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MECHANISMS OF RESISTANCE

Carbapenem-Resistant Hypervirulent *Klebsiella pneumoniae* of Sequence Type 36

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ABSTRACT Sequence type 36 (ST36) *Klebsiella pneumoniae* is distributed worldwide. We found an ST36 *K. pneumoniae* clinical isolate that was carbapenem resistant, carried bla_{KPC-2}, had mucoid regulator gene *rmpA*, and exhibited high virulence. The finding suggests the emergence of carbapenem-resistant hypervirulent *K. pneumoniae* of ST36, and surveillance of carbapenem-resistant hypervirulent *K. pneumoniae* is required.

KEYWORDS carbapenem resistance, hypervirulence, *Klebsiella pneumoniae*

Hypervirulent *Klebsiella pneumoniae* (hvKP) is a particular threat because it can cause severe infections in apparently healthy people, with high mortality rates (1). hvKP is usually susceptible to carbapenems, and its infections have been successfully treated using carbapenems (1, 2). However, some carbapenem-resistant *K. pneumoniae* strains of the widely distributed sequence type 11 (ST11) became hypervirulent by acquiring a pLVPK-like virulence plasmid (3). The combination of carbapenem resistance and hypervirulence significantly compromises the options of antimicrobial agents for treating the life-threatening infections caused by the carbapenem-resistant hvKP and therefore represents a major urgent challenge for clinical treatment, infection control, and public health (4). Carbapenem-resistant hvKP is not restricted to ST11 (5, 6), as such strains can emerge either from carbapenem-resistant strains by acquiring the virulence plasmid or from hvKP by acquiring carbapenem resistance. Here, we report the acquisition of carbapenem resistance in a non-ST11 hvKP strain.

Strain WCHKP13F2 was recovered in the intensive care unit of a hospital in Sichuan province in 2015 from the blood of a male patient in his late 40s with severe burns. The patient developed a health care-associated bloodstream infection, with rigors, high fever (temperature >39°C), headache, and shock, that was caused by strain WCHKP13F2. He received the combination of imipenem and amikacin plus surgical debridement. However, he had a poor outcome and was discharged with unresolved critical illness on his own will. The strain was identified as *K. pneumoniae* by Vitek II (bioMérieux, Marcy-l'Étoile, France) and was resistant to aztreonam (MIC, 128 μg/ml), ceftazidime (MIC, 16 μg/ml), gentamicin (MIC, 32 μg/ml), imipenem (MIC, 32 μg/ml), meropenem (MIC, 16 μg/ml), piperacillin-tazobactam (MIC, 256/4 μg/ml), sulfisoxazole (MIC, >512 μg/ml), and tigecycline (MIC, 4 μg/ml); intermediate to levofloxacin (MIC, 4 μg/ml); and susceptible to amikacin (MIC, 1 μg/ml) and colistin (MIC, 1 μg/ml), as determined by the microdilution method of the Clinical and Laboratory Standards Institute (CLSI) (7). The strain was mucoid on the agar plate. A string test was performed by stretching bacterial colonies using an inoculation loop with a hypervirulent ST23:K1 *K. pneumoniae* strain WCHKP030925 used as a control (1). Strain WCHKP13F2 formed a 2-mm viscous string, which is below the >5 mm that defines hypermucous conditions.

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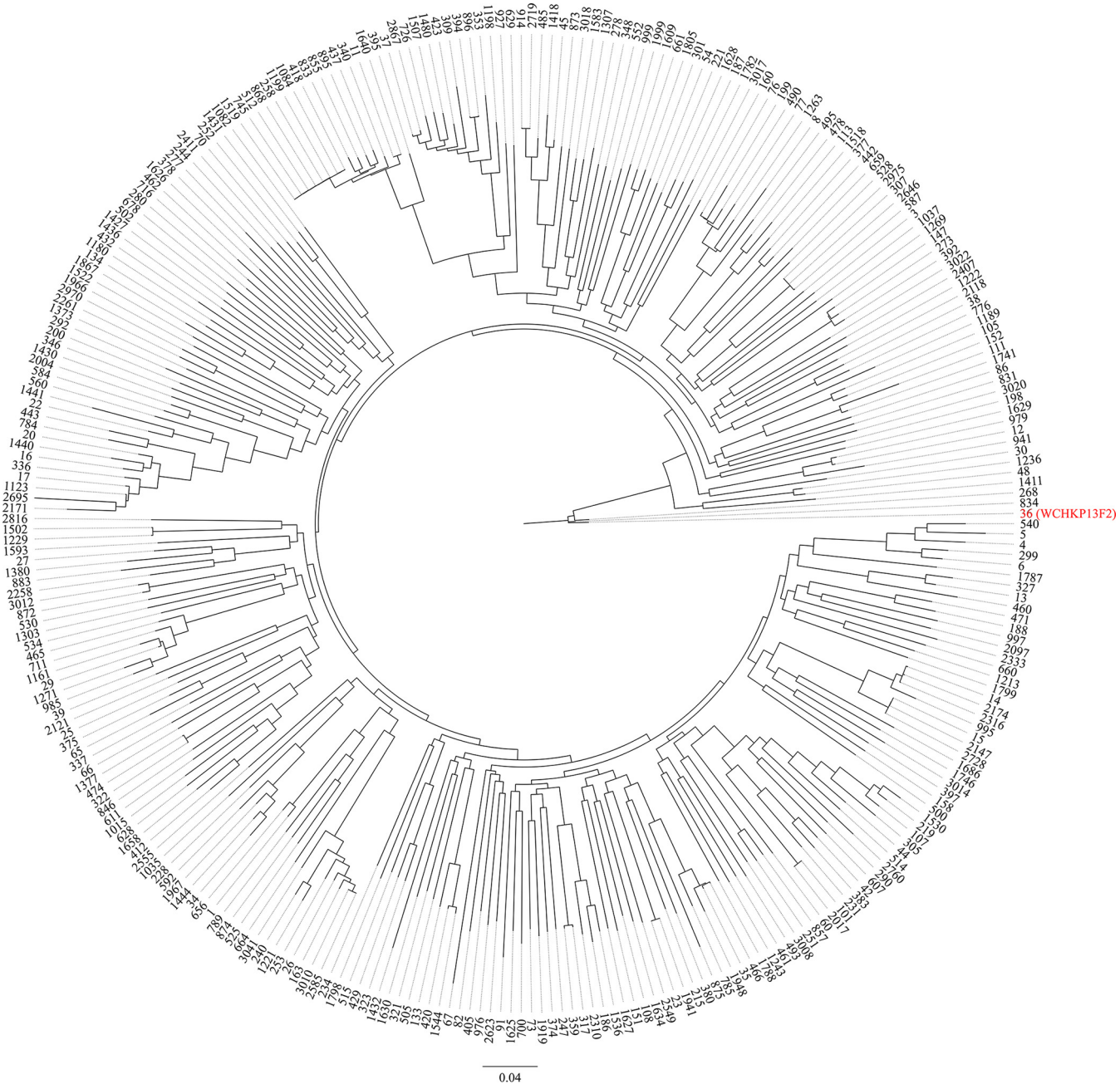

FIG 1 Core genome-based phylogenetic tree of *K. pneumoniae* belonging to different STs. ST36 is indicated in red. ST36 is closely related to ST268 and ST834 but is distinct from other STs.

The strain was subjected to whole-genome sequencing using both HiSeq X10 sequencer (Illumina, San Diego, CA), with the 150-bp paired-end protocol and 200× coverage, and the long-read MinION sequencer (Nanopore, Oxford, United Kingdom). The *de novo* hybrid assembly of both short and long reads was performed using Unicycler under the conservative mode for increased accuracy (8). The complete circular contigs generated were then corrected using Plion with Illumina reads for several rounds until no change was detected (9). The genome size of strain WCHKP13F2 was 5,749,649 bp, including a 5.38-Mb chromosome and two plasmids. Annotation was carried out using Prokka (10). Strain WCHKP13F2 belonged to ST36 (*gapA-infB-mdh-pgi-phoE-rpoB-tonB* allele number 2-1-2-1-7-1-7) and the capsular type K62, as determined using the assembled contigs to query the multilocus sequence typing (MLST)

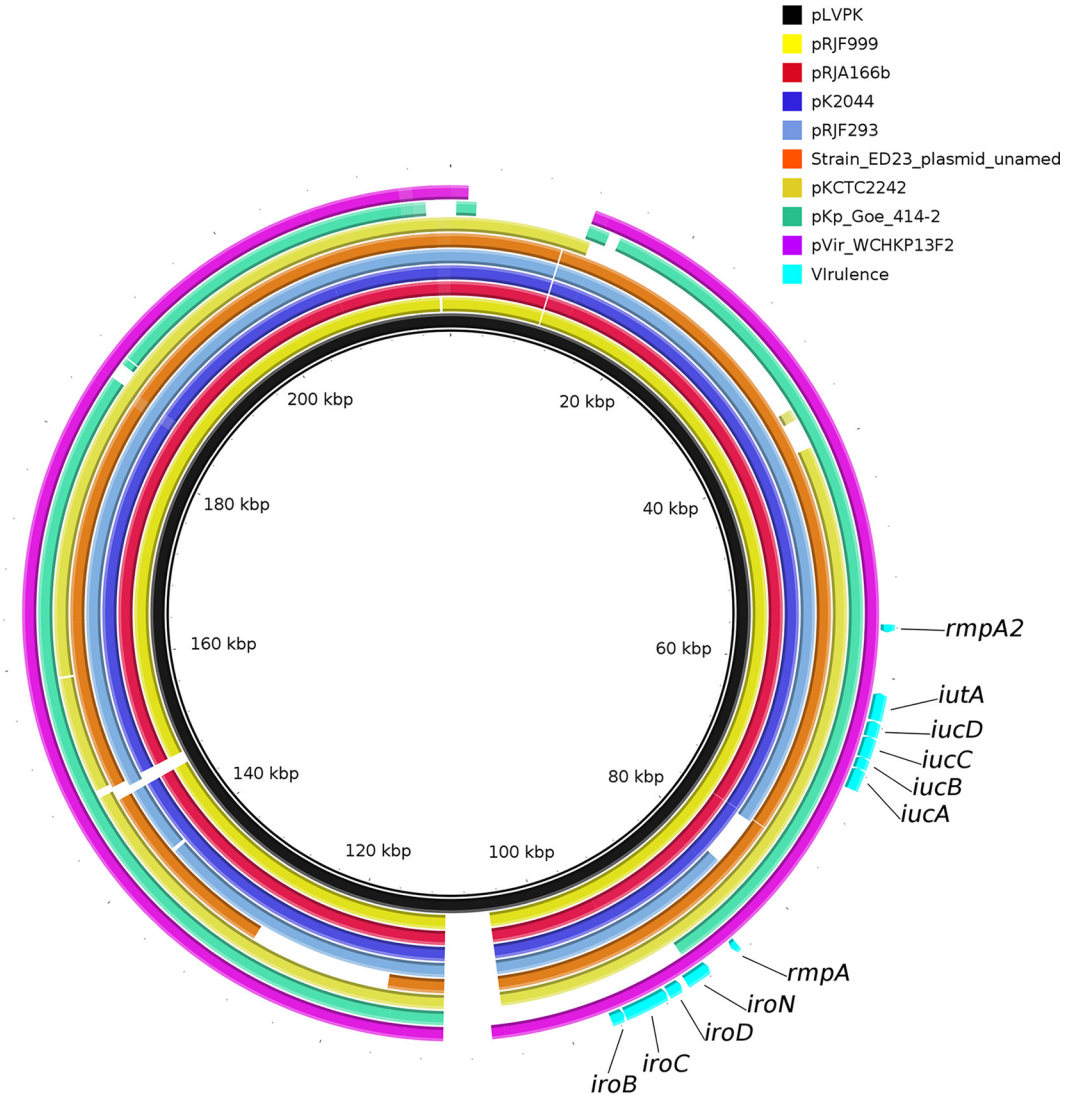

FIG 2 Alignment of pVir-KP13F2 and plasmids encoding hypervirulence. pLVPK is used as a reference. The alignment is a pairwise BLASTn alignment performed using BLAST Ring Image Generator (BRIG). Accession numbers for the plasmids are AP006726 (pK2044), AY378100 (pLVPK), CP016815 (unnamed plasmid of ED23), CP014011 (pRJF999), CP014009 (pRJF293), CP018338 (pKp_Goe_414-2), CP019049 (pRJA166b), CP002911 (pKCTC2242), and MF943217 (pVir-KP13F2). The locations of virulence genes *rmpA*, *rmpA2*, *iucABCD*, *iutA*, and *iroBCDN* are indicated.

and *wzi* allele databases (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). To study the phylogenetics of ST36 *K. pneumoniae* with those of other STs, all assembled *K. pneumoniae* genomes ($n = 3{,}423$; accessed by 27 March 2018) were retrieved from GenBank and were checked for quality and contaminations using Kraken with the MiniKraken 8-GB database (11). Assemblies with >200 contigs were discarded. MLST was performed using the script (https://github.com/tseemann/mlst). For each known ST, the complete genome or a draft one with a higher N50 value was selected as the representative, whereas the assemblies of unknown STs were excluded. A total of 299 assemblies with distinct STs were included and were annotated using Prokka (10), followed by identification of the core genome using Roary (12). A phylogenetic tree was inferred based on 86,396 SNP sites from 2,839 genes presented in all assemblies using RAxML with a GTRGAMMA model (13) (Fig. 1). ST36 is closely related to ST268 (2-1-2-1-7-1-81) and ST834 (2-1-2-1-7-1-25) with only one allele (*tonB*) variance among the three STs (Fig. 1).

It is known that ST36 *K. pneumoniae* is an hvKP that causes bacteremia (2) and has

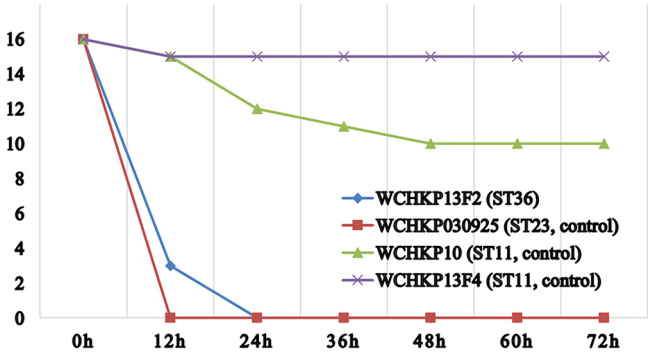

FIG 3 Survival of *G. mellonella* after infection by carbapenem-resistant strains. The effect of 1×10^6 CFU/ml of each hvKP isolate on survival of *G. mellonella* is shown, whereas those of other inoculums are shown in Table S1 in the supplemental material. WCHKP10 and WCHKP13F4 were two bla_{KPC-2}-carrying carbapenem-resistant *K. pneumoniae* clinical isolates of ST11 and were used as controls of low virulence. WCHKP030925 was a carbapenem-susceptible clinical isolate of ST23:K1 and was used as the control of hypervirulence.

worldwide distribution (14–16). Virulence genes were identified using the database available at http://bigsdb.pasteur.fr/klebsiella/klebsiella.html. Strain WCHKP13F2 had a number of virulence factors, including the regulator of mucoid phenotype (*rmpA*), aerobactin (*iucABCD*, *iutA*), colibactin (*clbA-R*), salmochelin (*iroN*, *iroBCDN*), yersiniabactin (*fyuA*, *irp2*, and *ybtAEPQTUX*) (17), and the type 3 fimbriae-encoding system (*mrkABCDFHIJ*) (18). Strain WCHKP13F2 also had a truncated *rmpA2* (another regulator of mucoid phenotype) gene, which was due to a mutation introducing a stop codon. The *rmpA*, *iroBCDN*, and *iucABCD* genes were carried by a 208,166-bp IncHI1/IncFIB plasmid, designated pVir-KP13F2, which was highly similar (97% coverage and 99% identity) to the known virulence plasmid pLVPK (GenBank accession no. AY378100) (Fig. 2). pLVPK (IncHI1/IncFIB) is 219,385 bp and possesses *rmpA*, *rmpA2*, *iucABCD*, *iutA*, and *iroBCDN*, while the recently identified IncHI1/IncFIB virulence plasmid pVir-CR-HvKP4 (178,154 bp) in ST11 carbapenem-resistant hvKP carries *rmpA2*, *iucABCD*, and *iutA* but not *rmpA* or *iroBCDN* (3).

Wax moth (*Galleria mellonella*) larvae weighing ~250 to 350 mg (purchased from Tianjin Huiyude Biotech Company, Tianjin, China) were used to assess the virulence of the strain. Overnight culture of strain WCHKP13F2 was washed with phosphate-buffered saline (PBS) and then adjusted with PBS to concentrations of 1×10^4 CFU/ml, 1×10^5 CFU/ml, 1×10^6 CFU/ml, and 1×10^7 CFU/ml. All experiments were performed in triplicate. An aliquot of 10 μl of each inoculum was injected into the hemocoel of 16 larvae via the last left proleg using 25-μl Hamilton syringes (19). The larvae were then incubated at 37°C in plastic containers. We counted the number of live larvae every 12 h for 3 days. Two carbapenem-resistant *K. pneumoniae* clinical isolates, WCHKP10 and WCHKP13F4, both of which carried bla_{KPC-2} and belonged to ST11, were used as the controls of low virulence. Carbapenem-susceptible hypermucous ST23:K1 *K. pneumoniae* strain WCHKP030925 was used as the control of hypervirulence. At an inoculum of 1×10^6 CFU/ml at 72 h after infection, survival of *G. mellonella* was 0% with strains WCHKP13F2 and WCHKP030925 and 62.5% and 93.8% with WCHKP10 and WCHKP13F4, respectively (Fig. 3; see Table S1 in the supplemental material). This suggests that strain WCHKP13F2 was truly hypervirulent.

Antimicrobial resistance genes were predicted using ResFinder (Center for Genomic Epidemiology, http://genomicepidemiology.org/). Strain WCHKP13F2 had the carbapenemase gene bla_{KPC-2}, which was carried by a 166,034-bp IncFII plasmid, designated pKPC-KP12F2. Conjugation was performed in broth using the azide-resistant *Escherichia coli* strain J53 as the recipient. Transconjugants were selected on agar plates containing 2 μg/ml meropenem and 150 μg/ml azide, and the presence of bla_{KPC-2} in transconjugants was examined by PCR. This suggests that pKPC-KP12F2 was a self-transmissible plasmid. Similar to its multidrug resistance phenotype, strain WCHKP13F2 had multiple

genes mediating resistance to aminoglycosides [*aac(6′)-Ib-cr*, *aac(3)-IId*, *aadA16*, *aph(3′)-Ia*, *strA*, *strB*], β-lactams (bla_{TEM-1}, bla_{SHV-11}), fosfomycin (*fosA*), macrolides [*mph*(A)], quinolones [*aac(6′)-Ib-cr*, *oqxA*, *oqxB*, *qnrS1*, *qnrB49*], rifampin (*arr3*), sulfonamides (*sul1*, *sul2*), tetracycline [*tet*(A)], and trimethoprim (*dfrA27*). bla_{SHV-11}, *fosA*, *oqxA*, and *oqxB* were located in the chromosome, whereas all other resistance genes were carried by pKPC-KP12F2.

The findings in the present study together with those from several recent reports (3, 5, 6) suggest that carbapenem-resistant hvKP isolates are diverse in clonal background and have emerged independently. As both carbapenem resistance and hypervirulence are encoded by plasmids, it is possible that carbapenem-susceptible strains may acquire plasmids carrying virulence genes or nonhypervirulent strains may acquire plasmids encoding carbapenem resistance to form carbapenem-resistant hvKP. Surveillance of carbapenem-resistant hvKP is urgently required to generate essential information for preventing their spread.

Accession number(s). Complete sequences of the chromosome of strain WCHKP13F2, plasmid pVir-KP13F2, and pKPC-KP12F2 have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers CP028389 to CP028391.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02644-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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