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Genome Note

Tigecycline-resistant *Klebsiella pneumoniae* strains from sewage in Norway carry heavy-metal resistance genes encoding conjugative plasmids

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ABSTRACT

Objectives: Tigecycline is a last-resort antibiotic used for treatment of infections with carbapenem-resistant *Klebsiella pneumoniae*. The aim of the study was to understand the genetic mechanism of resistance and the genetic context of resistance genes in two tigecycline-resistant *K. pneumoniae* strains isolated from sewage in Bergen, Norway.

Methods: Complete genome sequencing of the two strains was accomplished using a combination of short-read Illumina MiSeq-based and long-read Oxford Nanopore MinION-based sequencing. Conjugation experiments were performed using filter mating and a green fluorescent protein (GFP)-tagged *Escherichia coli* strain.

Results: The complete genome sequences of strain K6-320.1 and strain K7-325 were assembled into two contigs for each strain, one contig representing the complete circular chromosomes of 5 223 440 bp (K6-320.1) and 5 263 092 bp (K7-325), respectively, and the other representing plasmids with sizes of 276 509 bp (pK6-320.1) and 246 731 bp (pK7-325). Strain K6-320.1 belonged to sequence type (ST)869, whereas strain K7-325 belonged to the pathogenic ST307. Both plasmids belonged to the IncFIB(K)/IncFII(K) group and carried several antibiotic resistance genes (ARGs), including *tet*(A) and bla_{CTX-M}. Both plasmids (pK6-320.1 and pK7-325) were transferred to a GFP-tagged *E. coli* strain, leading to the acquisition of resistance against multiple classes of antibiotics. Several heavy-metal resistance genes (HMRGs) encoding resistance against silver (*sil*), copper (*pco*), and arsenic (*ars*) were also present on both plasmids.

Conclusions: Our study demonstrates the presence of multidrug-resistant *K. pneumoniae* strains carrying conjugative plasmids encoding both ARGs and HMRGs that have potential for persistence in the environment and human microbiota.

Extended-spectrum-β-lactamase (ESBL)-producing *Klebsiella pneumoniae* and carbapenem-resistant *K. pneumoniae* strains are a major health concern because of the limited available treatment options. Tigecycline is a last-resort antibiotic used for treatment of infections caused by carbapenem-resistant *K. pneumoniae* strains [1]. Two tigecycline-resistant and ESBL-producing *K. pneumoniae* strains (K6-320.1 and K7-325) were isolated from sewage in Bergen, Norway, in a previous study [2]. The aim of this study was to understand the genetic mechanism of resistance and the genetic context of antibiotic resistance genes (ARGs) in these strains, using long-read sequencing for determining the complete genome sequences of the strains.

The strains were isolated on Simmons Citrate Agar with myo-Inositol (SCAI), incubated at 37°C for 24–48 h. The resistance profiles were checked against 15 antibiotics, using the broth microdilution assay with Sensititre™ EUVSEC 3 plates (Thermo Scientific, USA), following the manufacturer's protocol. Resistance or sensitivity was determined using the EUCAST clinical breakpoint

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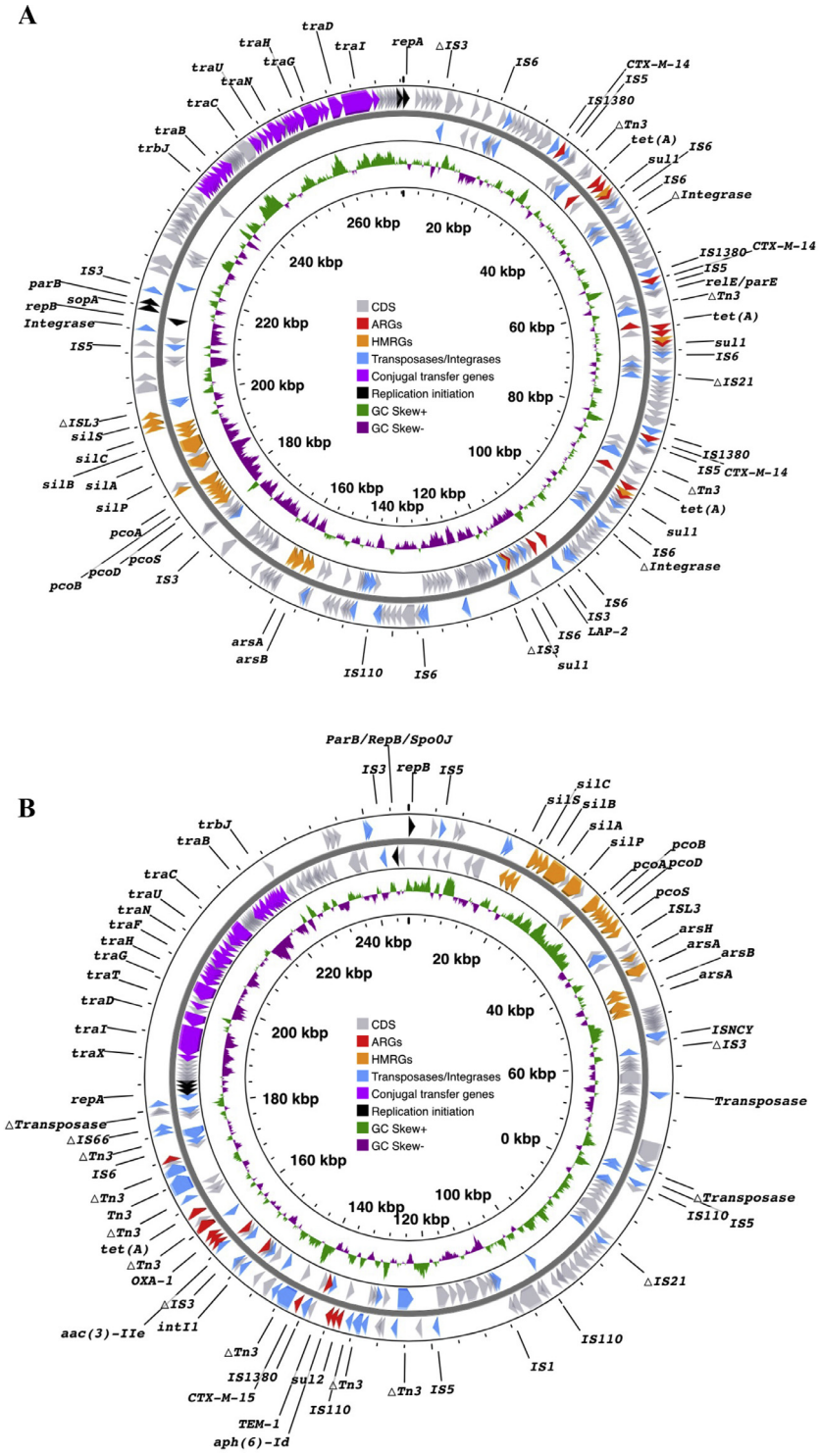

Fig. 1. (A) Genomic map of plasmid pK6-320.1 (GenBank accession no. CP119057). (B) Genomic map of plasmid pK7-325 (GenBank accession no. CP119059). The arrows indicate the sizes of the open reading frames (ORFs) and their orientations. Antibiotic resistance genes (ARGs) are shown in red, heavy-metal resistance genes (HMRGs) in orange, transposases/integrases in blue, conjugal transfer genes in purple, and replication initiation in black. All other genes are shown in grey.

tables v.13.0 [3]. The *K. pneumoniae* strains (K6-320.1 and K7-325) were resistant against different classes of antibiotics, including penicillins, cephalosporins, sulphonamides, fluoroquinolones and tetracyclines, thus classified as multidrug-resistant (Supplementary Table S1).

For obtaining the complete genome sequences, we performed Illumina MiSeq short-read and Oxford Nanopore MinION long-read sequencing, followed by hybrid *de novo* genome assemblies, which combined the short and the long reads. Both assemblies resulted in complete and closed genome sequences, each of them formed by two circular contigs. The genome sequences were annotated, using Prokaryotic Genome Annotation Pipeline (PGAP) v.4.13 (https://academic.oup.com/nar/article/44/14/6614/2468204). The overview of the complete genome sequences is presented in Supplementary Table S2. Strains were typed using Kleborate v.2.1.0 [4]. The plasmid replicon typing was per-

formed using PlasmidFinder v.2.0 (https://cge.food.dtu.dk/services/PlasmidFinder/), and complete overviews of the plasmids were obtained using CGView (https://proksee.ca/). ARGs were screened using the comprehensive antibiotic resistance database (CARD) v.3.2.6 (https://card.mcmaster.ca/) and the ResFinder database v.4.1 (https://cge.food.dtu.dk/services/ResFinder/).

Strain K6-320.1 belongs to the sequence type (ST)869, whereas strain K7-325 belongs to the high-risk pathogenic clone ST307. Strain K6-320.1 has a chromosome of 5 223 440 bp (GenBank accession no. CP119056) and a plasmid (pK6-320.1) with a size of 276 509 bp (CP119057). Strain K6-320.1 carries bla_{SHV-27}, *fosA5, oqxA, oqxB19* and one copy of ESBL $bla_{CTX-M-14}$ on the chromosome, whereas other ARGs are present on the plasmid pK6-320.1. This plasmid belongs to the IncFIB(K)/IncFII(K) family and carries a multidrug-resistance (MDR) determining region with three copies of *tet*(A), *dfrA1*, *qacE*Δ*1* and *sul1* flanked by a truncated (△) Tn3 and $bla_{CTX-M-14}$ flanked by a IS1380 family transposase on one side and an IS5 family transposase on the other. It also carries an additional copy of *sul1* outside the MDR region, thus encoding four copies of *sul1*. Apart from these ARGs, plasmid pK6-320.1 also carries one copy of bla_{LAP-2} and *qnrS1* (Fig. 1A).

The complete genome of strain K7-325 was assembled into a chromosome of 5 263 092 bp (CP119058) and a plasmid (pK7-325) of 246 731 bp (CP119059). The strain carries chromosomal bla_{SHV-28}, *fosA5, oqxA* and *oqxB19*. Plasmid pK7-325 belongs to the IncFIB(K)/IncFII(K) family and encodes several ARGs such as $bla_{CTX-M-15}$, bla_{OXA-1}, bla_{TEM-1}, *aac(3)-IIe, aac(6′)-Ib-cr5, aph(3″)-Ib, aph(6)-Id, qnrB1, sul2, tet*(A), *catB3* and *dfrA14* (Fig. 1B). These ARGs are present in a MDR-determining region between positions 134 013 bp and 172 453 bp that is flanked by IS6 family transposase. A genomic map of the multidrug-resistance determining region (MDR) of both plasmids are presented in Supplementary Fig. S1 (A and B).

Both plasmids (pK6-320.1 and pK7-325) have a similar backbone (approximately 82 kb in total), with nucleotide identity of 99 % spanning a region from nucleotide positions 176 262 to 261 108 bp for pK6-320.1 and nucleotide positions 200 037 to 243 668 bp and 1 to 38 232 bp for pK7-325, respectively. The plasmids pK6-320.1 and pK7-325 are conjugative and were transferred to a green fluorescent protein (GFP)-tagged *Escherichia coli* strain, at a transfer frequency of 2×10^{-5} per recipient cells, via conjugation [5], with transfer of resistance against several antibiotics (Supplementary Table S1). The transconjugants were sensitive to tigecycline, despite carrying the *tet*(A) gene. In *K. pneumoniae*, tigecycline resistance can be achieved through a different mechanism, including mutations and/or loss of *ramR*, leading to overexpression of the *ramA* gene in combination with *tet*(A). RamR is a repressor that regulates the expression of RamA. Overexpression of RamA may increase AcrAB pump expression and cause high-level multidrug resistance, including tigecycline resistance in *K. pneumoniae* [6,7]. Both *K. pneumoniae* strains in our study did not carry the *ramR* gene, thus explaining the tigecycline resistance in combination with *tet*(A).

Plasmid pK7-325 encodes RelE/ParE and Phd/YefM familytype II toxin-antitoxin system (TAs), while pK6-320.1 encodes TAs belonging to Phd/YefM, RelE/ParE and HigB family, thus suggesting the potential for spread and maintenance of these MDR plasmids within human microbiota.

Both plasmids, pK6-320.1 and pK7-325, carry several heavy-metal resistance genes (HMRGs), conferring resistance against silver (*sil*), copper (*pco*) and arsenic (*ars*) (Supplementary Table S3). In plasmid pK7-325, these genes were located between positions 16 868 bp (*silE*) and 50 498 bp (*arsR*), whereas for plasmid pK6-320.1, these genes were located between positions 156 245 bp (*ars*C) and 197 620 bp (*silE*). The co-localization of several ARGs and HMRGs on these plasmids suggest potential for co-selection of ARGs through heavy-metal selection.

In conclusion, our study shows presence of multidrug-resistant *K. pneumoniae* strains resistant against last-resort antibiotics in sewage from Norway. It further shows the presence of MDR-encoding conjugative plasmids that have the potential for persistence in the environment and in human microbiota. It also emphasizes the importance of sewage-based surveillance of antimicrobial resistance (AMR) in pathogens for understanding the emerging resistance against the last-resort antibiotics in low-prevalence countries such as Norway.

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Ethical Approval: Not required.

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Data availability: The assembled, complete genome sequences of the strains have been submitted to DDBJ/ENA/GenBank under the following genome accession numbers: CP119056-57 and CP119058-59.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2023.10.023.

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