Emergence of an NDM-5-Producing Hypervirulent *Klebsiella pneumoniae* Sequence Type 35 Strain with Chromosomal Integration of an Integrative and Conjugative Element, ICE*Kp1*

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ABSTRACT Here, we report an NDM-5-producing sequence type 35 (ST35) hypervirulent *Klebsiella pneumoniae* strain, isolated from the blood of a male patient. It showed a remarkable resistance to serum killing and neutrophil phagocytosis and high virulence in a mouse peritonitis infection model. Instead of carrying a pLVPK-like virulence plasmid, chromosomal integration of ICE*Kp1* (∼76 kb) was identified in a specific asparagine-tRNA gene, harboring the iron acquisition system salmochelin genes (*iroBCDN*), a yersiniabactin gene, and a variant of the *rmpA* gene.

KEYWORDS carbapenem-resistant hypervirulent *K. pneumoniae*, NDM-5, ICE*Kp1*, ST35

The emergence and global dissemination of carbapenem-resistant hypervirulent *Klebsiella pneumoniae* (CR-hvKp) pose a significant therapeutic challenge to public health, leaving few treatment options for the severe infections caused by these strains (1). Previous studies indicated that such strains were developed through two mechanisms. The first is acquisition by an hvKp strain of a conjugal plasmid that contains carbapenem resistance determinants (2, 3), while the second is acquisition of the hvKp virulence plasmid by carbapenem-resistant *K. pneumoniae* (CRKP) strains (4, 5). However, the species-specific integrative and conjugative element ICE*Kp*, harboring biosynthetic genes for siderophores, plays an important role in the pathogenesis and evolutionary history of *K. pneumoniae* (6, 7). The horizontal transfer of ICE*Kp* provides an important mechanism for virulence factors to spread within the general population of *K. pneumoniae* (6, 7). Here, we report an NDM-5-producing CR-hvKp strain, RJY9645, with chromosomal integration of ICE*Kp1*, harboring the iron acquisition system salmochelin genes (*iroBCDN*), a yersiniabactin gene, and a variant of the *rmpA* gene.

A total of 345 clinical CRKP isolates were recovered in our hospital over 3 years (2016 to 2018). All isolates were screened for carbapenemases (bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM}, and bla_{OXA-48}) and virulence genes (*iucABCDiutA*, *iroBCDN*, *rmpA,* and *rmpA2*) (see Table S1 in the supplemental material) (8, 9). Only one strain, *K. pneumoniae* RJY9645, displayed positive detection of bla_{NDM-5}, and the virulence genes *iroBCDN* and *rmpA* were also detected (Table 1). *K. pneumoniae* RJY9645 was resistant to ceftazidime, ceftriaxone, cefepime, piperacillin-tazobactam, imipenem, and mempenem, as determined using the CLSI reference broth microdilution method (10). In contrast, it displayed susceptibility to aztreonam, amikacin, gentamicin, ciprofloxacin, levofloxacin, tigecycline, and polymyxin B (Table S2).

K. pneumoniae RJY9645 was recovered in the Department of Liver Surgery from the blood of a male patient. The patient was discharged after liver transplantation, but he had intermittent fever 3 weeks later and was hospitalized for further treatment. He had been on combination therapy with imipenem and levofloxacin for 3 weeks after the

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TABLE 1 Microbiological characteristics of *K. pneumoniae* RJY9645 and the control strains

Strain	Isolation source	Carbapenemase	ST	Serotype	String test	Serum killing (grade)[a]	Phagocytosis rate[b]	Virulence genes present				LD$_{50}$ (CFU)
								iucABCDiutA	*iroBCDN*	*rmpA*	*rmpA2*	
RJY9645	Blood	NDM-5	ST35	K108	+	4	R	−	+	+	−	~10^4
HAP-Kp11	Sputum	KPC-2	ST11	K47	−	1	S	−	−	−	−	>10^6
LA-Kp23	Liver pus		ST23	K1	+	5	R	+	+	+	+	~10^2

[a]A strain was defined as serum sensitive at grades of 1 and 2, intermediately resistant at 3 and 4, and resistant at 5 and 6.
[b]R, phagocytic rate of <50% at 30 min; S, phagocytic rate of ≥50% at 30 min.

isolation of strain RJY9645 and was then discharged. However, he was admitted to the hospital again for the recurrent bloodstream infection only 1 week later, with the positive blood culture of *K. pneumoniae* RJY9699, which displayed identical pulsed-field gel electrophoresis (PFGE) patterns with RJY9645 and belonged to the same clone (Fig. S1). Since RJY9645 was resistant to imipenem (MIC, 16 μg/ml), the combination therapy of imipenem and levofloxacin is suboptimal and may contribute to the reoccurrence of the bloodstream infection. For the second episode of bloodstream infection, the patient received 3 weeks of antimicrobial therapy of aztreonam and levofloxacin and was discharged after 4 weeks of hospitalization. From then on, the patient was seen for follow-up visits once every 3 months, and he had no recent history of foreign travel.

K. pneumoniae RJY9645 showed a hypermucoviscous phenotype, with a positive string test. Strain virulence was estimated using serum killing, neutrophil phagocytosis, and mouse lethality assays. Serum resistance and neutrophil phagocytosis assays were performed as previously described (11, 12). The mouse lethality assay was performed for estimation of the *in vivo* virulence, and the 50% lethal dose (LD$_{50}$) was determined with pathogen-free, 6- to 8-week-old, female BALB/c mice. A 10-fold serial dilution of CFU was made from a starting concentration of 10^6 CFU/ml to 10^2 CFU/ml, and BALB/c mice were infected intraperitoneally with 0.1 ml of each concentration. A total of six mice were used as a sample population for each bacterial concentration. Symptoms and mortality rates were observed for 14 days. The exact inoculation dose was confirmed on LB agar, and the LD$_{50}$ was calculated using the SigmaPlot program (version 11.0). One KPC-2-producing *K. pneumoniae* strain, HAP-Kp11, which was associated with hospital-acquired pneumonia and belonged to sequence type 11 (ST11), was used as the low-virulence control (Table 1). In contrast, one ST23 *K. pneumoniae* strain, LA-Kp23, isolated from the abscess drainage of a patient with community-acquired liver abscess, was used as the hypervirulence control (Table 1).

K. pneumoniae RJY9645 exhibited grade 4 response to serum killing, with viable counts of >100% of the inoculum after both 1 and 2 h but <100% after 3 h (Fig. 1 and Table 1). Also, RJY9645 had a survival rate of >50% after incubation with human neutrophils for 30 min, and the survival of both RJY9645 and LA-Kp23 was significantly higher than that of HAP-Kp11 at each time point ($P < 0.05$ by one-way analysis of variance [ANOVA], Fig. 1). The mouse lethality assay indicated that RJY9645 showed high virulence, with an LD$_{50}$ of 10^4 CFU (Table 1). When infected intraperitoneally with 5×10^4 CFU, RJY9645 killed four of the six mice tested within 4 days (Fig. 1).

Strain RJY9645 was subjected to whole-genome sequencing using a MiSeq platform (Illumina, USA) and long-read PromethION platform (Nanopore, Oxford, United Kingdom). Nanopore reads were assembled using CANU version 1.7, which returned four contigs. The sequences were polished by Illumina reads to decrease the error rates. The genome size was 5,587,671 bp, including a circular chromosome of 5,269,438 bp, with an average GC content of 57.6% and three circular plasmids, pY9645-166 (166,344 bp), pY9645-105 (105,728 bp), and pY9645-NDM5 (46,161 bp). The sizes of the plasmids were confirmed using S1-PFGE (Fig. S1). *K. pneumoniae* RJY9645 belonged to ST35 and capsular type K108 (*wzi_allele* 194), as determined using the assembled contigs to query the multilocus sequence typing (MLST) and *wzi* allele (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html) databases. Phylogenetic analysis of RJY9645 was performed using RAxML version 8.0 (13) with the whole genomes of other representative *K.*
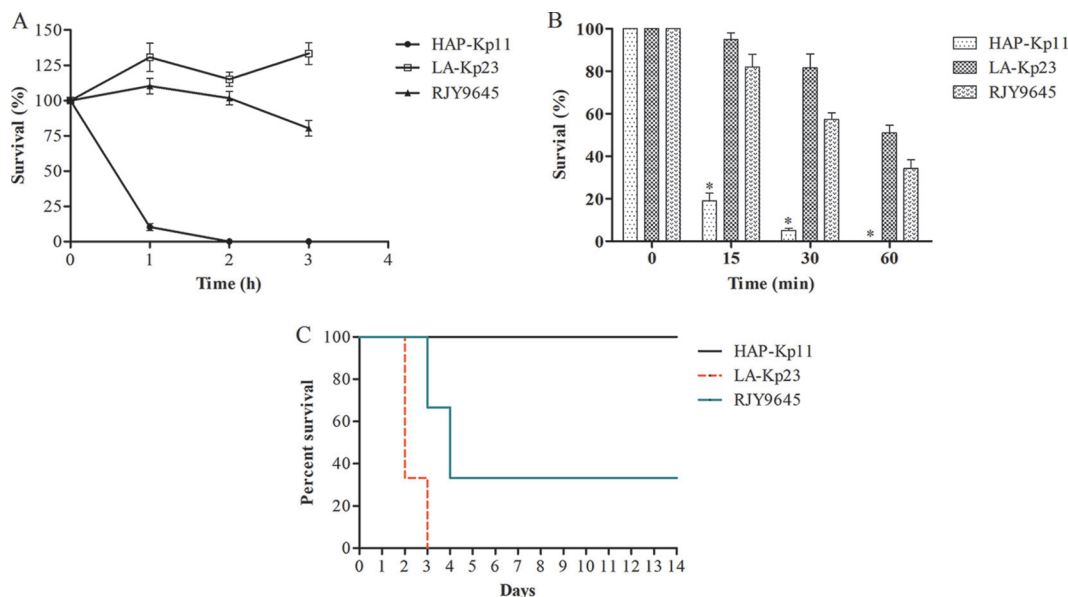
FIG 1 Virulence characteristics of *K. pneumoniae* RJY9645. (A) Serum resistance assay. *K. pneumoniae* RJY9645 exhibited a grade 4 response, with viable counts of >100% of the inoculum after both 1 and 2 h but <100% after 3 h. The hypervirulence control LA-Kp23 and the low-virulence control HAP-Kp11 exhibited grade 5 and grade 1 responses, respectively. (B) Human neutrophil phagocytosis assay. The data are presented as the means ± standard deviation. The survival of RJY9645 and LA-Kp23 was significantly higher than that of HAP-Kp11 at each time point (*, $P < 0.05$, one-way ANOVA). (C) Survival curves for *K. pneumoniae* RJY9645-infected mice. Mice were intraperitoneally infected with 5×10^4 CFU of each strain. Six mice were used as a sample population for each strain. Symptoms and mortality rates were observed for 14 days.

pneumoniae strains available in GenBank (Table S3). RJY9645 was grouped with classical *K. pneumoniae* (cKp) strains, such as OXA-232-producing UCLAOXA232KP and KPC-2-producing HS11286 (Fig. S2). In contrast, RJY9645 was distant from the clades grouped by ST23 or K2 (ST66 or ST86) hvKp strains.

Unlike the previously reported CR-hvKp strains, no pLVPK-like virulence plasmid was identified in the genome of *K. pneumoniae* RJY9645. Instead, the chromosomal integration of the integrative and conjugative element ICE*Kp1* (~76 kb) was spotted in a specific asparagine-tRNA gene. ICE*Kp1* was divided into three regions, with one similar to the high-pathogenicity island of *Yersinia* species encoding the siderophore yersiniabactin, followed by a middle region similar to a part of the large virulence plasmid pLVPK (GenBank accession no. AY378100), encoding the siderophore salmochelin (*iroBCDN*) and the capsular polysaccharide regulator RmpA, while the third region encoded the type IV secretion system (T4SS) (Fig. 2A). The genetic context of salmochelin located on ICE*Kp1* was distinct from pLVPK-carried *iro* lineage, with an inverted and preamble *iroN* sequence in gene cluster (Fig. 2B). In order to exclude genome misassembly events, PCRs verifying the junctions were performed between the virulence cluster (*iroBCDN* and *rmpA*) and ICE*Kp1* and between the intact ICE*Kp1* and chromosome, respectively (Table S1). Apart from ICE*Kp1*, another four chromosomally borne ICE variants were identified by VRprofile, with default parameters (14), including two different type VI secretion system (T6SS) loci and two prophages encoding the prophage core component proteins (Table S4). Other chromosomally borne virulence genes, such as type 1 fimbrial genes (*fimACDGH*) and an enterobactin gene cluster (*entABCEF*), have also been identified.

The *bla*$_{NDM-5}$ gene was located on an IncX3 plasmid of 46,161 bp, designated pY9645-NDM5, which could be transferred to *Escherichia coli* strain J53 through a conjugation assay. The *bla*$_{NDM-5}$-carrying plasmid could be transferred at a frequency of 10^{-4} transconjugants per donor cell. This plasmid displayed 100% coverage and 99% identity to the epidemic *bla*$_{NDM-5}$-carrying plasmid pP768-NDM5 in China (GenBank accession no. MF547510) (15), and only two nucleotide substitutions were identified.
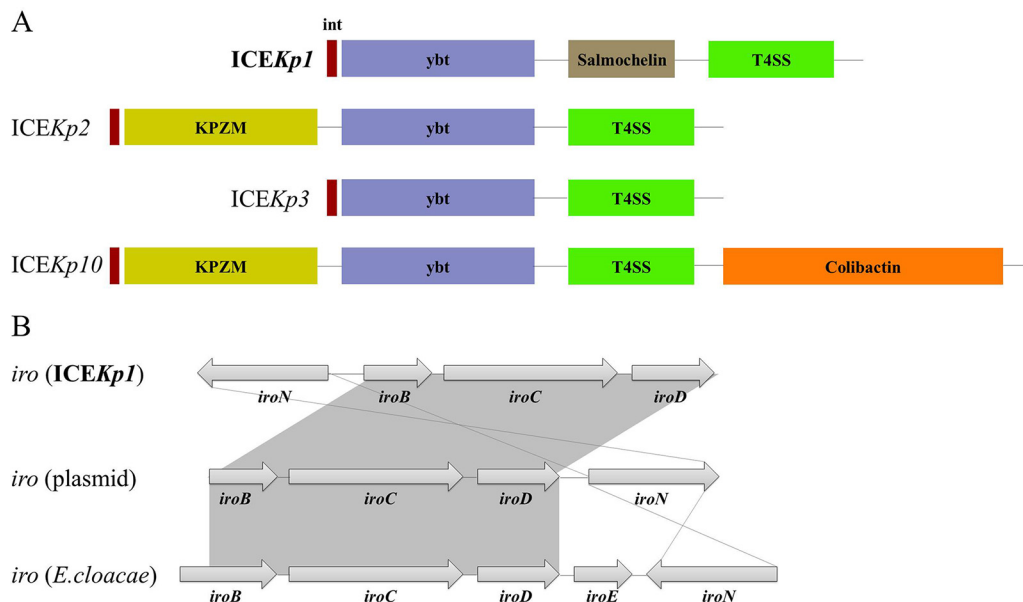
FIG 2 Structures of the representative ICE*Kp* variants and salmochelin (*iro*) locus. (A) The representative ICE*Kp* structures. *ybt*, yersiniabactin synthesis locus; T4SS, type 4 secretion system; KPZM, Zn^{2+}/Mn^{2+} metabolism module. (B) Salmochelin (*iro*) locus. The gray area indicates that this region was identical between different sequences. The *iro* lineages were based on the sequences of ICE*Kp1*, plasmid pLVPK, and *Enterobacter cloacae iroBCDEN* under GenBank accession numbers AB298504, AY378100, and CP032832.

The pY9645-NDM5 plasmid did not carry resistance genes other than *bla*$_{NDM-5}$. In addition, most of the *bla*$_{NDM-5}$-carrying plasmids reported in China belonged to the IncX3 type and showed highly identical nucleotide sequences (15, 16). The IncX3-type plasmids facilitate the rapid dissemination of *bla*$_{NDM-5}$ among *Enterobacteriaceae* in China. Other resistance genes of *K. pneumoniae* RJY9645 were predicted using Res-Finder (Center for Genomic Epidemiology, http://genomicepidemiology.org/). A chromosomally borne broad-spectrum β-lactamase gene (*bla*$_{SHV-33}$), a fosfomycin resistance determinant gene (*fosA*), and efflux pump genes (*oqxAB*) were identified, and strain RJY9645 was found to display resistance to fosfomycin through antimicrobial susceptibility testing using a disk diffusion method.

Instead of carrying a pLVPK-like virulence plasmid, chromosomal integration of ICE*Kp1* was identified in *K. pneumoniae* RJY9645. ICE*Kp* is the most common virulence-associated mobile genetic element of *K. pneumoniae*, and 14 different structural variants have been reported to date (6). ICE*Kp1* was first described in hvKp strain NTUH-K2044 and was more prevalent in hvKp than in cKp strains, suggesting a role in hvKp pathogenesis (17). The sequence of ICE*Kp1* in RJY9645 displayed 100% coverage and 99% identity to that of strain NTUH-K2044. Though all ICE*Kp* variants carried yersiniabactin, only ICE*Kp1* harbored the salmochelin biosynthesis gene cluster (6). Yersiniabactin has been shown to be an important factor for cKp in mouse infection models (18). Salmochelin is hvKp specific and does not just act as a siderophore. In conjunction with microcin E492, salmochelin is also a critical mediator of hvKp colonization (19). Together with the *rmpA* gene, the chromosomal integration of ICE*Kp1* may facilitate genetic diversity and contribute significantly to the hypervirulence in *K. pneumoniae* RJY9645.

ST35 *K. pneumoniae* is an international multidrug-resistant clone. It has worldwide distribution and has been reported in Tunisia (20), Spain (21), Denmark (22), France (23), and Yemen (24). Various extended-spectrum β-lactamases (ESBLs) have been detected in ST35 *K. pneumoniae* strains, such as CTX-M-15 and SHV-12 (20, 21). Recently, one NDM-1-producing ST35 *K. pneumoniae* strain was recovered from the blood of a 5-year-old boy in a pediatric intensive care unit (24). However, little attention has been paid to the pathogenicity of ST35 *K. pneumoniae*. To the best of our knowledge, this is

the first report of ST35 NDM-5-producing CR-hvKp with chromosomal integration of ICE*Kp1*.

A previous comparative analysis of 2,498 whole-genome sequences of *K. pneumoniae* indicated that independent ICE*Kp* integration events were detected, affecting hundreds of phylogenetically distinct lineages, including CRKP and hvKp strains (6). The horizontal gene transfer of ICE*Kp1* provided an important mechanism for the yersiniabactin, salmochelin, and *rmpA* virulence genes to spread within *K. pneumoniae* populations. Also, NDM-5-positive *K. pneumoniae* strains are distributed across a number of sequence types (STs), with no predominant lineages (25). Furthermore, RJY9645 exhibited a cKp genetic background distant from the clades formed by K1 (ST23) or K2 (ST66 or ST86) hvKp strains. Taken together, the development of CR-hvKp strain RJY9645 might be attributed to chromosomal integration of ICE*Kp1* and acquisition of the bla_{NDM-5}-carrying plasmid.

All procedures performed in this study involving human participants and animals were in accordance with the ethical standards of the institutional review board ethics committee of Renji Hospital, School of Medicine, Shanghai Jiaotong University. For this type of retrospective study, formal consent is not required.

Data availability. The complete sequences of the chromosome of strain RJY9645 and plasmids pY9645-166, pY9645-105, and pY9645-NDM5 have been deposited in GenBank under accession numbers CP041353, CP044029, CP044030, and MN064714, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.
SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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