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ORIGINAL RESEARCH

Genomic and Phylogenetic Analysis of a Multidrug-Resistant *bla*$_{NDM}$-carrying *Klebsiella michiganensis* in China

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Objective: *Klebsiella michiganensis* is an emerging hospital-acquired bacterial pathogen. However, there is a dearth of knowledge on the antimicrobial resistance and transmission of *K. michiganensis*. Here, we characterized the microbiological and genomic features of a carbapenem-resistant *K. michiganensis* strain harboring the *bla*$_{NDM-1}$ gene in China.

Methods: *K. michiganensis* strain 2563 was recovered from the sputum sample of a hospitalized patient with pulmonary infection. Whole-genome sequencing of *K. michiganensis* strain 2563 was conducted using both the short-read Illumina and long-read MinION platforms to thoroughly characterize the genetic context of *bla*$_{NDM}$-carrying plasmid in *K. michiganensis* 2563. Furthermore, BacWGSTdb server was utilized to perform in silico multilocus sequence typing (MLST), identify antimicrobial resistance genes, and conduct genomic epidemiological analyses of the closely related isolates deposited in the public database.

Results: *K. michiganensis* 2563 was resistant to piperacillin, aztreonam, meropenem, imipenem, amoxicillin-clavulanic acid, ampicillin, cefotaxime, cefazolin, ampicillin/sulbactam, cefepime, piperacillin-tazobactam, and ceftazidime. It belonged to sequence type (ST) 43, and the *bla*$_{NDM-1}$ gene was found to be located on the plasmid p2563_NDM (54,035 bp). This plasmid showed remarkable similarity to other *bla*$_{NDM-1}$-encoding plasmids found in various Enterobacterium species in the public database. The occurrence of global ST43 *K. michiganensis* was primarily sporadic, and the closest relative of *K. michiganensis* 2563 was another ST43 isolate 12,084 recovered from China in 2013, which differed by 171 SNPs.

Conclusion: Our study reports the genome characteristics of a carbapenem-resistant *K. michiganensis* strain carrying the *bla*$_{NDM-1}$ gene in China, highlighting the need for ongoing surveillance of this pathogen in clinical settings.

Keywords: whole-genome sequencing, *Klebsiella michiganensis*, *bla*$_{NDM}$, multidrug-resistant, phylogenetics

Introduction

Multidrug-resistant Enterobacteriaceae infection is a severe threat to public health that is worsening globally and is associated with increasing mortality rates.[1] *Klebsiella pneumoniae* is a significant pathogen that contributes to the dissemination of multidrug-resistant strains in healthcare environments.[2] *K. michiganensis* is a novel specie of pathogenic *Klebsiella* genus. Since its first discovery in 2013, *K. michiganensis* has continued to expand in the clinical setting and has been discovered colonizing patients and causing nosocomial epidemics.[3,4] Carbapenems play a critical role in managing severe infections caused by multidrug-resistant Gram-negative bacteria. Currently, the increased incidence of carbapenem-resistant Enterobacteriaceae (CRE), particularly *E. coli* and *K. pneumoniae* isolates, is overburdening worldwide healthcare systems.[5,6]

The carbapenem resistance 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, which are found on mobile genetic elements and conjugative plasmids, are associated with the widespread dissemination of carbapenem resistance.[7]

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K. michiganensis producing bla_{KPC},[4,8,9] bla_{VIM},[10] bla_{NDM}[11,12] and bla_{OXA-48}[12] have been found and they have been distributed in countries, such as China,[4] Canada,[8] Italy,[9] Japan,[11] Switzerland,[10] and South Africa.[12] However, only sporadic cases have been reported, and the prevalence and transmission patterns of multidrug-resistant *K. michiganensis* are poorly understood. Further research is required to investigate the genomic traits and antimicrobial resistant characteristics exhibited by the *K. michiganensis*.

Here, we report the genomic traits of a multidrug-resistant ST43 *K. michiganensis* strain discovered in China. In addition, we performed a comprehensive genomic epidemiology analysis to date with all the available genome sequences of ST43 *K. michiganensis* isolates.

Materials and Methods

The *K. michiganensis* strain 2563 was isolated in a sputum sample collected from a woman upon admission to the hospital with pulmonary infection. The culture of *K. michiganensis* 2563 was obtained from the sputum sample within 24 hours of collection. The isolate was first identified by VITEK 2 (bioMérieux, Marcy-l'Étoile, France) and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS, Bruker, Billerica, MA, USA). *K. michiganensis* 2563 was tested for antimicrobial susceptibility with different antimicrobial agents, including piperacillin, gentamicin, imipenem, amoxicillin-clavulanic acid, cefotaxime, cefepime, ceftazidime, amikacin, ciprofloxacin, levofloxacin, moxifloxacin, trimethoprim-sulfamethoxazole, aztreonam, meropenem, piperacillin-tazobactam, ampicillin/sulbactam, cefazolin, chloramphenicol, and tetracycline. The Clinical and Laboratory Standards Institute (CLSI) 2021 guidelines were used for interpretation of the results. Antimicrobial susceptibility testing was quality-controlled using *Escherichia coli* (ATCC 25922).

The genome of *K. michiganensis* strain 2563 was sequenced using the 150 bp paired-end protocol on the HiSeq X10 platform (Illumina, San Diego, CA, USA) and the MinION platform (Nanopore, Oxford, UK). Unicycler v 0.5.0[13] was utilized for the hybrid assembly of both Illumina short-reads and Nanopore long-reads. The genome annotation was conducted employing the NCBI Prokaryotic Genome Annotation Pipeline. On January 19, 2023, we retrieved genome sequences and strain metadata of 577 *K. michiganensis* from the National Center for Biotechnology Information database. We conducted in silico multilocus sequence typing (MLST) analysis using our BacWGSTdb server.[14] The NCBI AMRFinderPlus tool[15] and VFDB database (http://www.mgc.ac.cn/VFs/) were utilized to conduct a search for antimicrobial resistance genes and virulence genes. Easyfig[16] was utilized in order to conduct an analysis of the genetic context of antimicrobial resistance genes, while BRIG[17] was used to compare the complete sequences of bla_{NDM}-carrying plasmids. The global distributed *K. michiganensis* strains share the same sequence type with the *K. michiganensis* strain 2563 were selected for the phylogenetic analysis (Table S1). Snippy was used to construct phylogenetic trees based on recombination-free core genome SNPs (https://github.com/tseemann/snippy). rhierBAPS[18] was used to define clusters of global ST43 *K. michiganensis* isolates. The interactive Tree of Life (iTOL) V5 web server[19] was used to visualize and interpret the phylogenetic trees and display the presence/absence of different classes of antimicrobial resistance genes.

Results

The genome of *K. michiganensis* strain 2563 was assembled into six contigs, with a total length of 6,720,981 bp and an overall G+C content of 55.46%. The contig 1 consisting of 5,961,931 bp was recognized as the chromosome, while the remaining six contigs were associated with different plasmids at 362,448 bp, 183,276 bp, 105,902 bp, 54,035 bp and 53,269 bp, respectively. A total of 6130 protein-coding sequences, 84 tRNA genes, 118 rRNA genes, and one CRISPR array were identified by the PGAP server. *K. michiganensis* 2563 displayed a high degree of antimicrobial resistance to multiple β-lactam antibiotics, including piperacillin, amoxicillin-clavulanic acid, aztreonam, meropenem, imipenem, ampicillin, ampicillin/sulbactam, cefotaxime, cefazolin, cefepime, piperacillin-tazobactam and ceftazidime. However, it remained susceptible to amikacin, gentamicin, levofloxacin, ciprofloxacin, moxifloxacin, trimethoprim-sulfamethoxazole, chloramphenicol, and tetracycline (Table 1). *K. michiganensis* 2563 harbored multiple antimicrobial resistance genes, including aminoglycosides [*aph(3')-Ia*], carbapenem [bla_{NDM-1}], ESBL [bla_{SHV-12}], other β-lactams [bla_{OXY-1} and ble_{MBL}], fosfomycin [*fosA*], and fluoroquinolones [*oqxA10* and *oqxB9*]. Furthermore, multiple virulence genes have

Table 1 Minimal Inhibitory Concentrations (MICs) to Different Antimicrobial Agents of *K. michiganensis* 2563

Antimicrobials	MIC (μg/mL)	Susceptibility
β-lactams		
Ampicillin	>16	R
Piperacillin	>64	R
Aztreonam	>16	R
Meropenem	>8	R
Imipenem	>8	R
Amoxicillin-clavulanic acid	>16/8	R
Ampicillin/sulbactam	>16/8	R
Piperacillin-tazobactam	>64/4	R
Cefotaxime	>32	R
Cefazolin	>16	R
Cefepime	>16	R
Ceftazidime	>16	R
Aminoglycosides		
Amikacin	<=8	S
Gentamicin	<=2	S
Fluoroquinolones		
Levofloxacin	<=1	S
Ciprofloxacin	<=0.5	S
Moxifloxacin	<=1	S
Others		
Trimethoprim-sulfamethoxazole	<=0.5/9.5	S
Chloramphenicol	<=4	S
Tetracycline	<=2	S

Abbreviations: R, resistance; S, sensitivity.

been identified on the chromosome of this strain, including *entA, entB, fyuA, irp1, irp2, mgtB, ompA, yagZ/ecpA, ybtA, ybtE, ybtP, ybtQ, ybtS, ybtT, ybtU* and *ybtX*.

The $bla_{\text{NDM-1}}$ gene was located on an IncX3 type plasmid (p2563-NDM). In addition to the resistance gene $bla_{\text{NDM-1}}$, the IncX3 plasmid harbored the ESBL resistance gene $bla_{\text{SHV-12}}$ and other β-lactam gene ble_{MBL} (Figure 1A). Five insertion sequences (ISs), namely Tn*3*, IS*3000*, IS*Aba125*, IS*5* and IS*30*, were identified upstream of the $bla_{\text{NDM-1}}$ gene, and the ble_{MBL}, *trpF, tat*, IS*91* and IS*26* genes were detected downstream. Sequence comparison using BLAST showed that the p2563-NDM plasmid exhibited 100% query coverage and 100% identity with various bla_{NDM}-bearing plasmids, such as pCQ17X3 from an *E. cloacae* strain CQ17, which was isolated from a sputum sample in China (NCBI GenBank accession number: CP103970.1); pNDM1_020049 in *K. pneumoniae* strain SCKP020049 from a human pus sample in China (NCBI GenBank accession number: CP028786.2) and pC46-NDM11 was identified in *C. freundii* strain C46 isolated from urine in China (NCBI GenBank accession number: MW269623.1) (Figure 1B).

According to an in silico MLST investigation, *K. michiganensis* strain 2563 belongs to ST43. To investigate the global genomic epidemiology of *K. michiganensis* strains, we analyze the phylogenetic relationships between *K. michiganensis* 2563 and other 27 ST43 *K. michiganensis* strains retrieved from the NCBI GenBank database. ST43 *K. michiganensis* isolated from Japan (n=7), Switzerland (n=6), Australia (n=5), China (n=5), the United States (n=4), and Germany (n=1). The most prevalent source of ST43 *K. michiganensis* (n=23, 82.14%) was clinical samples, followed by several samples from sink (n=2), livestock (n=1), soil (n=1), and serpentes (n=1). The isolation rate showed an increasing trend yearly, from only one strain in 2013 to ten strains in 2018 (Figure 2A). Phylogenetic analysis suggested that the global ST43 *K. michiganensis* could be divided into four clusters, and the strain 2563 isolated was located in cluster 1. The average SNP difference between these strains was 291 SNPs, whereas 16 pairs had differences of less than 21 SNPs (Figure 2B). These highly homologous strains appeared in the Chinese sink and Japanese human origins, but did

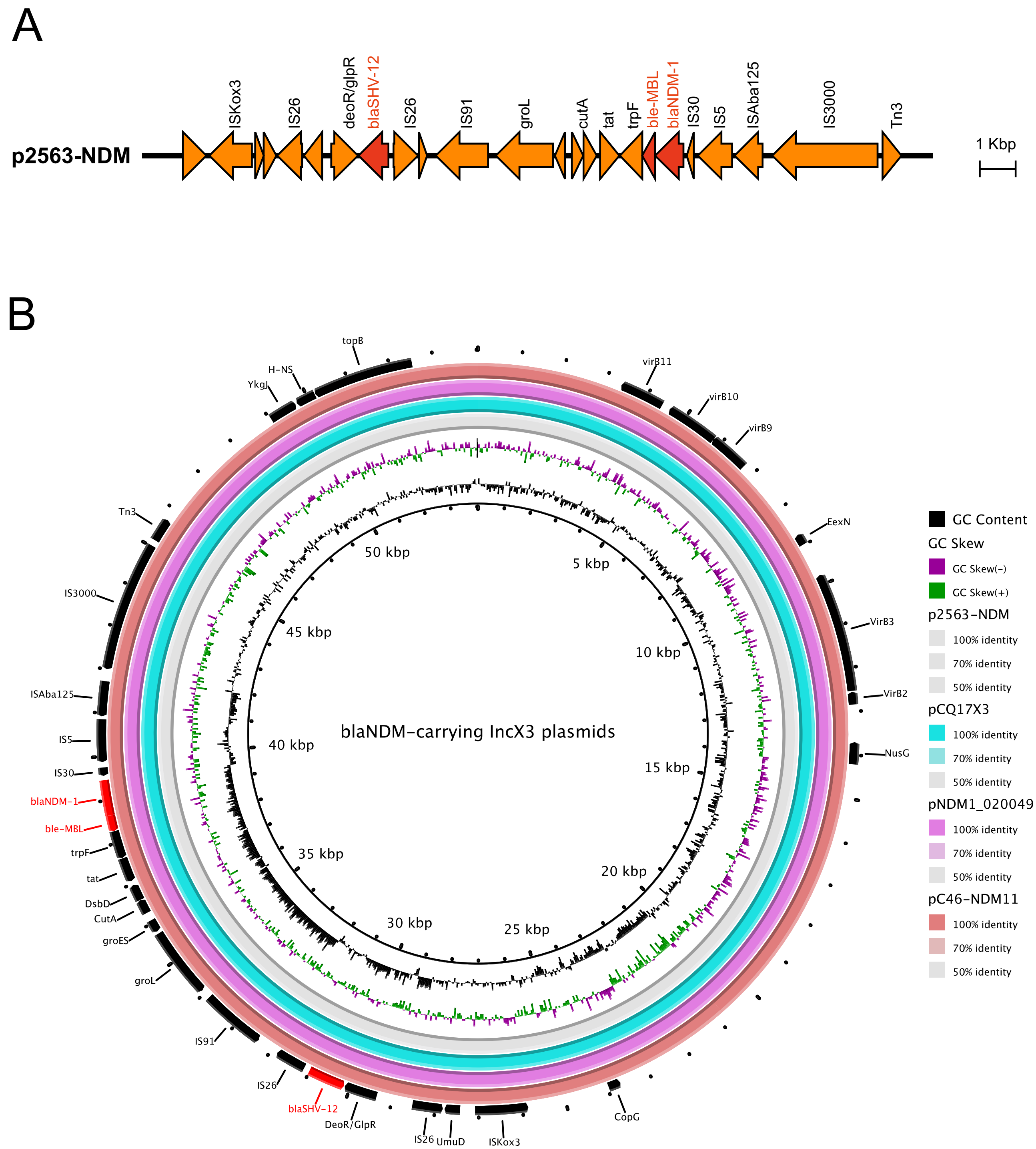

Figure 1 Genetic organization of plasmids harboring the bla_{NDM} gene. (**A**) Genetic environment of the bla_{NDM-1} flanking region in p2563-NDM. The red arrows represent the β-lactam resistance genes, the Orange arrows represent additional coding sequences (CDSs). (**B**) Circular comparison of p2563-NDM and IncX3 bla_{NDM} carrying plasmid. The homology between these plasmids is shown by the percentage identity in the figure legend, whereas the absence of or a similarity value of <50% is indicated on the circular map as a white gap. The red text highlighted in the figure represent antimicrobial resistance genes.

not occur transnational or cross-host transmission. The closest relative strain of ST43 *K. michiganensis* strain 2563 was strain 12,084, which recovered from clinical specimens in China in 2013, with 171 SNPs differences. This strain only carried five antimicrobial resistance genes, ie, *aph(3')-Ia*, bla_{OXY-1}, *fosA, oqxA10*, and *oqxB9*. The range of antimicrobial resistance genes harbored by ST43 *K. michiganensis* ranged between 4 and 19, including aminoglycoside [*ant(2")-Ia*

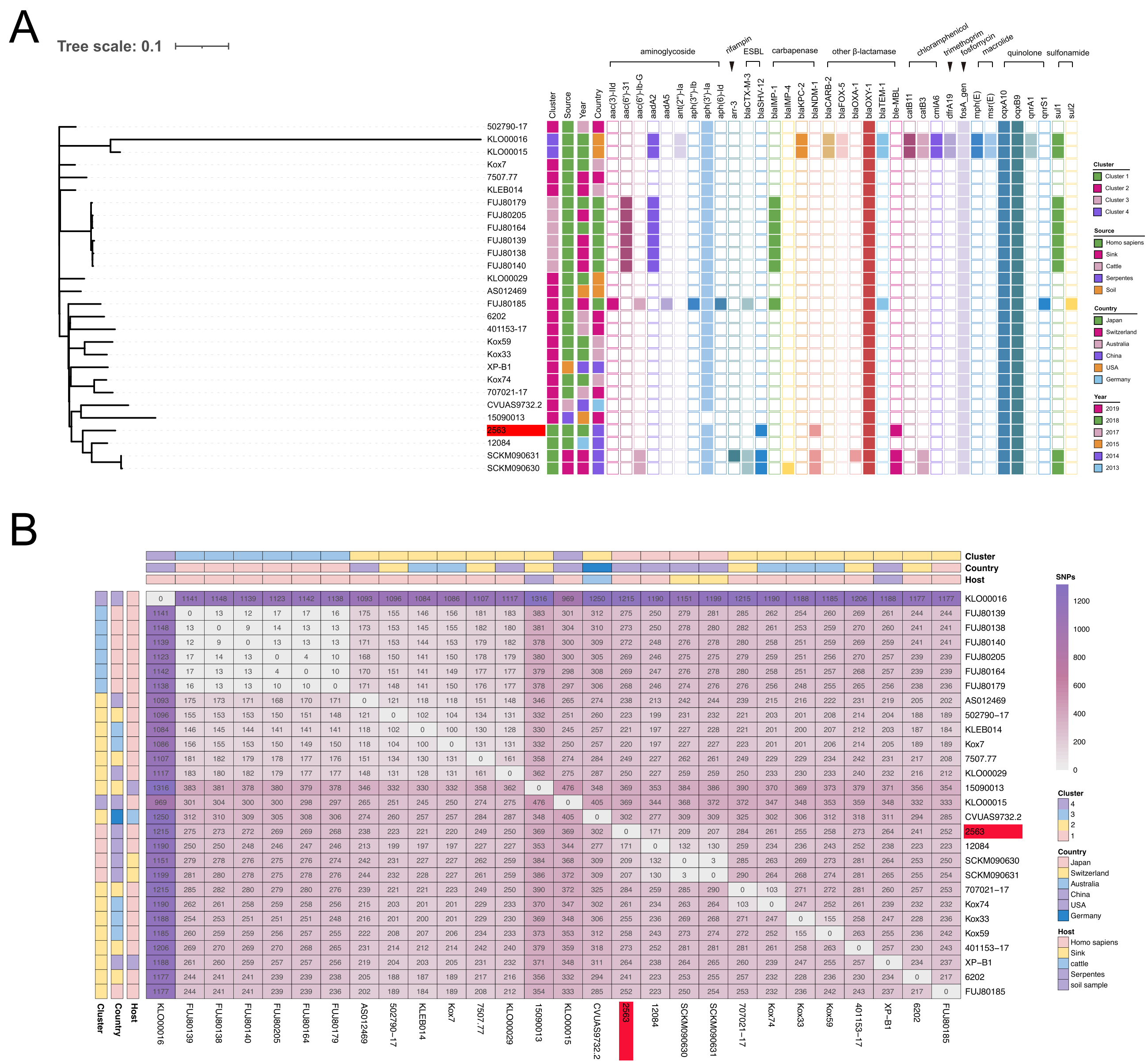

Figure 2 Phylogenetic relationship of global ST43 *K. michiganensis* strains. (**A**) The phylogenetic tree was generated based on the differences of core genome single nucleotide polymorphisms, annotated by iTOL webserver. The figure provides information regarding the antimicrobial resistance genes, isolation source, time, and country of these strains. (**B**) Visualization heat map of the differences of core genome single nucleotide polymorphisms among global ST43 *K. michiganensis* strain. The number in the square indicates the number of core genome single nucleotide polymorphisms between strains. The source, country and cluster of strains are also colored.

(7.14%), *aph(3')-Ia* (96.42%), *aadA2* (28.57%), *aph(3")-Ib* (3.57%), *aac(6')-31* (21.43%), *aac(6')-Ib* (10.71%), *aadA5* (3.57%), *aph(6)-Id* (3.57%), and *aac(3)-IId* (3.57%)], rifampin [*arr-3* (3.57%)], ESBL [$bla_{\text{CTX-M-3}}$ (10.71%), $bla_{\text{SHV-12}}$ (10.71%)], carbapenems [$bla_{\text{IMP-1}}$ (25%), $bla_{\text{IMP-4}}$ (3.57%), $bla_{\text{KPC-2}}$ (7.14%), and $bla_{\text{NDM-1}}$ (10.71%)], other β-lactamase [$bla_{\text{CARB-2}}$ (7.14%), $bla_{\text{FOX-5}}$ (7.14%), $bla_{\text{OXA-1}}$ (3.57%), $bla_{\text{OXY-1}}$ (100%), $bla_{\text{TEM-1}}$ (10.71%), ble_{MBL} (10.71%)], chloramphenicol [*catB11* (7.14%), *catB3* (14.29%), *cmlA6* (7.14%)], trimethoprim [*dfrA19* (7.14%)], fosfomycin [*fosA* (100%)], macrolide [*mph(E)* (7.14%), *msr(E)* (7.14%)], quinolone [*oqxA10* (100%), *oqxB9* (100%), *qnrA1* (7.14%), and *qnrS1* (3.57%)], and sulfonamide [*sul1* (42.96%), *sul2* (3.57%)] (Figure 2A). All ST43 *K. michiganensis* strains carried the same virulence genes, namely *entA, entB, fyuA, irp1, irp2, mgtB, ompA, yagZ/ecpA, ybtA, ybtE, ybtP, ybtQ, ybtS, ybtT, ybtU* and *ybtX*, except for strain SCKM090630 which lacks *entA* and *entB*.

Discussion

CRE is a serious challenge in the hospital settings, with high frequency of treatment failures due to a high resistance rate to various antimicrobial agents.[20] In our investigation, we identified a bla_{NDM-1}-carrying *K. michiganensis* strain and characterized its genomic characteristics. In addition, we conducted a global genomic epidemiological investigation of ST43 *K. michiganensis* to aid health departments in their efforts to monitor carbapenem-resistant pathogens more effectively. *K. michiganensis* 2563 was a multidrug-resistant strain, which was resistant to β-lactam antimicrobial agents, including meropenem and imipenem. Fortunately, our findings indicated that amikacin, gentamicin, levofloxacin, ciprofloxacin, moxifloxacin, trimethoprim-sulfamethoxazole, chloramphenicol, and tetracycline can still be effectively used to treat the infection. A previous study indicated that despite harboring the *fosA* gene, the strain remained susceptible to fosfomycin, which was consistent with our findings.[21] The carbapenemase gene can be transferred horizontally between antimicrobial-resistant and -susceptible strains via transposable element and conjugative plasmids. This is the primary factor contributing to the rising incidence of CRE infections in both hospital and community settings.[22] The bla_{NDM-1} is located on an IncX3-type plasmid in *K. michiganensis* 2563, which is the main replicon type of bla_{NDM}-carrying and bla_{KPC}-carrying plasmid in China.[23,24] We also investigated the genetic surroundings of bla_{NDM-1} and discovered an abundance of mobile genetic elements, which shared a highly conserved sequence structure to those described before.[25,26] We discovered that the bla_{NDM}-carrying IncX3 plasmid was highly similar among different species, indicating that the antimicrobial resistance determinant carried by the IncX3 plasmid may be capable of horizontal transmission between species, posing a significant danger to the prevention and management of nosocomial infection. Moreover, multiple virulence genes were also identified in *K. michiganensis* 2563, including enterobactin and yersiniabactin, which act as siderophores to promote bacterial invasion and infection of the host.[27]

Whole-genome sequencing offers useful data for epidemiological surveillance on account of its strong discriminatory power and low cost.[28] Through phylogenetic analysis, we found that the global population of ST43 *K. michiganensis* population was expanding continuously since it was first discovered in a case reported in 2013. We hypothesized that for highly probable local transmission, the differences between isolates were ≤21 SNPs.[7] The clonal transmission of ST43 *K. michiganensis* has occurred locally in Japan and China, which should attract the attention to avoid the expansion and development of transcontinental or trans-host transmission. *K. michiganensis* 2563 is most closely related to strain 12,084, which lacks the carbapenem resistance gene and the IncX3-type plasmid, implying that the acquisition of the bla_{NDM-1} gene by *K. michiganensis* 2563 may have occurred through horizontal transmission.

In summary, we characterized the genomic features of a *K. michiganensis* strain carrying bla_{NDM-1} from China and conducted an epidemiological study of ST43 *K. michiganensis* at the global scale. The implementation of an active surveillance programs to control the further transmission of this emerging multidrug resistant pathogen is an absolute requirement.

Data Sharing Statement

The complete genome sequence of *K. michiganensis* 2563 has been deposited into NCBI GenBank database under the accession numbers CP116214-CP116219. The data supporting the findings of this study are available within the article or its Supplementary Materials.

Institutional Review Board Statement

The study was performed in compliance with the Declaration of Helsinki and was approved by the Ethics Committee of Sir Run Run Shaw Hospital, Zhejiang University School of Medicine (Approval No.:2022-227). Written informed consent was waived by the Ethics Committee of Sir Run Run Shaw Hospital, Zhejiang University School of Medicine as the study exclusively focused on bacteria.

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 declare no conflict of interest.

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