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The coexistence of three *bla*$_{KPC-2}$ genes on an IncF/IncR plasmid in ST11 *Klebsiella pneumoniae*

Running title: three blaKPC-2 genes on a plasmid

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Highlights

- Three copies of *bla*$_{KPC-2}$ co-existed on a large IncFII/IncR plasmid of a carbapenem-resistant ST11 *Klebsiella pneumoniae*.
- Three copies of the IS*26*-IS*Kpn27*-*bla*$_{KPC-2}$-IS*Kpn6*-IS*26* unit were present in tandem.
- Multiple copies of IS*26* are one key factor to generate genetic plasticity and could mediate the multiplication of resistance genes.

Abstract

Background: We found three copies of *bla*$_{KPC-2}$ on a plasmid of a *Klebsiella pneumoniae* strain and report the findings here.

Methods: A carbapenem-resistant *K. pneumoniae* clinical strain, SCEC020002, was subjected to whole genome sequencing using both short-read Illumina X10 platform and long-read MinION sequencer. Hybrid assembly was performed using Unicycler and contigs were then corrected using Plion. Based on the whole genome sequence, sequence type, capsular type, plasmid replicon type and plasmid multi-locus sequence type were determined and virulence and antimicrobial resistance genes were identified. Mating was performed to obtain a self-transmissible plasmid mediating carbapenem resistance.

Results: Strain SCEC020002 was resistant to imipenem (MIC, 64 μg/ml) and meropenem (128 μg/ml). This strain SCEC020002 had a 5,477,148-bp circular chromosome, two small ColRNAI-like plasmids (5,596-bp and 10,060-bp, and one large plasmid (177,508-bp, designated pKPC2_020002) containing an IncR and an FII replicon. Surprisingly, there are three copies of the carbapenemase-gene *bla*$_{KPC-2}$ on pKPC2_020002, which was not self-transmissible. Each of the *bla*$_{KPC-2}$ genes was located in the same genetic context with insertion sequence IS*Kpn27* upstream and IS*Kpn6* downstream, which was bracketed by IS*26*. The three copies of the IS*26*-IS*Kpn27*- *bla*$_{KPC-2}$-IS*Kpn6*-IS*26* unit were present in tandem.

Conclusion: We report the surprising co-existence of three copies of *bla*$_{KPC-2}$ on an IncR/IncF plasmid, which was due to the action of IS*26*. Multiple copies of IS*26* are one key factor to generate genetic plasticity and could mediate the multiplication of resistance genes.

Keywords: carbapenem resistance; plasmids; KPC-2; Klebsiella pneumoniae.

1. Introduction

Carbapenem-resistant *Klebsiella pneumoniae* has emerged as a major challenge for clinical management and infection control for human health [1] and is labelled as “Critical” in the WHO list of antimicrobial-resistant "priority pathogens" that pose the greatest threat to human health [2]. Carbapenem resistance in *K. pneumoniae* is largely due to the production of carbapenem-hydrolysing enzymes (carbapenemases) [3]. KPC is the most common type of carbapenemase in *K. pneumoniae*. The KPC-encoding gene *bla*$_{KPC}$ is commonly present in a single copy on plasmids and sometimes on the chromosome. Here we report the presence of three copies of *bla*$_{KPC-2}$ on a single plasmid of a carbapenem-resistant *K. pneumoniae*.

2. Materials and Methods

2.1 Strain, susceptibility and string tests

Strain SCEC020002 was recovered in China in August 2016 from sputum of a male patient with pneumonia, which was sent for routine clinical microbiology examinations. MICs of amikacin, aztreonam, aztreonam-avibactam, ceftazidime, ceftazidime-avibactam, ciprofloxacin, colistin, imipenem, meropenem, piperacillin-tazobactam,

trimethoprim-sulfamethoxazole and tigecycline were determined using the broth microdilution method of the Clinical and Laboratory Standards Institute (CLSI) [4]. As there are no breakpoints for colistin and tigecycline from CLSI, those defined by EUCAST (http://www.eucast.org/) were applied. Viscous strings were measured in length when their colonies were stretched by an inoculation loop in the string test [5] with a hypermucous ST23:K1 *K. pneumoniae* strain WCHKP030925 as the control.

2.2 Genomic sequencing and analysis

Genomic DNA of strain SCEC020002 was prepared using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) and was subjected to whole genome sequencing using both Illumina HiSeq X10 platform (Illumina, San Diego, CA, USA) and the long-read MinION Sequencer (Nanopore, Oxford, UK). The *de novo* hybrid assembly of both short Illumina reads and long MinION reads was performed using Unicycler v0.4.3 [6] under conservative mode for increased accuracy. Complete circular contigs were then corrected using Pilon v1.22 [7] with Illumina reads for several rounds until no further improvements were reported.

Sequence type was determined using the genome sequence to query the multi-locus sequence typing database available at http://bigsdb.pasteur.fr/klebsiella/klebsiella.html. Capsular typing was performed using Kaptive (github.com/katholt/Kaptive). Virulence genes were identified by querying the database available at http://bigsdb.pasteur.fr/klebsiella/klebsiella.html. Antimicrobial resistance genes were identified from genome sequences using the ABRicate program (https://github.com/tseemann/abricate) to query the ResFinder database (http://genomicepidemiology.org/). Plasmid replicon type and plasmid multi-

locus sequence type were determined using the PlasmidFinder and pMLST tools at http://genomicepidemiology.org/.

Nucleotide sequence accession numbers. The complete sequence of p1_020002, p2_020002, pKPC2_020002 and the chromosome of strain SCKP020002 has been deposited into GenBank under the accession no. CP028539-CP028542. The short reads of strain SCKP020002 has been deposited into the SRA database under the no. SRR6955424.

2.3 Mating

Conjugation experiments were carried out in broth and on filters with the azide-resistant *E. coli* strain J53 as the recipient at both 25 and 37 ºC. Potential transconjugants were selected on LB agar plates containing 4 μg/ml meropenem and 150 μg/ml azide.

3. Results and discussion

Strain SCEC020002 was resistant to amikacin (MIC, >512 μg/ml), aztreonam (>512 μg/ml), ceftazidime (256 μg/ml), ciprofloxacin (256 μg/ml), imipenem (64 μg/ml), meropenem (128 μg/ml), and piperacillin-tazobactam (>512/4 μg/ml), but was susceptible to aztreonam-avibactam (1/4 μg/ml), ceftazidime-avibactam (2/4 μg/ml) colistin (2 μg/ml), tigecycline (0.5 μg/ml), and trimethoprim-sulfamethoxazole (<0.5/9.5 μg/ml).

Hybrid assembly of the short and long reads revealed that strain SCEC020002 had a 5,477,148-bp circular chromosome, two small ColRNAI-like plasmids (5,596-bp and

10,060-bp, designated p1_020002 and p2_020002) and one large plasmid (177,508-bp, designated pKPC2_020002) containing an IncR and an IncFII(pHN7A8) replicon. Strain SCEC020002 belongs to ST11, the predominant ST of CRKP in China [8], and the KL64 capsular type. Strain SCEC020002 was not hypermucous in the string test. With respect to virulence, strain WCHKP020002 had genes encoding yersiniabactin (*fyuA*, *irp1*, *irp2*, *ybtAEPQSTUX*) and a type 3 fimbriae (*mrkABCDFHIJ*) but had no *rmpA* or *rmpA2*, both of which encode the hypermucous phenotype and serve as the marker for hypervirulence. All of the virulence factors were located on the chromosome of strain SCEC020002.

The strain had 13 types of antimicrobial resistance genes mediating resistance to aminoglycosides (*ant(2'')-Ia*, *aadA2*, *rmtB*), β-lactams ($bla_{\text{CTX-M-65}}$, $bla_{\text{SHV-158}}$, $bla_{\text{KPC-2}}$, $bla_{\text{TEM-1b}}$), fosfomycin (*fosA3*, *fosA6*), quinolones (*oqxA*, *oqxB*), rifampicin (*catA2*), and sulphonamides (*sul1*). Among these resistance genes, $bla_{\text{CTX-M-65}}$, $bla_{\text{KPC-2}}$, $bla_{\text{TEM-1b}}$, *fosA3*, *rmtB*, and *catA2* were carried by the large plasmid pKPC2_020002 (Figure 1), while the remaining genes were located on the chromosome. Surprisingly, there are three copies of $bla_{\text{KPC-2}}$ on pKPC2_020002. The coverage of $bla_{\text{KPC-2}}$ was 690 ×, while that of *rmtB* and $bla_{\text{CTX-M-65}}$, both of which were carried on pKPC2_020002, was 230 × and 243 ×, respectively. This verified that there were three copies of $bla_{\text{KPC-2}}$. The co-existence of two copies of $bla_{\text{KPC-3}}$ on a single plasmid has been reported before [9] but not for three copies of the same bla_{KPC} gene. As we demonstrated recently, Illumina sequencing alone was unable to reliably reveal the presence of multiple copies of the same gene, while the combination with MinION sequencing is able to resolve the copy numbers of genes and therefore untangle the complicated genetic context of antimicrobial resistance genes.

Each of the *bla*$_{KPC-2}$ genes was located in the same genetic context with insertion sequence IS*Kpn27* upstream and IS*Kpn6* downstream, which was bracketed by IS*26* (Figure 2). The three copies of the IS*26*-IS*Kpn27*-*bla*$_{KPC-2}$-IS*Kpn6*-IS*26* unit were present in tandem. The insertion of IS*26* could generate 9-bp direct target repeats (DR). However, there were no 9-bp DR present around any copy of IS*26* and between any two copies of this insertion sequence. This suggests that the tandem multiplication of IS*26*-IS*Kpn27*-*bla*$_{KPC-2}$-IS*Kpn6* was due to homologous recombination. It is well known that two copies of IS*26* can form a composite transposon, which may be excised from plasmids to form a circular intermediate [10, 11]. IS*26* provides a region for homologous recombination and therefore could serve as a Trojan horse [12]. In the presence of IS*26* on a plasmid, the intermediate may be integrated into the plasmid via homologous recombination to generate tandem repeats. Previous studies have shown that IS*26* is involved in the dissemination and amplification of *bla*$_{KPC}$ [13], although the co-existence of three copies of *bla*$_{KPC}$ has been seen before.

Despite repeated attempts, no transconjugants were obtained, suggesting that pKPC2_020002 was not self-transmissible. Although all conjugative genes were present on pKPC2_020002, *traI*, which encodes the TraI DNA relaxase and is required for conjugative transfer[14], was truncated by the insertion of IS*26*. This explains why pKPC2_020002 was not self-transmissible.

4. Conclusion

We report the co-existence of three copies of *bla*$_{KPC-2}$ on an IncR/IncF plasmid, which was due to the action of IS*26*. Multiple copies of IS*26* could mediate the multiplication

of resistance genes to generate complicated genetic context of antimicrobial resistance genes.

Declarations

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Competing Interests: None

Ethical approval: Not required.

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Figure legends

Figure 1. The circular map of pKPC2_020002. The locations of antimicrobial resistance genes, plasmid replicon genes and genes encoding conjugation (*tra* and *trb*) are shown.

Figure 2. The genetic context of *bla*$_{KPC-2}$ on pKPC2_020002. Three copies of the IS*26*-Tn*3*Δ-IS*Kpn27*-Tn*3*Δ-*bla*$_{KPC-2}$-IS*Kpn6* unit are arranged in tandem.