla*_{OXA-10}. In silico analysis found that it was K2 serotype. AST results revealed that DY2009 was resistant to carbapenems, cephalosporins, ciprofloxacin, chloramphenicol, and colistin but remained susceptible to aztreonam, gentamicin, amikacin, and tigecycline. Through the whole-genome analysis, a variety of virulence determinants were identified, including *rmpA*. Plasmid analysis confirmed that the *mcr-1* and *bla*_{NDM-1} gene harbored a ~33 kb IncX4 plasmid and a ~44 kb IncX3 plasmid. In contrast, *bla*_{OXA-10} was encoded by chromosome.

Conclusion: To the best of our knowledge, we first report the clinical hypervirulent *K. pneumoniae* isolate co-producing MCR-1, NDM-1, and OXA-10 causing bacteremia. We found that *mcr-1* and *bla*_{NDM-1} genes were located on two self-conjugative epidemic plasmids, contributing to the widespread MCR-1 and NDM-1 in China. The results of this work improve our understanding of the genetic background of colistin- and carbapenem-resistant *K. pneumoniae* isolate from bacteremia and the resistance mechanisms. Our findings highlight the urgent need for infection control of such strain to prevent it from becoming an extensive-drug resistant clone.

Keywords: *Klebsiella pneumoniae*, NDM-1, MCR-1, OXA-10, *rmpA*, bacteremia

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has been recognized as one of the most worrying threats to human health globally.¹⁻⁵ Due to the limited therapeutic options, infections caused by CRKP result in high mortality.⁶ Polymyxins, including colistin, are a significant “last-line” treatment for infections caused by CRKP.⁶ However, since the first plasmid-mediated colistin resistance gene *mcr-1* was identified in China, many *mcr* genes

have been detected in Enterobacteriaceae species.^{7–9} The emergence of *mcr* genes in CRKP represents a real challenge to clinical treatments.

A low prevalence of *mcr-1* in *K. pneumoniae* was observed,¹⁰ although several *mcr* genes and their variants have been detected in *K. pneumoniae*.^{11,12} In this work, we reported a bloodstream infection caused by a colistin-resistant CRKP strain. Whole-genome sequencing (WGS) and microbiological analysis revealed the emergence of colistin-resistant CRKP clinical strain co-producing NDM-1, MCR-1, and OXA-1.

K. pneumoniae strain DY2009 was recovered from an 85-years old female patient with bacteremia in January 2020 from Dongyang, China. The bacterial identification was conducted using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight mass-spectrometer (MALDI-TOF-MS) (Bruker Daltonics, Germany).¹³ Antimicrobial susceptibility testing was performed by the broth microdilution method. The results were interpreted according to the Clinical and Laboratory Standards Institute standards,¹⁴ except for tigecycline and colistin, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (<https://www.eucast.org/>). *K. pneumoniae* strain DY2009 was resistant to imipenem (MIC = 4 mg/L), meropenem (MIC = 4 mg/L), cefotaxime (MIC = 128 mg/L), ceftazidime (MIC ≥128 mg/L), ceftazidime (MIC = 32 mg/L), piperacillin/tazobactam (MIC ≥128 mg/L), ciprofloxacin (MIC = 8 mg/L), chloramphenicol (MIC ≥128 mg/L), and colistin (MIC = 32 mg/L). However, this strain was susceptible to aztreonam (MIC = 0.25 mg/L), gentamicin (MIC = 2 mg/L), amikacin (MIC = 2 mg/L), and tigecycline (MIC = 1 mg/L) (Table 1). The patient was treated with piperacillin/tazobactam empirically and changed to amikacin after obtaining susceptibility results. The patient recovered gradually and discharged from the hospital.

Genomic DNA of *K. pneumoniae* DY2009 was extracted using the Genra Puregene Yeast/Bact. Kit (Qiagen, Germany) according to the manufacturer's instructions. The sequencing library was prepared by using Illumina Nextera XT kit and sequenced using the Illumina HiSeq X 10-PE150 platform (Illumina, San Diego, CA, USA). A-tailed fragments were ligated with paired-end adaptors and PCR-amplified with a 500-bp insert. A mate-pair

Table 1 Antimicrobial Susceptibilities of Strain *Klebsiella pneumoniae* DY2009

Antibiotics	MIC (µg/mL)	Interpretation
Imipenem	4	R
Meropenem	4	R
Cefotaxime	128	R
Ceftazidime	>128	R
Ceftazidime	32	R
Piperacillin/Tazobactam	>128	R
Aztreonam	0.25	S
Ciprofloxacin	8	R
Chloramphenicol	>128	R
Gentamicin	2	S
Tobramycin	1	S
Amikacin	2	S
Fosfomycin	<0.25	S
Tigecycline	1	S
Colistin	32	R

library with an insert size of 5 kb was used for library construction, at the Beijing Novogene Bioinformatics Technology Co., Ltd. PCR adapter reads and low-quality reads from the paired-end and mate-pair library were filtered during a quality control step using the Novogene pipeline. To investigate the genetic environment of plasmids carrying *bla*_{NDM-1} and *mcr-1* genes, DY2009 was further analyzed by Nanopore sequencing (Oxford, UK). Unicycler was used for hybrid assembling *K. pneumoniae* DY2009 genome from Illumina short reads and Nanopore long reads.¹⁵ Three modes (conservative, normal and bold) of Unicycler were run to generate the best-assembled results. Antimicrobial resistance genes (ARGs) were identified using the ResFinder 3.1 database.¹⁶ Plasmid Finder 1.3 was used to determine the incompatibility type of the plasmids.¹⁷ We identified the virulence loci with the assembled genome sequences using the BIGSdb *Klebsiella* genome database (<http://bigsd.b.pasteur.fr/klebsiella/>).

By searching against the *K. pneumoniae* locus/sequence definitions database (<https://cge.cbs.dtu.dk/services/MLST/>), we found that *K. pneumoniae* DY2009 was classified as ST5571. An IncX4 plasmid-mediated colistin resistance gene, *mcr-1*, was identified using ResFinder 3.1 (Figure 1A). A total of 19 ARGs was found in DY2009 (Table 2). Of note, one class D β -lactamase encoding gene, *bla*_{OXA-10}, was found in the chromosome of strain DY2009. In contrast, NDM-1 was encoded by an IncX3 plasmid (Figure 1B). PCR detection and Sanger sequencing confirmed that *K. pneumoniae* DY2009 was positive for *bla*_{NDM-1} and *mcr-1*. OXA-10 type was known to have narrow spectrum β -lactamase activity and shown to be weak carbapenemases.¹⁸ These results are consistent with the antimicrobial profile observed in *K. pneumoniae* DY2009. Additionally, screening of virulence factors found that this isolate harbored several virulence determinants. These virulence factors include the regulator of the mucoid phenotype (*rmpA*), ABC transporter (*iroC*), yersiniabactin polyketide synthase (*irp1* and *irp2*), type VI secretion protein (*icmF*), enterobactin synthase subunit F (*entF*), acriflavine resistance protein B (*acrB*), ferric aerobactin receptor (*iutA*), aerobactin siderophore biosynthesis protein (*iucC*), and aerobactin synthetase (*iucA*) encoded genes. Furthermore, in silico analysis indicates that DY2009 is capsule type K2. It is well known that a significant virulence factor for *K. pneumoniae* is the capsule, and capsule type K2 isolates produce a capsule that causes hypermucoidy. These results revealed that DY2009 is likely a hypervirulent strain.

S1 Nuclease-Pulsed Field Gel Electrophoresis (S1-PFGE) and Southern blotting was conducted to determine the location of *mcr-1* and *bla*_{NDM-1} with a digoxigenin-labeled *mcr-1* and *bla*_{NDM-1} probe.¹³ Transconjugation assay was performed by mating *E. coli* J53 as the recipient strain. Transconjugants were selected on agar (OXOID, Hampshire, UK) medium at a 200 mg/L sodium azide concentration with two mg/L colistin/meropenem. Finally, MALDI-TOF-MS was used to identify transconjugants, and PCR verified the target gene. Nanopore sequencing revealed that DY2009 carried seven plasmids (Table S1). S1-PFGE and Southern blotting confirmed the existence of 33.3 kbp IncX4 pDY2009-MCR-1 and 44.6 kbp IncX3 pDY2009-NDM-1 (Figure 2). The transconjugants carrying the 33.3 kbp IncX4 pDY2009-MCR-1 plasmid showed a MIC of colistin of 4 mg/L and 44.6 kbp IncX3 pDY2009-NDM-1 plasmid showed a MIC of imipenem of 4 mg/L. These data confirmed that IncX4 pDY2009-MCR-1 plasmid and 44.6 kbp IncX3 pDY2009-NDM-1 are both conjugative and responsible for colistin resistance and carbapenem resistance, respectively. Importantly, eight mutations were found in *phoP* gene and three mutations were found in *phoQ* gene, which result in increased colistin resistance in DY2009.

By Blastn against NCBI database, we found that pDY2009-MCR-1 shared the highest similarity (99.99% identity with 99% coverage) with pT16R-3 (CP046719), pCP55-IncX4 (CP053735), pMUB-MIN6-MCR (CP069695), and pMUB-MIN10-MCR (CP069680). Of note, plasmids pT16R-3 and pCP55-IncX4 were identified from animal-originated *Escherichia coli* isolates in China, in contrast, pMUB-MIN6-MCR and pMUB-MIN10-MCR were identified from clinical *E. coli* isolates in Poland.¹⁹ Lo and colleagues proved that 33 kb IncX4 plasmid is highly transmissible, showing ~100-fold higher transfer frequencies relative to epidemic IncFII plasmid.²⁰ In vitro assay confirmed the fitness advantage of *mcr-1*-harboring IncX4 plasmids.²¹ These results suggest that self-transmissible IncX4-type plasmids may contribute to the global dissemination of the *mcr-1* gene.^{22,23} Our study further highlights

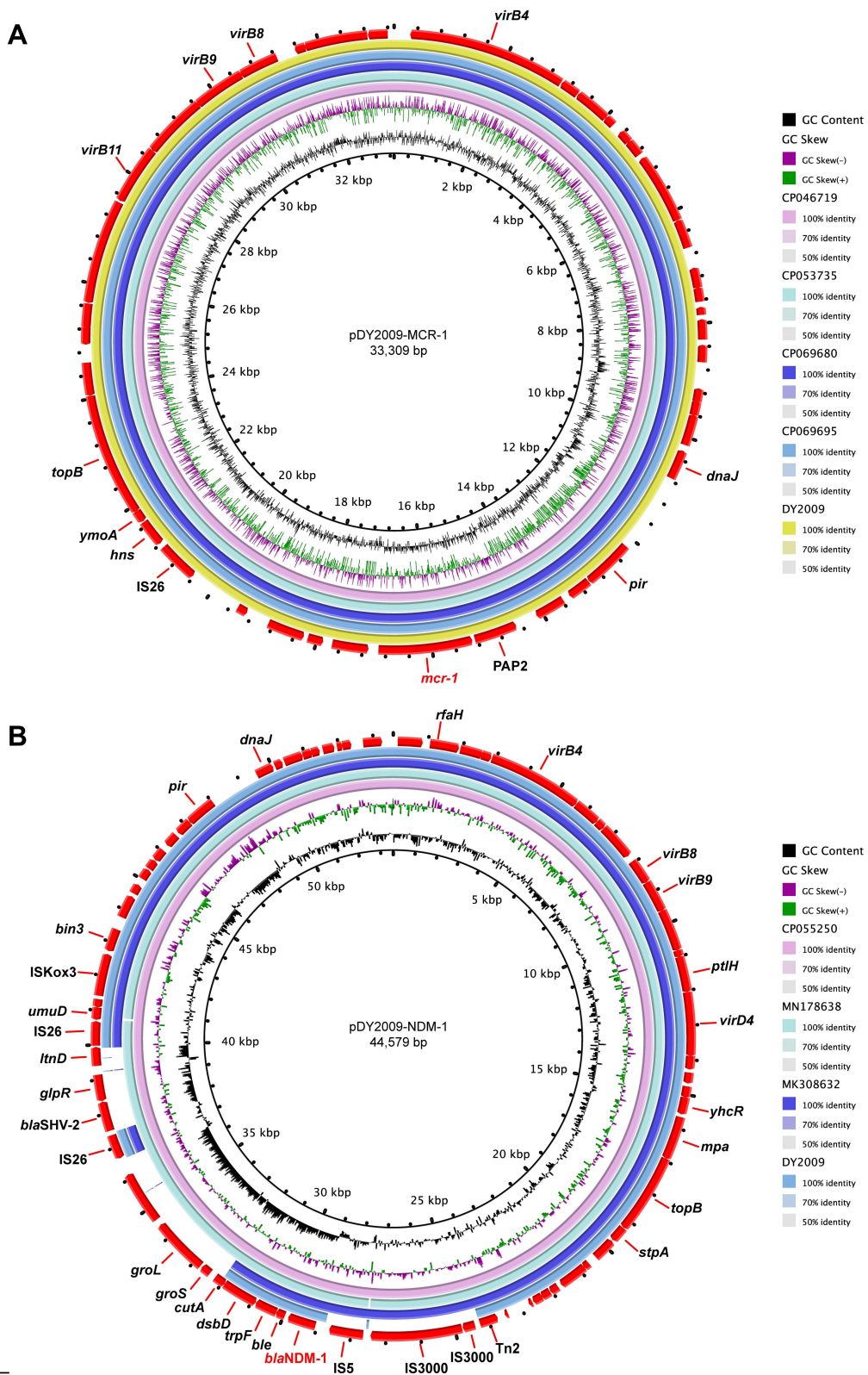


Figure 1 Alignment of *mcr-I/bla_{NDM-1}*-harboring plasmids detected in this study. **(A)** Genomic map of the *mcr-I*-carrying IncX4 pDY2009-MCR-1 plasmid with four closely related plasmids (CP046719, CP053735, CP069680, CP069695). **(B)** Circular genome alignment of pDY2009-NDM-1 with three *bla_{NDM-1}*-bearing IncX3 plasmids (CP055250, MN178638, MK308632). Arrows portrayed genes. The circular map was generated with the BLAST Ring Image Generator.

Table 2 Antimicrobial Resistance Genes Encoded by *Klebsiella pneumoniae* DY2009

Antimicrobial Agents	Resistance Gene
Streptogramin B	<i>erm(B)</i>
Aminoglycoside	<i>aadA1</i>
	<i>aph(6)-Id</i>
	<i>aph(3'')-Ib</i>
Polymyxin	<i>mcr-1.1</i>
Rifamycin	<i>ARR-2</i>
Sulfonamides and trimethoprim	<i>sul2</i>
	<i>dfrA14</i>
Quinolone	<i>qnrS1</i>
	<i>OqxA</i>
	<i>OqxB</i>
Fosfomycin	<i>fosA</i>
Tetracycline	<i>tet(A)</i>
β-lactam	<i>bla_{NDM-1}</i>
	<i>bla_{OXA-10}</i>
	<i>bla_{LAP-2}</i>
	<i>bla_{SHV-110}</i>
Amphenicol	<i>cmIA1</i>
	<i>floR</i>

that an effective prevention strategy should be taken to restrain the further dissemination of *mcr-1*-bearing IncX4 plasmids.

Blastn search further revealed that pDY2009-NDM-1 shared the highest similarity (100% identity with 91% coverage) with pZY-NDM1 (CP055250), pNDM1_SCW13 (MN178638), and pNDM5-LDR (MK308632). A conserved gene-environment around *bla_{NDM-1}* (IS5- *bla_{NDM-1}*-*trpF-dsbC-cutA1-groEL*) was observed in pDY2009-NDM-1 (Figure 1B). Previous studies indicated that most of the IncX3 plasmids were present in *E. coli*, followed by *K. pneumoniae*.²⁴ Of note, *bla_{NDM}* has been rapidly spreading in China mainly due to the national dissemination of *bla_{NDM}*-bearing IncX3 plasmid.^{25,26} Our study highlights that IncX3 plasmid may accelerate the occurrence of the carbapenem-resistant *K. pneumoniae* isolate in China.

In general, we isolated a hypervirulent colistin-resistant ST5571 CRKP strain DY2009 from a blood sample in China. The WGS and microbiological analysis were performed to elucidate its antimicrobial resistance mechanisms, which found that this strain contained 19 ARGs associated with its resistance phenotype. This is the first report in China of a clinical bacterial isolate co-producing MCR-1, NDM-1, and OXA-10, highlighting the necessity of active surveillance efforts for colistin- and carbapenem-resistant organisms in clinical practice settings. Thus, our findings improve the understanding of the genetic context of colistin- and carbapenem-resistant *K. pneumoniae* isolate from bacteremia and the resistance mechanisms.

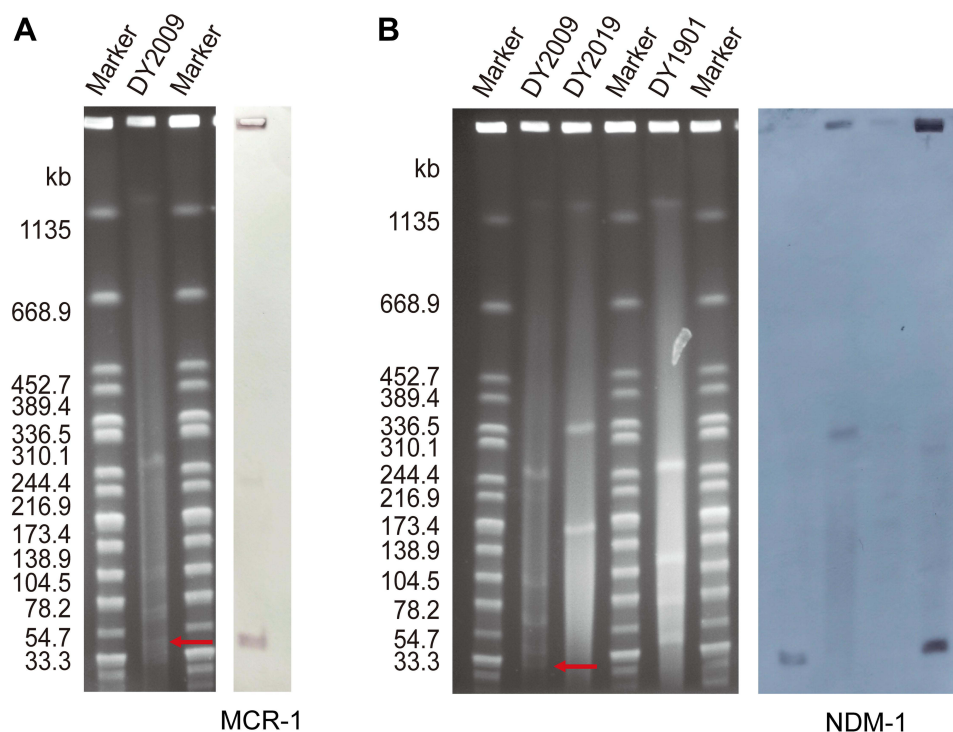


Figure 2 Plasmid analysis of the ST5571 colistin-resistant CRKP strain DY2009 via SI-PFGE and Southern blotting using *mcr-I* and *bla_{NDM-1}* probes. **(A)** Analysis of pDY2009-MCR-I. **(B)** Analysis of pDY2009-NDM-I. Due to the similar plasmid size of pDY2009-MCR-I and pDY2009-NDM-I, NDM-I-producing clinical isolates DY2019 and DY1901 recovered from the same hospital were used as control. The red arrows indicate the locations of the resistance plasmid.

Nucleotide Sequence Accession Numbers

The whole-genome sequencing of *K. pneumoniae* strain DY2009 has been deposited into DDBJ/EMBL/GenBank under the Biosample accession number SAMN25509743.

Ethics Approval and Consent to Participate

This study was conducted following the Declaration of Helsinki and obtained approval from the Medical Ethics Committee at The First Hospital of Jiaying. The patient provided written informed consent to allow the case details to be published.

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Disclosure

The authors report no conflicts of interest in this work.

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