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Short Communication

A hybrid plasmid formed by recombination of a virulence plasmid and a resistance plasmid in *Klebsiella pneumoniae*

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ABSTRACT

Objective: The emergence of multidrug-resistant (MDR) and hypervirulent *Klebsiella pneumoniae* (hvKP) facilitates simultaneous dissemination of virulence and resistance in a single event, which poses serious threat to public health.

Methods: This study characterized the multidrug-resistant and moderately virulent ST11 K64 *K. pneumoniae* strain HB25-1 from a clinical case with microbiological and genomic approaches. Plasmids from strain HB25-1 were subjected to whole plasmid sequencing using both the Illumina NextSeq 500 sequencing platform and Nanopore MinION sequencer platforms. *Klebsiella pneumoniae* HB25-1 was subjected to a conjugation experiment and *Galleria mellonella* infection model to evaluate the transmission and virulence potential.

Results: We report the emergence of an ST11, serotype K64 *K. pneumoniae* isolate, which is resistant to third-generation cephalosporin and exhibited a moderate level of virulence. WGS revealed that this strain harboured a plasmid, pHB25-1, which carried multidrug resistance genes (bla_{DHA-1}, *qnrB4*, *dfrA12*, *aadA2*, *sul1*, *aac(3)-lld*, bla_{TEM-1}, *mph(E)*) and virulence-encoding genes (the regulator of mucoid phenotype A gene *rmpA2* and the aerobactin gene cluster *iutAiucABCD*). Genomic analysis indicated that pHB25-1 was formed through co-integration of structural regions located in two different plasmids, enabling it to encode both resistance and virulent phenotypes.

Conclusion: Findings in this study provide evidence of active plasmid evolution in *K. pneumoniae* and suggest that surveillance of multidrug-resistant and hypervirulent *K. pneumoniae* is urgently needed.

1. Introduction

In the past few decades, the emergence of multidrug-resistant (MDR) and hypervirulent *Klebsiella pneumoniae* (hvKP), which pose a serious threat to public health, has been reported worldwide [1–7]. Hypervirulent and MDR isolates of *K. pneumoniae* were largely considered not to overlap before the recent emergence of outbreak strains that are simultaneously hypervirulent and MDR [8–12]. Such convergent clones, namely MDR-hvKP, primarily arise after acquisition of virulence genes encoded on the pLVPK-like virulence plasmid [13] by an MDR strain, or acquisition of MDR genes by a hypervirulent strain via uptake or transposition of mobile genetic elements such as plasmids, transposons and integrons [14]. A recent study showed that the selective pressure imposed by the use of antimicrobial agents had an important effect on acquisition and loss of antimicrobial resistance genes in *K. pneumoniae* [15], leading to constant evolution of plasmids in *K. pneumoniae*. Studies reporting carbapenem-resistant and/or hypervirulent *K. pneumoniae* isolates are the most frequently published. But understanding epidemic traits and dynamics of plasmid acquisition is important to help anticipate future impacts on human health. Understanding the genetic basis of convergence of MDR and hypervirulence phenotypes in *K. pneumoniae* isolates is essential for development of effective strategies for further control of such superbugs. In this study, we characterized the genetic basis of a *K. pneumoniae* isolate that exhibited both

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third-generation cephalosporin resistance and moderate virulence phenotypes by plasmid sequencing and bioinformatics analyses. The virulence phenotype was examined using the string test and *Galleria mellonella* infection model.

2. Materials and methods

2.1. Bacterial strains

Klebsiella pneumoniae strain HB25-1 was identified in the Second Affiliated Hospital of Zhejiang University in 2015 in a nationwide antimicrobial resistance surveillance project [16], subject to species confirmation with Vitek 2 system (bioMérieux, Marcy-l'Etoile, France) and MALDI-TOF MS apparatus (Bruker Microflex LT, Germany).

2.2. Antibiotic susceptibility testing

Antimicrobial susceptibility testing was conducted by the microdilution method according to the guidelines suggested by the Clinical and Laboratory Standards Institute [17]. Antimicrobial agents including cefotaxime, ceftazidime, amikacin, kanamycin, ciprofloxacin, polymyxin E, cefepime and meropenem were tested.

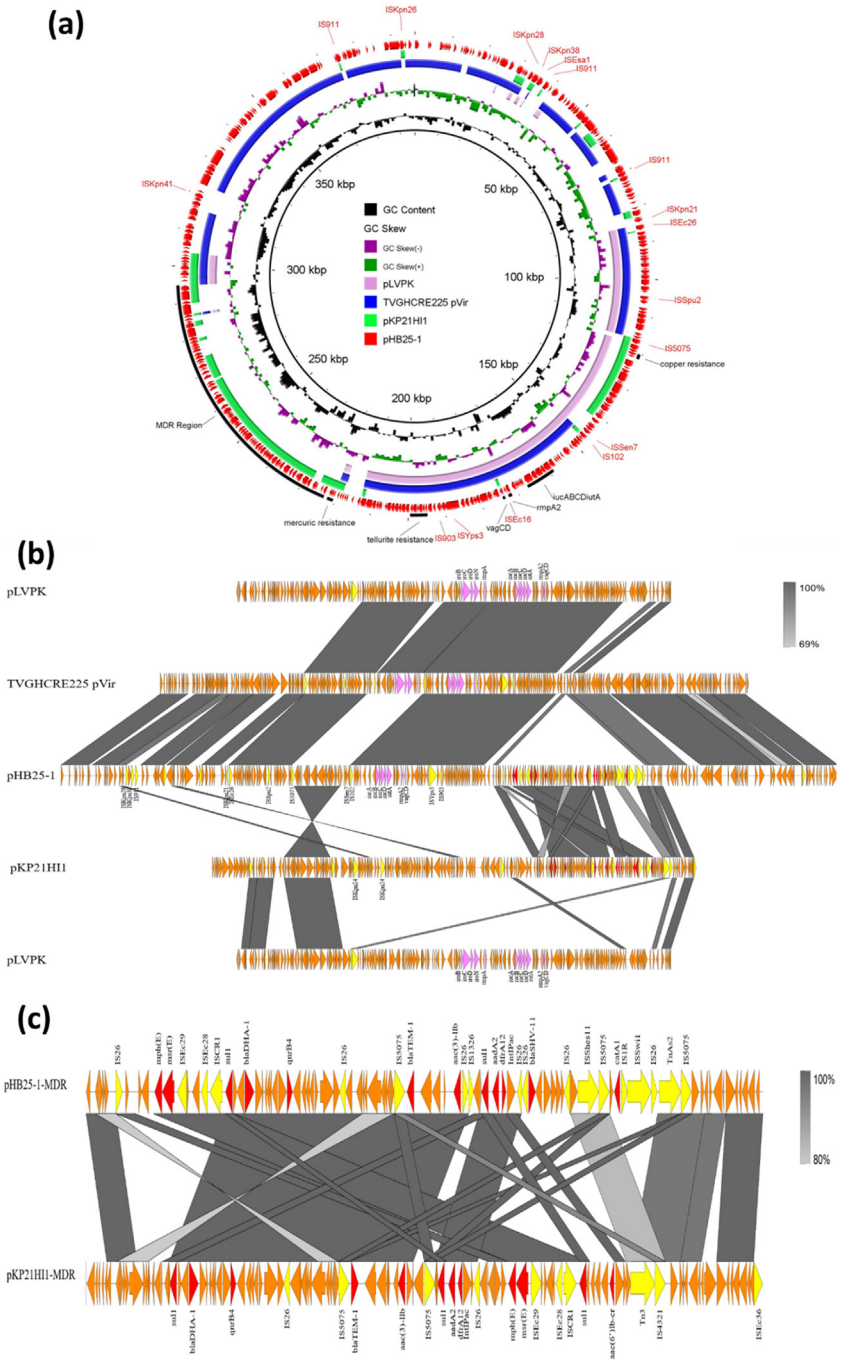

Fig. 1. The hybrid plasmid pHB25-1 harboured by *Klebsiella pneumoniae* strain HB25-1. (a) Circular maps of plasmid pHB25-1 recovered from *K. pneumoniae* HB25-1 with plasmids pVir, pLVPK and pKP21HI1 recorded in the NCBI database. This figure was constructed with BLAST Ring Image Generator (BRIG) [31]. (b) Linear alignment of plasmid pHB25-1 with plasmids pVir, pLVPK and pKP21HI1 using EasyFig [32]. (c) Linear alignment of the multidrug-resistance (MDR)-encoding region in plasmid pHB25-1 with that in plasmid pKP21HI1. Orange, yellow, pink and red arrows denote open reading frames, insertion sequences, virulence genes and antimicrobial genes, respectively.

2.3. String test

Strain HB25-1 was cultured on agar plates supplemented with 5% sheep blood and incubated at 37 °C overnight. The string test was regarded as positive when a viscous string of >5 mm could be formed by stretching a single colony using a bacteriology loop [18]. A hypervirulent ST11 K47 *K. pneumoniae* HvKP4 reported previously [19] was used as a positive control.

2.4. Conjugation

Conjugation was conducted with the filter-mating method as previously reported [20], using a rifampin-resistant *Escherichia coli* EC600 as the recipient. In brief, strain HB25-1 and the recipient strain EC600 were cultured to logarithmic phase (OD600 ~0.6) at 37 °C in Luria-Bertani (LB) broth. Next, strain HB25-1 and the recipient EC600 were mixed at a volume ratio of 1:4 and inoculated gently on a 0.45 μm membrane placed on the surface of an LB agar plate. After incubation at 37 °C for 16–18 h, the mixture on the membrane was gathered, resuspended in saline and serially diluted 10-fold with saline. The diluted mixture was then spread onto China Blue agar plates supplemented with 4 μg/mL potassium tellurite (K_2TeO_3) (Sigma–Aldrich) and 600 μg/mL rifampin to select the transconjugant.

2.5. Galleria mellonella infection model

Virulence potential of *K. pneumoniae* HB25-1 was evaluated in a *Galleria mellonella* infection model. *Galleria mellonella* weighing approximately 300 mg (Tianjin Huiyude Biotech Company, Tianjin, China) was infected with 1×10^6 CFU of each *K. pneumoniae* strain as described previously [21]. The survival rates of *G. mellonella* were recovered 48 h post-infection. An ST11 carbapenem-resistant hypervirulent *K. pneumoniae* isolate HvKP4 and a classical carbapenem-resistant *K. pneumoniae* isolate FJ8 [19] were also included as high virulence control and low virulence control, respectively. Results were analysed and visualized using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

2.6. Whole-genome sequencing and bioinformatics analysis

Whole-genome sequencing was performed to investigate the complete circular sequence of plasmids in *K. pneumoniae* HB25-1 using both the Illumina NextSeq 500 platform and the long-read MinION sequencer platforms [22]. The rapid sequencing kit (SQK-RBK001) and flow cell type R9.4 were applied for Nanopore MinION sequencing. Hybrid assembly of short Illumina reads and long MinION reads was performed using Unicycler v.0.3.0 [23]. The complete circular plasmid sequences were modified with Pilon v.1.22 [24] and annotated with RAST tool [25].

3. Results and discussion

3.1. Phenotypic characteristic of K. pneumoniae strain HB25-1

Klebsiella pneumoniae HB25-1 was subjected to antimicrobial susceptibility tests and found to be resistant to cefotaxime (MIC, 128 μg/mL), ceftazidime (MIC, 32 μg/mL), amikacin (MIC, >256 μg/mL), kanamycin (MIC, >128 μg/mL), ciprofloxacin (MIC, 32 μg/mL) and potassium tellurite (MIC, >128 μg/mL), but remained susceptible to polymyxin E (MIC, 2 μg/mL), cefepime (MIC, 1 μg/mL) and meropenem (MIC, 0.12 μg/mL). hvKP isolates normally exhibit hypermucoviscous phenotypes, which can be semi-quantitatively defined by a positive 'string test' [18]. Here, the string test was conducted with a hypervirulent ST11 K47 *K. pneumoniae* HvKP4 used as a positive control. *Klebsiella pneumoniae* HB25-1 generated a 2 mm viscous string, which was below the criterion (>5 mm) used to define hyperviscosity.

3.2. Genomic characteristic of K. pneumoniae HB25-1

Klebsiella pneumoniae strain HB25-1 was then subjected to whole-genome sequencing using the Illumina NextSeq 500 platform (Illumina, San Diego, CA, USA) and Nanopore MinION sequencer platforms. Strain HB25-1 was found to harbour a circular chromosome with the size of 5,439,115 bp and comprised 5263 predicted open reading frames (ORFs), with a G + C content of 57.4%. It was shown to belong to ST11 based on Multilocus Sequence Typing and K64 serotype based on capsular typing by wzi allele [26,27], a sequence- and sero-type which was recently reported to exhibit extensive phenotypic drug resistance and cause fatal bacteraemia in Brazil [28]. Drug-resistance genes including bla_{SHV-11}, $bla_{CTX-M-3}$, *oqxA* and *oqxB* genes were detectable in the chromosome. The resistance phenotype to third-generation cephalosporins could be conferred by the chromosomal-encoded $bla_{CTX-M-3}$ gene.

Two circular plasmids with sizes of 396,420 bp (designated as pHB25-1) and 88,581 bp (designated as pHB25-1-88K), respectively, were recovered from *K. pneumoniae* strain HB25-1. The pHB25-1 plasmid contained the IncHI1/FIB replicon and comprised 320 predicted coding sequences, with a G + C content of 48.1%. A BLASTN search against the NCBI nucleotide database showed that plasmid pHB25-1 was 99.89% identical to plasmid pVir in clinical *K. pneumoniae* strain TVGHCRE225 (GenBank accession no. CP023723) at 66% coverage, and 99.95% identical to the MDR-encoding plasmid pKP21HI1 (GenBank accession no. CP031563), also from a clinical *K. pneumoniae* strain, at 29% coverage (Fig. 1a), suggesting that pHB25-1 was generated by genetic recombination of the two plasmids. A further BLAST analysis indicated that pHB25-1 harboured a backbone region (312,039 bp), which was 99.95% identical to the virulence plasmid pVir at 80% coverage, and an MDR region (MDR-pHB25-1, 84,381 bp) located between two hypothetical protein-encoding genes, which was 99.95% identical to an MDR region located in pKP21HI1, at 83% coverage.

The backbone region of pHB25-1 harboured genes responsible for plasmid replication, virulence, and genes encoding for heavy metal resistance. Virulence-related genes, including the regulator of mucoid phenotype A gene *rmpA2* and the aerobactin gene cluster *iutAiucABCD*, were detectable in the backbone. The major difference between this region and pVir was the absence of a 23,079 bp element encoding virulence factors salmochelin (*iroBCDN*) and regulator of the mucoid phenotype (*rmpA*) in pHB25-1 (Fig. 1b).

The MDR region of plasmid pHB25-1, which was flanked by the gene *yadA* and a gene encoding a hypothetical protein, comprises 72 ORFs, with a G + C content of 52.9%. Mobile genetic elements including six copies of IS*26* and two copies of IS*5075* were identified in the MDR region of plasmid pHB25-1, suggesting that this region has been inserted into the backbone of pHB25-1 via transposition activities of mobile genetic elements. Comparative genomic analysis showed that pHB25-1-MDR and pKP21HI1 shared multiple genes that confer resistance to aminoglycosides (*aac(3)-lld*), β-lactams (bla_{TEM-1}, bla_{DHA-1}), macrolides (*mph(E)*), quinolones (*qnrB4*) and a class I integron In369 (*intI1-dfrA12-aadA2-qacED1-sul1-*△*orf5*) (Fig. 1c). Compared with the MDR region in plasmid pKP21HI1, pHB25-1 harboured an extra transposable unit with a structure of IS*26*-bla_{SHV-11}-*deoR-orf-orf-orf-orf*-IS*26* located between IS*26* and IS*Shes11*, which exhibited 99% similarity with the chromosomal fragment in *K. pneumoniae* strain HB25-1, indicating that such a unit was acquired as a result of mobilization activities of the IS*26*-flanked transposable unit.

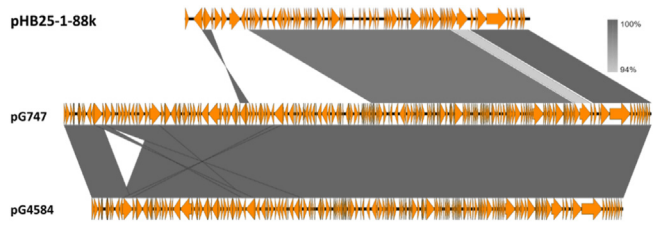

Fig. 2. Genetic similarity of plasmid pHB25-1-88K to known plasmids in the Genbank. Linear alignment of plasmid pHB25-1-88K with its blast hits pG747 (CP034137) and pG4584 (CP034131) in the NCBI database using EasyFig.

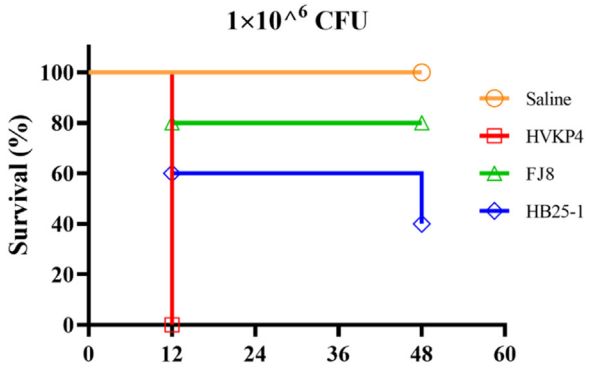

Fig. 3. Virulence potential of *Klebsiella pneumoniae* HB25-1. Virulence potential of strain HB25-1 was measured in a *Galleria mellonella* infection model.

The transferability potential of the hybrid plasmid pHB25-1 was tested by conjugation using a rifampin-resistant *E. coli* EC600 strain as the recipient strain. Transconjugants were selected on China Blue Agar plates containing 4 μg/mL K_2TeO_3 and 600 μg/mL rifampin as the hybrid plasmid pHB25-1 harboured genes *terABCD* encoding resistance to tellurite. However, plasmid pHB25-1 could not be transferred to the recipient *E. coli* EC600 via conjugation; this finding was consistent with that of Dong et al., in which the hybrid virulence plasmid pKP70-2 could not be transferred to an *E. coli* recipient through conjugation [29].

Another plasmid harboured by *K. pneumoniae* HB25-1, designated pHB25-1-88K, is an IncFII type plasmid that does not harbour any antimicrobial resistance genes. It is 88,581 bp with a G + C content of 52.9%, which comprises 90 predicted coding sequences. BLASTN results showed that it was 99.26% identical to plasmid pG747 (CP034137) at 85% coverage and 99.47% identical to plasmid pG4584 (CP034131) at 85% coverage (Fig. 2).

3.3. Virulence potential of K. pneumoniae HB25-1

The virulence potential of *K. pneumoniae* HB25-1 was tested in a *Galleria mellonella* infection model. Upon being infected for 48 h with an inoculum of 1×10^6CFU of these strains, the survival rate of *G. mellonella* was 40% for strain HB25-1, while 0% survival was observed for the hypervirulence control HvKP4 and 80% survival for strain FJ8 (Fig. 3). Both the string test and *Galleria mellonella* infection models suggested a moderate virulence of strain HB25-1. Combined with the genetic structure of plasmid pHB25-1, the absence of the 23 kb element encoding virulence factors salmochelin (*iroBCDN*) and regulator of the mucoid phenotype A (*rmpA*) could potentially contribute to the attenuated virulence of strain HB25-1. Of note, previous studies have demonstrated that a *G. mellonella* infection model cannot differentiate accurately between hypervirulent and classical *K. pneumoniae* [30]. Animal models such as mouse, and neutrophil assays could be conducted to further validate these results.

4. Conclusion

In conclusion, we reported the phenotypic and genomic characteristics of an ST11, serotype K64, third-generation cephalosporin-resistant, moderately virulent *K. pneumoniae* strain, which emerged upon acquisition of an MDR-encoding fragment by the virulence plasmid that it harboured, as a result of transposition activities of mobile elements. Findings in this study indicate that plasmids in the MDR-hvKP strains can undergo active genetic recombination and pose a severe threat to human health in an era when development of new antibiotics lags far behind the rate of emergence and dissemination of antimicrobial-resistant bacterial strains.

Accession numbers

The complete genome sequence of *K. pneumoniae* strain HB25-1 has been deposited in the NCBI database under accession numbers CP039524 (chromosome), CP039526 (pHB25-1) and CP039525 (pHB25-1-88K).

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Conflict of interest

None declared.

Ethical approval

Not required.

Author contributions

MMX and ND performed the experiments and drafted the manuscript; KCC helped with animal experiments; LWY and XMY helped with genome and plasmid sequencing; EWCC edited the manuscript; RZ helped with the study design and collection of clinical strains and data; SC supervised the whole project and wrote the manuscript.

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