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First Report of Carbapenem-Resistant *Klebsiella michiganensis* Co-Harboring bla_{KPC-2} and *TmexCD2-ToprJ2* Isolated from Wastewater at a Tertiary Hospital in Beijing

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Background: *Klebsiella michiganensis* is an emerging human pathogen that causes nosocomial infections. Its prevalence and spread in the environment should not be ignored. This study identified and characterized *Klebsiella michiganensis* co-harboring bla_{KPC-2} and *TmexCD2-ToprJ2* in hospital wastewater samples.

Methods: Twelve *K. michiganensis* strains were isolated from wastewater samples collected at a tertiary hospital in Beijing, China. The genomic characteristics of *K. michiganensis* strains were analyzed using whole-genome sequences, providing information on the comparison between the genome of *K. michiganensis* strains and the reference genome, antibiotic resistance genes (ARGs), virulence genes, secretion systems, and mobile genetic elements (plasmids, insertion sequences [ISs], and prophages).

Results: Genome analysis showed that the twelve multi-drug resistant (MDR) strains carried a variety of ARGs and virulence genes, as well as four macromolecular secretion systems (T1SS, T2SS, T5aSS, T5bSS, and T4aP). The genetic environments of both the *TmexCD2-ToprJ2* gene cluster and bla_{KPC-2} gene contained ISs. The plasmids carrying *TmexCD2-ToprJ2* gene cluster of nine strains in clade 1 and two strains in clade 2 were annotated as IncR plasmid and rep_cluster_1254 type, respectively. The plasmids carrying bla_{KPC-2} in 10 strains in clade 1 were identified as IncU, and the plasmids carrying bla_{KPC-2} in the k11 and k12 strains in clade 2 were IncU and IncX6. The phylogenetic tree and heatmap revealed that the secretion system of type VI (T6SSi) existed in 10 strains in clade 1, and Type IV (T4SS) only existed in the k11 strain in clade 2. In addition, *K. michiganensis* strains carried 13 plasmids, 14 ISs, and 138 prophages.

Conclusion: In this study, the whole genome sequencing demonstrated the diversity of *K. michiganensis* genome despite 12 *K. michiganensis* strains from a hospital wastewater, which lays the foundation for further genetic research and drug resistance gene transmission.

Keywords: *Klebsiella michiganensis*, hospital wastewater, whole-genome sequencing, bla_{KPC-2}, *TmexCD2-ToprJ2*

Introduction

The *Klebsiella* group is widely distributed in humans, livestock, plants, soil, water and wild animals, with genetic and ecological diversity.[1] *Klebsiella michiganensis* (*K. michiganensis*) is a novel species of the pathogenic *genus Klebsiella*, and it shares a high degree of homology and similarity with *Klebsiella pneumoniae* in molecular characteristics.[2] Meanwhile, a review summarized the phylogenetic relationships between *Klebsiella pneumoniae* and other select members of the

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Klebsiella genus and family *Enterobacteriaceae* based on whole-genome sequence, which showed *K. michiganensis* and *Klebsiella oxytoca* (*K. oxytoca*) are very close to the tree.[3]

K. michiganensis was first discovered in a toothbrush holder from Michigan in 2013 and is an emerging Gram-negative opportunistic pathogen.[4] Several studies have reported that *K. michiganensis* has been isolated from a variety of animals and environments.[5–8] In addition, this potential pathogen has also been reported in clinical samples in many countries, often associated with numerous hospital-acquired infections.[9,10]

Carbapenems have become the last line of defense owing to the widespread use of antibiotics in recent years. However, the number of carbapenem-producing *Klebsiella* strains is gradually increasing, which may lead to higher morbidity and mortality rates, posing a significant threat to global public health.[11,12] Surveillance is key to the effective treatment and management of antibiotic resistance and provides guidance for clinical treatment. Hospital wastewater contains a variety of microorganisms, antibiotics, and antibiotic metabolites, which are important for the spread of drug-resistant bacteria and drug-resistant genes to the environment.[13,14] Surveillance of hospital wastewater has become an alternative to traditional clinical drug resistance detection, with the potential to reflect the prevalence of MDR bacteria in clinical settings.[15–17] There has been considerable research on carbapenem-resistant *Klebsiella pneumoniae* derived from wastewater.[18,19] The carbapenem-resistant mechanism in Gram-negative bacteria is primarily attributed to the expression of carbapenemase enzymes, such as *KPC, VIM, OXA-48*, and *NDM*. These β-lactamase genes are harbored on mobile genetic elements and conjugated plasmids, which are associated with the widespread dissemination of carbapenem resistance.[20] However, the genomic characteristics of *K. michiganensis* in hospital wastewater are yet to be clarified.

In this study, we identified 12 carbapenem-resistant *K. michiganensis* strains from hospital wastewater in Beijing, China, all of which harbored abundant ARGs and virulence genes. The purpose of this study was to understand the overall genomic characteristics of *K. michiganensis* in hospital wastewater, lay the foundation for further studies of *K. michiganensis*, and provide a reference for public health risk assessment.

Materials and Methods

Setting

The Fifth Medical Center of the PLA General Hospital (Beijing, China) is a tertiary hospital with more than 2000 beds, comprising 80 departments that treats approximately 100,000 patients annually. There are two hospital campuses: the wastewater treatment station in the northern hospital campus generally discharges approximately 300 tons of wastewater per day and one of the wastewater treatment stations in the southern hospital campus discharges around 600 tons of wastewater per day.

Isolation and Identification of Bacterial Strain

In June–July 2023, effluent samples were collected at different depths (0.5 to 1 meter below the surface at the inlet of the wastewater treatment facilities in both hospital campuses, using 500 mL sterile sampling bags). After collection, samples were stored on ice for transportation to the laboratory. Each effluent sample was aspirated 100μL of suspension applied to 2μg/mL Meropenem on MacConkey agar plates and incubated at 37°C for 18–24 h. Individual colonies were selected and subcultured on MacConkey agar plates for purification according to colony size, color, and morphology. Each strain was preserved in a 40% sterile glycerol solution and stored at −80°C for further study. Preliminary species identification was performed using an automated VITEK 2 system (bioMérieux; Balmes-Les-Grottes, France). Genome annotation was performed using GENOME TAXONOMY DATABASE (GTDB) v2.1.0.[21]

Antimicrobial Susceptibility Test

The VITEK-2 compact system with a vitek2 Gram-negative bacterial drug sensitivity card (AST-N335; bioMérieux) was used to test the antimicrobial susceptibility of the strains, which included 14 antibiotics: piperacillin/tazobactam, ceftazidime, cefepime, aztreonam, imipenem, meropenem, amikacin, tobramycin, ciprofloxacin, levofloxacin, doxycycline, minocycline, tigecycline, and trimethoprim/sulfamethoxazole. *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as quality control strains for testing, and the results were interpreted according to the Clinical and Laboratory Standards Institute criteria (CLSI, 2023).[22]

Whole Genome Sequencing and Assembly

To further analyze the strain, whole-genome sequencing was performed by Novogene Co., Ltd. (Beijing, China) using the Illumina NovaSeq platform with the PE150 strategy. The sequencing data quality was checked using FastQC v0.11.9.[23] Then, clean data were assembled using Spades v3.13.0,[24] Shovill v1.1.0 (https://github.com/tseemann/shovill) and Unicycler v0.5.0,[25] respectively. The assembly results were evaluated using Quast v5.2.0,[26] and the completeness and contamination were assessed using CheckM v1.2.2.[27] The best-assembled results were selected for further analysis.

Phylogenetic Analysis

The Genomic sequence data were retrieved from all *K. oxytoca* and *K. michiganensis* with complete assembly levels in the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/genome/), and CheckM was also used for quality assessment. Core genome was determined by Roary v3.13.0[28] after annotating assembled genome by Prokka v1.14.6.[29] The phylogenetic tree was constructed by IQ-tree2 with 1000 ultra-fast bootstrap replicates.[30] Based on the position of the strains on the tree and the mash distances between strains, the strains isolated in this study were identified. Additionally, a strain from NCBI that was closest in distance to the strains in this study was selected as a reference genome for follow-up analysis.

A phylogenetic tree was constructed using IQ-TREE 2[30] and visualized using FigTree v1.4.4 graphical viewer (http://tree.bio.ed.ac.uk/software/Figtree/).

Bioinformatic Analysis

To understand the genome structure relationship of different strains, we used the Mauve software to perform covariance comparisons between the whole genome of the strains and the reference genome (GCA_025263805.1).[31]

ARGs, virulence factor genes, and plasmid replicons were identified using Abricate v1.0.1, Resfinder, VFDB, and PlasmidFinder database, respectively.[32–34] The heat map was visualized using TBtools software.[35] ISs were predicted by ISEScan v1.7.2.3.[36] Secretion systems (T1SS to T6SS) were detected by MacSyFinder v2 with the TXSScan model and visualized using Easyfig v2.2.5.[37,38]

Plasmid and chromosome sequences in the genome were predicted by Mlplasmids and Platon v1.7,[39,40] while the classification of contigs harboring the bla_{KPC-2} and *TmexCD2-ToprJ2* clusters was determined using Mlplasmids and MOB-suite v3.0.3.[41] The coverage of specific plasmids was calculated using SAMtools v1.20, determine their inter-relationships.[42] Plasmid comparisons were performed in the PLSDB database (search strategy: mash dist) to find the closest plasmid sequences to the reference plasmids[43] and visualized by the Proksee v1.0.0a6.[44]

DBSCAN-SWA software was used to predict and analyze the prophage carriage of the 12 strains,[45] and the possible ARGs and virulence genes in the prophage genomes were predicted using Abricate software with the Resfinder and VFDB databases.

Results

Identification and Comparison of K. Michiganensis

From June to July 2023, samples of 12 bacterial strains were collected from untreated wastewater at the northern campus (n = 1) and southern campus (n = 11) of the Fifth Medical Center of the PLA General Hospital. Initial species characterization, using Vitek 2, identified twelve strains as *K. oxytoca*. QUAST assessed the assembly results of the three software packages, with Unicycler yielding the best assembly effectiveness, which was subsequently used for further analysis (Figure S1 and Table S1). The best assembled sequence showed high completeness and low contamination (Table S2). According to GTDB classification annotations, 12 strains were identified as *K. michiganensis*. Meanwhile, all *K. oxytoca* strains and *K. michiganensis* strains downloaded from NCBI were re-annotated as 42 *K. oxytoca* strains, 84 *K. michiganensis* strains and 9 other strains (*Klebsiella grimontii, Klebsiella oxytoca_C, Cedecea michiganensis_A*) (Table S3). They were used to construct the phylogenetic tree (Figure 1). Finally, 12 bacteria were identified as *K. michiganensis* and *K. michiganensis* BSI-KPN166 strain (GCA_025263805.1)[2] was selected as the reference genome. Sequence coverage exceeded 90% (Table S2). Using *K. michiganensis* BSI-KPN166 strain (km48) as the reference genome, multiple genome alignments were performed after reordering the order of contigs for each strain.

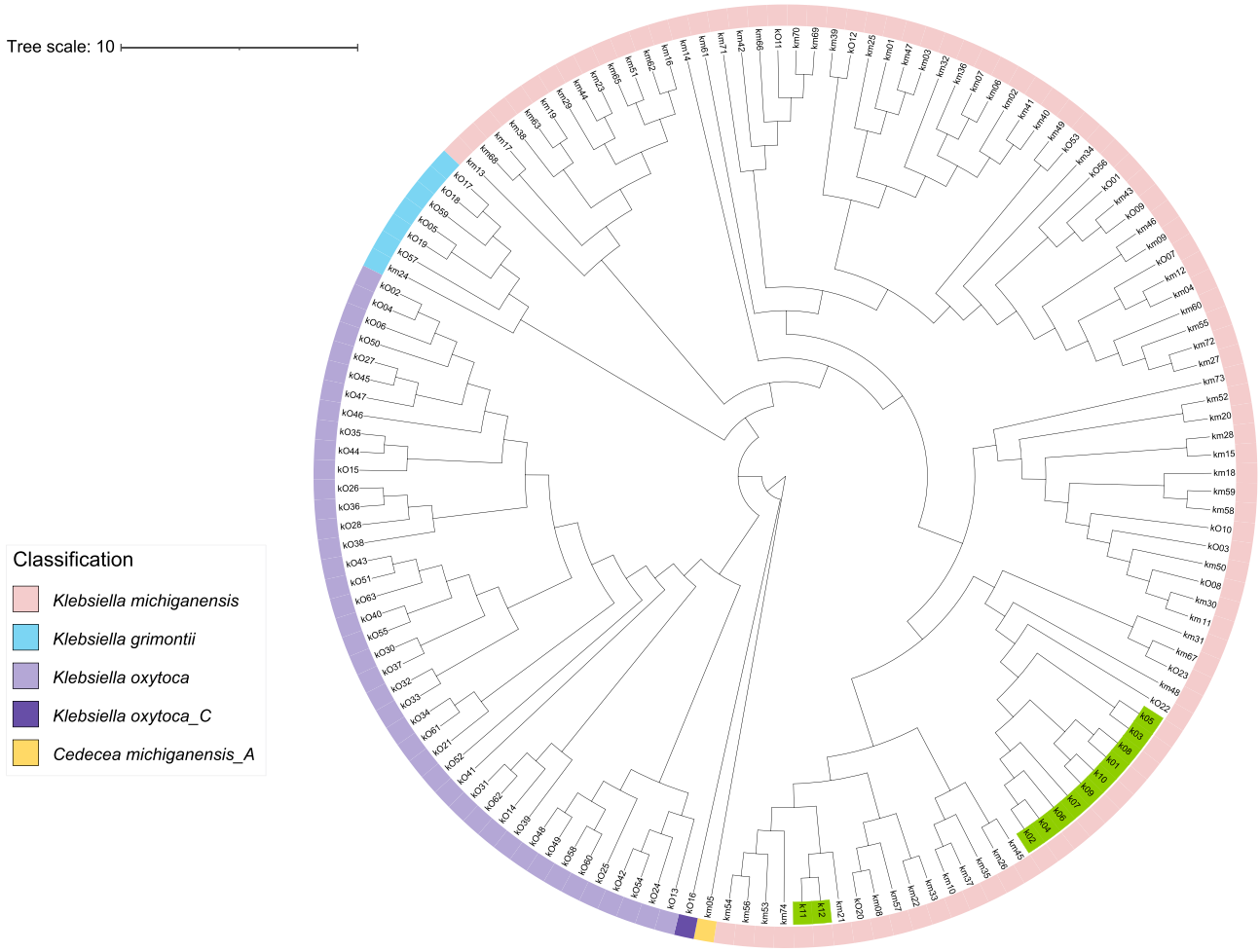

Figure 1 The phylogenetic tree and the GTDB classification annotations. The sequencing data of 12 *K. michiganensis* strains in this study (green strip) and 42 *K. oxytoca* strains, 84 *K. michiganensis* strains and 9 other strains (*Klebsiella grimontii, Klebsiella oxytoca_C, Cedecea michiganensis_A*) downloaded from NCBI were used to construct the phylogenetic tree. Each color represents a different species.

The 12 strains and the reference genome had many locally collinear blocks (LCBs) of the same color, indicating that the genomes were highly consistent (Figure S2).

Results of Antimicrobial Susceptibility Test

Twelve *K. michiganensis* strains were MDR; resistant to piperacillin/tazobactam, ceftazidime, cefepime, aztreonam, imipenem, meropenem, tobramycin, ciprofloxacin, and levofloxacin; and all were intermediate to tigecycline. The detection rate of drug resistance to doxycycline and minocycline was 8.3% (1/12). The results of antimicrobial susceptibility tests are presented in Table S4.

Characterization of Antimicrobial Resistance Genes

The genomes of 12 *K. michiganensis* strains encoded 25 ARGs in 10 different antibiotic classes (Table S5), including aminoglycoside resistance (*aadA5, aph(3')-Ia, armA*), beta-lactams ($bla_{\text{CTX-M-3}}$, $bla_{\text{KPC-2}}$, $bla_{\text{NDM-1}}$, $bla_{\text{OXA-1}}$, $bla_{\text{OXY-1}}$), diaminopyrimidine (*dfrA1, dfrA14*), fluoroquinolone (*aac(6')-Ib-cr, OqxA, OqxB, qnrS1*), fosfomycin (*fosA, fosA3*), macrolides (*mphE, msrE*), phenicol (*catB3*), rifampicin (*arr-3*), sulfonamide (*sul1, sul2*), and tetracyclines (*tmexC2, tmexD2, TOprJ2*). Each strain contained 14 to 21 ARGs. Notably, each strain carried $bla_{\text{KPC-2}}$. The phylogenetic tree comprised two lineages: clade 1 and clade 2. ARGs, including *aadA5, aph(3')-Ia, armA,* $bla_{\text{CTX-M-3}}$, *dfrA1, fosA3, mph(E), msr(E), sul2* were only located in 10 strains of clade 1 (Figure 2). $bla_{\text{OXA-1}}$ and *catB3* were located in only two strains in clade 2.

Figure 2 The heatmap of secretion systems, virulence genes, ARGs and plasmids mapped to the phylogenetic tree. From left to right, distribution of secretion systems, virulence genes, ARGs, and plasmid replicons. Gray squares represent none.

Genetic Environment of TmexCD2-ToprJ2 Gene Cluster and bla_{KPC-2} Gene

We analyzed the genetic environment of bla_{KPC-2}, *tmexC2, tmexD2*, and *TOprJ2* genes. The *TmexCD2-ToprJ2* gene clusters were inserted into *umuC* with an IS881 family element located upstream (Figure 3A). Upstream of the bla_{KPC-2} gene is *klcA*, which encodes an anti-restriction protein, accompanied by numerous genes coding for hypothetical proteins. There were two main genetic environments for bla_{KPC-2} genes. One was that the downstream was the IS481 family, and the other was that the downstream contained the IS481 and IS3 families (Figure 3B).

Characterization of Plasmids Harboring TmexCD2-ToprJ2 and bla_{KPC-2} Genes

The contigs carrying *TmexCD2-ToprJ2* gene cluster of 11 strains were identified as plasmid sequences using Platon and Mlplasmids software. To clarify the relationship between the 11 plasmids, the maximum contig (k12-contig34, 58417bp) was selected as the reference sequence to calculate the coverage of each contig carrying the gene cluster (Table S6). The coverage of k11-contig45 was 100%, indicating a high level of similarity between plasmids carrying *TmexCD2-ToprJ2* gene cluster in k11 and k12. Coverage of the remaining plasmids in the nine strains was 82.1%. Although the k08 strain lacked *TmexCD2-ToprJ2* cluster, its coverage was 81.5% (Table S7). The plasmids carrying *TmexCD2-ToprJ2* gene cluster of nine strains in clade 1 and two strains in clade 2 were annotated as IncR plasmid and rep_cluster_1254 type, respectively. The alignments of the closest plasmids from the PLSDB database, *K. pneumoniae* strain ARLG-4866 plasmid pC597_5 (complete sequence, CP067603.1, respiratory, USA: South), *K. pneumoniae* strain S245 plasmid pS245-2 (complete sequence, NZ_CP114855.1, blood, China: Guangzhou), and 12 *K. michiganensis* strains in the two clades were visualized using Proksee (Figures S3A and B, Table S8).

Contigs carrying bla_{KPC-2} from the 12 strains were identified as plasmids. The coverage of k11-contig42 was 100% based on the maximum contig (k12-contig47, 30234bp), and the coverage rate of the remaining 10 strains was 38.08% (Table S7). After annotation, the contigs carrying bla_{KPC-2} from the 10 strains in clade 1 were annotated as IncU plasmids, and the contigs carrying bla_{KPC-2} of k11 and k12 in clade 2 were annotated as IncU and IncX6, respectively. The alignments among the closest plasmids, including *K. pneumoniae* strain KP424 plasmid pKPC-2-KP424 (complete sequence, NZ_CP109990.1, stool, China: Hangzhou) and *K. pneumoniae* strain KPN55602 plasmid pK55602_2 (complete sequence, NZ_CP042976.1, blood, China: Anhui) and 12 *K. michiganensis* strains, are shown in Figures S3C and D and Table S8.

Characterization of Virulence Genes and Macromolecular Secretion Systems in K. Michiganensis Genome

Twelve *K. michiganensis* strains encoded 49 different virulence genes (Table S9) that were associated with adherence, antimicrobial activity/competitive advantage, biofilms, effector delivery systems, exotoxins, nutritional/metabolic factors, and regulation. The virulence genes clpV/tssH and east1 were only located in Clade 1. The *dotU/tssL, tssF, vgrG/tssI,*

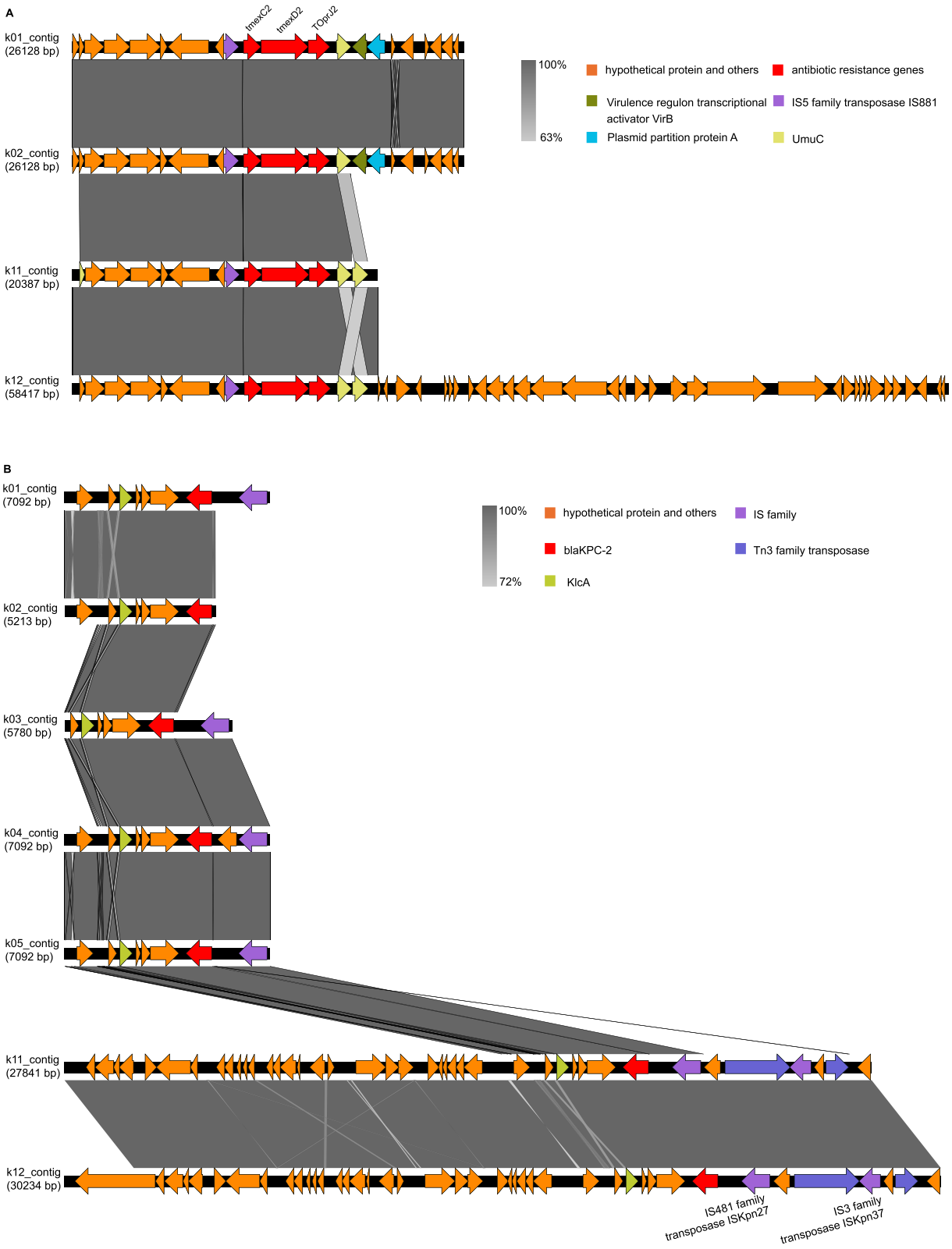

Figure 3 Comparison analysis of the genetic context of specific ARGs. (**A**) Comparative analysis of the genetic context of *tmexCD2-toprJ2* clusters with closely related sequences. (**B**) Comparison analysis of the genetic context of bla_{KPC-2} with that of closely related sequences. The red arrows indicate target ARGs, the purple arrows indicate IS families, the light green and green arrows indicate *Umuc* and *KlcA* genes respectively, the yellow arrows indicate hypothetical protein and others. The gray shaded regions indicate homology.

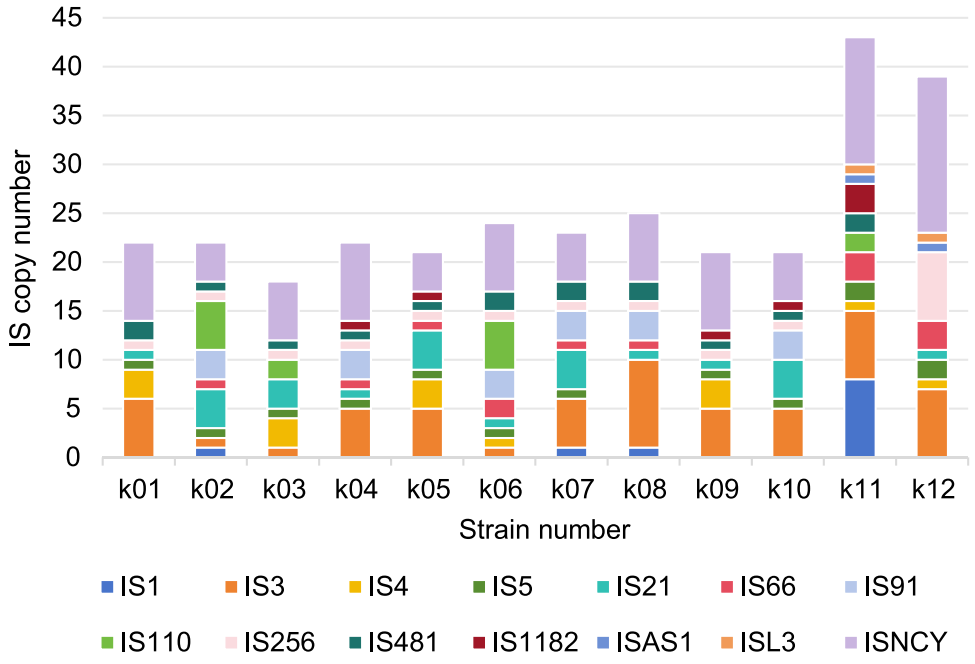

Figure 4 IS family distributions of *K. michiganensis* strains. The color codes represent the IS family.

vipA/tssB, vipB/tssC virulence genes were only located in Clade 2. Although *hcp/tssD* and *gndA* virulence genes were present in all strains, they all carried two virulence genes in 10 strains of clade 1. In addition, the secretion system results showed that all 12 strains had type I (T1SS), type II (T2SS), type V (T5aSS and T5bSS), and type IVa pilus (T4aP) pili (Figure 3). The type VI secretion system (T6SSi) was present in only 10 strains in clade 1. Type IV (T4SS) was present only in the k11 strain in clade 2.

Mobile Genetic Elements in K. Michiganensis Genome

There were 13 plasmids including Col(pHAD28), IncFII(p14), IncU, IncFIA(HI1), IncFIB(pQil), repB(R1701), IncR, Col440II, IncFIB(K), IncX6, IncFII(pKP91), IncFII(Yp), IncN in 12 *K. michiganensis* strains (Figure 3). All 12 strains carried IncU plasmids. Notably, both strains of clade 2 carried two Col (pHAD28) plasmids. IncFIB (pQil) and repB (R1701) plasmids were found only in clade 1, whereas Col440II, IncFIB (K), and IncX6 plasmids were found only in clade 2. The ISs belonged to 14 IS families (IS1, IS3, IS5, IS4, IS21, IS66, IS91, IS110, IS256, IS481, IS1182, ISL3, ISAS1, and ISNCY), the most prevalent being IS3 and ISNCY (Figure 4). ISL3 and ISAS1 were only present in clade 2. In total, 138 prophages were predicted using DBSCAN-SWA. No ARG was found in the prophage genome predicted by Abricate using the Resfinder database for the k12 strain. The other 11 strains were contained to 5–9 kinds of ARGs. Furthermore, *aac(6')-Ib-cr,* $bla_{\text{CTX-M-3}}$, *fosA3* and *sul2* were found in the prophage genomes of 10 strains in clade 1. Three virulence genes (*gndA, ugd, east1*) were identified in the prophage genome as predicted by the VFDB database (Table S10).

Discussion

Recently, with the extensive use of carbapenem antibiotics, carbapenem-resistant strains have become widespread, thereby posing a serious public health problem in many countries. ARGs, such as $bla_{\text{KPC-2}}$, $bla_{\text{NDM-1}}$ and the RND efflux pump gene cluster, appear in a variety of aquatic environments, including lake water, rivers, well water, and wastewater treatment plants.[46–48] The main source of ARGs at these sites is likely to be hospital wastewater. The sources of hospital wastewater are complex, including medical and domestic wastewater.[49] Hospital wastewater contains highly resistant bacteria, which is a suitable reservoir for horizontal gene transfer (HGT).[50] There was a study that reported the prevalence of *Klebsiella spp*. in hospital wastewater, but it did not specify the presence of *K. michiganensis*.[51] To our

knowledge, this was the first report of carbapenem-resistant *K. michiganensis* co-harboring bla_{KPC-2} and *TmexCD2-ToprJ2* gene cluster isolated from hospital wastewater.

In this study, we examined the drug sensitivity and genomic characteristics of 12 *K. michiganensis* strains isolated from hospital wastewater samples in detail. All strains were MDR, and 12 were resistant to most antibiotics. Although the rate of resistance to tetracycline-class antibiotics in our antimicrobial susceptibility test was less than 10%, the tetracycline resistance gene cluster *TmexCD2-ToprJ2* was found in 11 strains, which was inconsistent. There were some discrepancies in antimicrobial resistance (AMR) between the detection of ARGs and the actual phenotype among strains, indicating that AMR predictions relying on in silico analysis of a sequenced genome should always be accompanied by AMR testing to reliably determine the AMR of microorganisms.[52,53] It is necessary to acknowledge the limitations of the short-read sequencing in our study. To mitigate these inaccuracies, we categorized the ISs positions identified in 12 strains into three groups based on the length of their flanking sequences: The first category includes ISs that constitute the entire contig, accounting for 259 cases (28.0%). The second category consists of IS elements located near the ends of contigs, where the flanking sequences on either side of the ISs are shorter than 1000 base pairs, comprising 365 cases (39.5%). The third category encompasses IS elements situated in the middle of contigs, with flanking sequences on both sides being at least 1000 base pairs long, totaling 301 cases (32.5%). We have incorporated the 301 ISs from the third category into our final set of reliable identification results. In our future work, we intend to combine short-read and third-generation long-read technology for analysis to improve accuracy.

As the efflux pump gene cluster, *TmexCD2-ToprJ2* has attracted our special attention, which is an orthologous variant of *TmexCD-ToprJ*.[54] It has been reported that there was IncHI1B plasmid encoding efflux pump *TmexCD2-ToprJ2* in carbapenem-resistant *K. michiganensis* strains.[55] In our study, *K. michiganensis* strains carrying plasmids encoding *TmexCD2-ToprJ2* gene cluster were also found in hospital wastewater samples. A previous study has shown that *TmexCD2-ToprJ2* functions as an efflux pump system in efflux inhibition experiments. *TmexCD2-ToprJ2* can be resistant to a variety of drugs, including tigecyclines.[54] The rapid expansion of *TmexCD-ToprJ* cluster has been attributed to various mobile genetic elements, such as integrative and conjugative elements (ICEs), transposons, or IS elements.[55–58] The upstream of the gene cluster also contains an IS element. To avoid the further expansion of *K. michiganensis* strains harboring *TmexCD-ToprJ2* gene cluster carrying mobile genetic elements in the environment, it is necessary to implement more stringent and effective disinfection measures for hospital wastewater. In addition, we found that umuC was located near the gene cluster. A study showed that a larger putative transposon comprised 34827bp harboring *TmexCD2-ToprJ2* was also inserted into the umuC-like gene.[55] The umuC gene seems to be the "hotspot" for *TmexCD2-ToprJ2* integration, and the specific molecular mechanism of site-specific integration should be worthy of further study.

There are few reports of *K. michiganensis* strains harboring bla_{KPC-2} in hospital settings. The global evolutionary analysis of *K. michiganensis* conducted by Zhang et al showed that only 22 of 446 *K. michiganensis* strains carried the bla_{KPC-2} gene, indicating that the bla_{KPC-2} carriage rate was low.[2] However, all 12 *K. michiganensis* strains carried bla_{KPC-2} which was located on plasmids, and the surrounding genetic environments contained IS and/or Tn3 family transposases. More attention should be paid to further transfer and dissemination of *K. michiganensis* harboring bla_{KPC-2} with the help of mobile elements such as plasmids, IS, and transposons. In a study by Zhang et al, *K. michiganensis* strains carrying bla_{KPC-2} were isolated from a blood sample of a patient at a tertiary hospital in Beijing, whereas our *K. michiganensis* strain harboring bla_{KPC-2} was isolated from wastewater samples at another hospital in Beijing. This reminds us to guard against further spread of *K. michiganensis* harboring bla_{KPC-2} gene.

In this study, four secretion systems were identified in all the genomes. The secretion systems encoded by strains of the bacteria confer adaptive advantages according to the niche occupied.[59] Macromolecular secretion systems are equipment implanted on the cell membrane and secrete effector factors, which are involved in key biological processes, including nutrition acquisition, environmental adaptation, inter-communication, and virulence gene expression.[60] It indicates that these secretion systems play an important role in adaptability and pathogenicity. The T6SS secretion system was present in 10 *K. michiganensis* strains in clade 1, and the T4SS secretion system was present only in the genome of one strain in clade 2. The type VI secretion system (T6SS) is a multiprotein complex that delivers effector proteins to the extracellular environment or directly to eukaryotic or prokaryotic cells. T6SSs are widely distributed in gram-negative bacteria and are crucial for bacterial virulence and the interaction between bacteria and other microorganisms or the environment.[59] Type IV secretion system (T4SS) can secrete

effector molecules, mediate conjugation and transformation, and play an important role in HGT and improve survival and pathogenicity.[61]

Bacteria can acquire ARGs through mobile genetic elements such as plasmids, transposons, integrons, and IS elements.[62] Due to their mobile nature, these genetic elements can easily spread horizontally across many species of bacterial populations.[63,64] ISs typically contribute to bacterial genetic diversity by excising or copying themselves and inserting into different parts of the genome.[65] The genetic environment the *TmexCD2-ToprJ2* and bla_{KPC-2} genes had IS elements inserted, suggesting a close relationship between ARGs and IS elements. Plasmids can carry functional genes such as ARGs, allowing bacteria to survive in adverse environments. All the strains in this study carried IncU plasmids. The IncU plasmid incompatibility group is a unique group of mobile elements with highly conserved backbone functions and variable ARG cassettes.[66] IS and plasmids further promoted the transmission of ARGs. In this study, all *K. michiganensis* strains were found to carry prophages, and ARGs and virulence genes were detected. Prophage induction and mobility, which are often increased by the action of antibiotics, can favor the dissemination of ARGs and other mobile genetic elements such as pathogenicity islands, thereby promoting bacterial evolution.[67]

Hospital wastewater is usually mixed with municipal wastewater, treated at wastewater treatment plants, and discharged into the aquatic environment. Due to the dilution effect, micropollutants are not completely removed, and some pathogens can survive, which can persist in the environment and finally be transferred to human, fish, and animal pathogens.[68,69] Therefore, it is necessary to continuously monitor hospital wastewater and take more stringent disinfection measures. Despite there are few reports about *K. michiganensis* harboring bla_{KPC-2} from hospital wastewater, in low epidemic environments, monitoring hospital wastewater can be used as an early warning system for the emergence and spread of opportunistic pathogens such as carbapenemase-producing *K. michiganensis*.

To sum up, the detailed genomic characteristics of carbapenem-resistant *K. michiganensis strains* co-harboring bla_{KPC-2} and *TmexCD2-ToprJ2* were described in this study. The most similar plasmids from clinical respiratory or blood samples were identified when finding the similar plasmids carrying bla_{KPC-2} or *TmexCD2-ToprJ2*. It indicated the cross-transmission between environmental and clinical *K. michiganensis* strains and the robust transfer of plasmids between strains, further highlighting the potential risk of infection and transmission in hospitals. Because *K. michiganensis* and *K. oxytoca* have the resemblance of protein spectra, highly comparable phenotypes, biochemical reactions, and 16S rRNA sequences (99% similarity in the nucleotide sequence), they cannot be distinguished in conventional detection. However, our study reported that *K. michiganensis* has been present in hospital wastewater, suggesting that routine monitoring of *K. michiganensis* is urgently needed to reduce associated problems. The ability to monitor *K. michiganensis* has been hampered, and further investigation is required in this area.

Abbreviations

K. michiganensis, Klebsiella michiganensis; K. oxytoca, Klebsiella oxytoca; GTDB, GENOME TAXONOMY DATABASE; NCBI, National Center for Biotechnology Information; ARGs, Antibiotic resistance genes; ISs, Insertion sequences; LCBs, Locally collinear blocks; HGT, Horizontal gene transfer; ICE, Integrative and conjugative elements.

Data Sharing Statement

The data were deposited at the National Microbiology Data Center (NMDC) under the accession number NMDC10018827 (https://nmdc.cn/resource/genomics/project/detail/NMDC10018827).

Ethical approval

Given that all the strains in this experiment were bacteria isolated from hospital wastewater samples, this study did not involve patients' private information or data. Therefore, this study was exempt from ethical approval by the Ethics Committee of Hospital.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically

reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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