Evolution of Carbapenem-Resistant Serotype K1 Hypervirulent *Klebsiella pneumoniae* by Acquisition of *bla*_{VIM-1}-Bearing Plasmid

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ABSTRACT We report the identification of a carbapenem-resistant, hypervirulent *Klebsiella pneumoniae* (hvKp) strain which produced the carbapenemase VIM-1. Genomic analysis showed that the strain belonged to sequence type ST23 and serotype K1, a major hvKp clone, and harbored three resistance-encoding plasmids. Among them, a *bla*_{VIM-1}-bearing plasmid was found to possess a mosaic structure presumably generated by multiple gene mobilization events. This finding indicates that hvKp actively acquires mobile resistance-encoding elements, facilitating simultaneous expression of hypervirulence and carbapenem-resistance.

KEYWORDS *Klebsiella pneumoniae*, VIM, carbapenem resistance, hypervirulence

The emergence of carbapenem-resistant, hypervirulent *Klebsiella pneumoniae* (CR-hvKp) strains as a result of acquisition of a virulence plasmid by carbapenem-resistant *K. pneumoniae* of a specific sequence type, such as ST11, or of uptake of a carbapenemase-producing plasmid by hypervirulent clones, such as ST23, poses a serious threat to public health (1). Three major types of carbapenemase are known to be produced by carbapenem-resistant *Enterobacteriaceae*, namely, Ambler classes A (KPCs), B (NDMs, IMPs, VIMs), and D (OXA-48) β -lactamases (2). KPC- and NDM-producing hypervirulent *K. pneumoniae* (hvKp) have been reported by countries all over the world (3, 4), whereas VIM-producing *K. pneumoniae* isolates are mainly confined to Italy and Greece. hvKp strains producing VIM are rarely reported, and hence, little information regarding the *bla*_{VIM-1}-bearing plasmids that are transmissible among hvKp strains is available (5, 6). In this study, we used the whole-genome sequencing (WGS) approach to perform comprehensive characterization of both the chromosome and plasmids of a VIM-1-producing hvKp strain which caused nosocomial infections in China.

A retrospective genomic study led to the identification of a VIM-1-producing strain, R210-2, which was originally isolated in May 2018 from a feces sample from a 5-month-old male infant who suffered from upper respiratory tract infection and diarrhea. R210-2 was identified as *K. pneumoniae* using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), and the species identity was further confirmed by sequencing of the 16S rRNA gene. Antimicrobial susceptibility was determined using the broth microdilution method and interpreted according to the CLSI guidelines (7). The strain exhibited a multi-drug resistant (MDR) profile involving imipenem (4 μ g/ml), meropenem (4 μ g/ml), cefmetazole (16 μ g/ml), ceftazidime (>128 μ g/ml), cefotaxime (>128 μ g/ml), piperacillin-tazobactam (256/4 μ g/ml), cefoperazone-sulbactam (256/128 μ g/ml), ceftazidime-avibactam (>64/4 μ g/ml), cefepime (>64 μ g/ml), and aztreonam (64 μ g/ml) but remained sus-

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ceptible to ertapenem ($\leq 2 \mu\text{g/ml}$), polymyxin B ($\leq 0.5 \mu\text{g/ml}$), tigecycline ($\leq 0.25 \mu\text{g/ml}$), ciprofloxacin ($\leq 1 \mu\text{g/ml}$), and amikacin ($\leq 4 \mu\text{g/ml}$) (http://www.eucast.org/clinical_breakpoints/).

Strain R210-2 was hyper-mucoviscous and exhibited a positive string test by forming a viscous string of $>5 \text{ mm}$ upon touching and stretching the colonies with a loop. The virulence level of strain R210-2 was tested in wax moth (*Galleria mellonella*) larvae weighing about 300 mg ($n = 16$ in each group) at the bacterial infection concentration of $1 \times 10^6 \text{ CFU}$ and in 3- to 4-week-old healthy male NIH mice ($n = 4$ in each group) using methods described previously (8, 9). Mice were injected at the infection concentration of $1 \times 10^7 \text{ CFU}$ through the tail vein. The numbers of dead larvae and mice were recorded every 12 h for 48 h. CR-hvKp strain *K. pneumoniae* 4 and the carbapenem-resistant nonhypervirulent strain PC-*K. pneumoniae* 4, both from a previous study, were used as positive and negative controls, respectively, for the virulence assays (10). At the inoculum of $1 \times 10^6 \text{ CFU/ml}$ at 48 h postinfection (hpi), survival rates of *G. mellonella* were 10% with both strain R210-2 and the strain *K. pneumoniae* 4, while the survival rate with strain PC-*K. pneumoniae* 4 was 70% (Fig. S1A). In the mouse infection model, both R210-2 and hypervirulent control strain *K. pneumoniae* 4 led to 100% mortality at 48 hpi with the inoculum of $1 \times 10^7 \text{ CFU/ml}$, while low-virulence control strain FJ8 did not cause any mortality in mice, suggesting that R210-2 is hypervirulent (Fig. S1B) (10).

WGS was carried out by using both the Illumina NextSeq 500 (Illumina, USA) and Oxford Nanopore MinION (Oxford Nanopore Technologies [ONT], UK) platforms. All reads were *de novo* assembled using the Unicycler software v0.4.4 and polished with Pilon (11). The multilocus sequence type (MLST), serotype, virulomes, and resistomes were screened for using Kleborate (12). The plasmid incompatibility type and mobile elements were determined using the bioinformatics tools available from the Center for Genomic Epidemiology (<http://genomicepidemiology.org/>). *K. pneumoniae* strain R210-2 was found to harbor a circular chromosome of 5,464,394 bp and three plasmids with sizes of 229,648 bp (pR210-2-vir), 144,158 bp (pR210-2-VIM), and 113,569 bp (pR210-2-CTX). Bioinformatics analysis revealed that R210-2 belonged to sequence type 23 (ST23) and serotype K1, which is a major clone of hvKp (13). Regarding virulence, the *mrkABCDJHIH* (type 3 fimbriae), *clbABCDEFGHIJKLMNPOQR* (colibactin), *fyuA-irp1-irp2-ybtSXQPA* (yersiniabactin), *mceABCDEJIHG* (microcin E492), *kfuABC* (ABC iron transport system), *arcC* (carbamate kinase), *allABCDRS* (associated with allantoin metabolism), and *glxKR* (glycerate pathway) genes were detected in the chromosome of strain R210-2. The chromosome of this strain was also found to harbor the *bla*_{SHV-190} gene, which confers penicillin resistance, and the *oqxAB* gene, which is associated with quinolone resistance.

Plasmid pR210-2-vir was found to comprise 263 predicted open reading frames (ORFs), with a G+C content of 50.1%. It was an IncHI1B/IncFIB_K plasmid which was highly homologous to a virulence plasmid (pSGH10) recovered from an ST23 hvKp strain, with 99% similarity at 100% coverage (Fig. S2). The plasmid carried numerous virulence genes, including *iutAiuABCD* (aerobactin), *iroBCDN* (salmochelin), *rmpA*, and *rmpA2* (activator for capsular polysaccharide synthesis) and was therefore regarded as being strongly associated with the hypervirulence phenotype of strain R210-2 (10). The second plasmid that strain R210-2 harbored, pR210-2-VIM, was found to belong to the IncA group of plasmids, comprised 165 ORFs, and exhibit a G+C content of 50%. BLAST analysis showed that pR210-2-VIM was 99% identical to plasmid pKC-BO-N1-VIM (GenBank accession number [MG228427](#)), which was recovered from a *Kluyvera cryocrescens* strain, and plasmid pIncAC-KP4898 (GenBank accession number [KY882285](#)), which was recovered from an ST104 *K. pneumoniae* strain, both at 99% coverage (Fig. S3) (14, 15). The main structural difference between pR210-2-VIM and pKC-BO-N1-VIM lies in the resistance island (RI) (Fig. 1A). The RI of pR210-2-VIM (RI-R210-2-VIM) was 29,498 bp in size and comprised two intact copies of IS26, one copy each of IS6100 and IS4321R, and several mobile genetic elements. Therefore, the mosaic structure of the RI in pR210-2-VIM was possibly generated as a result of multiple gene mobilization events. On the other hand, the RI of pKC-BO-N1-VIM (RI-KC-BO-N1-VIM) was 55,739 bp in size

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Tn402, bracketed by 27-bp (ATCTCGACTATGCTCAATACTCGTGTG) inverted repeats was observed (15, 16). The In1461 element, which carries the cassette array (*int1*, *bla*_{VIM-1}, *aacA4*, *aphA15*, *aacA4*, *aphA15*, *aadA1b*, and *catB2*) and 5' and 3' conserved sequences (CSs) was found located within the Tn402 in the RI-R210-2-VIM fragment (Fig. 1B). Conjugation experiments were performed as previously described to test the transferability of the plasmid pR210-2-VIM using a rifampin-resistant *Escherichia coli* EC600 (Rif^r) strain as the recipient, and MacConkey agar supplemented with 600 µg/ml rifampin and 0.03 µg/ml meropenem was used to select transconjugants (17). pR210-2-VIM was transferred via conjugation, and the transconjugant also exhibited resistance toward most of the antibiotics tested, including imipenem (4 µg/ml), meropenem (4 µg/ml), ertapenem (4 µg/ml), cefmetazole (32 µg/ml), ceftazidime (>128 µg/ml), cefotaxime (>128 µg/ml), piperacillin-tazobactam (>256/4 µg/ml), cefoperazon-sulbactam (256/4 µg/ml), ceftazidime-avibactam (>64/4 µg/ml), and cefepime (>64 µg/ml) but remained susceptible to polymyxin B (≤0.5 µg/ml), tigecycline (≤0.25 µg/ml), ciprofloxacin (≤1 µg/ml), amikacin (≤4 µg/ml), and aztreonam (≤4 µg/ml).

The third plasmid harbored by strain R210-2, pR210-2-CTX, was an IncFII plasmid which comprised 137 ORFs with a G+C content of 50.9%. It was 99% identical to plasmid pSCM96-1 (GenBank accession number [CP028717](#)), which was recovered from a clinical *K. pneumoniae* strain, at 90% coverage (Fig. S4) (18). The major structural difference between the two plasmids lies in the MDR region. The MDR region in pR210-2-CTX was found to harbor the resistance genes *qnrS*, *bla*_{CTX-M-15}, and *bla*_{TEM-1C} and was bounded by the IS26 and a truncated Tn3 element, indicating that this element was acquired as a result of activities of mobile genetic elements. The *bla*_{CTX-M-15} and *bla*_{TEM-1C} genes were within the genetic structure ΔTn3-ISEcp1-*bla*_{CTX-M-15}-orf-ΔTn3(*bla*_{TEM-1C}).

In conclusion, we report the identification of an emerging carbapenem-resistant, hypervirulent *K. pneumoniae* strain which produced the carbapenemase VIM-1. Genomic analysis showed that the *bla*_{VIM-1}-bearing plasmid and other MDR-encoding plasmids could be readily acquired by a typical ST23 hvKp strain through horizontal gene transfer. The emergence of genetically diverse CR-hvKp isolates highlights an urgent need to monitor plasmid-mediated convergence of hypervirulence and carbapenem-resistance in *K. pneumoniae* in clinical settings.

Nucleotide sequence accession numbers. The complete nucleotide sequences of strain R210-2 have been deposited in GenBank under the accession numbers [CP034082](#) to [CP034085](#).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.01056-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

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We declare no conflict of interest.

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