Epidemiology of *Klebsiella michiganensis* Carrying Multidrug-Resistant IncHI5 Plasmids in the Southeast Coastal Area of China

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**Purpose:** This study aimed to explore the genomic characterization of multidrug-resistant IncHI5-carrying *Klebsiella michiganensis* strains and detailed genomic dissection of the IncHI5 plasmids.

**Materials and Methods:** Through whole-genome sequencing, the IncHI5 plasmid pK92-qnrS was obtained from a single clinical *K. michiganensis* isolate K92. All complete genomes of *K. michiganensis* strains from the Genome database of NCBI were collected and used to construct a maximum likelihood (ML) phylogenetic tree. The epidemiology and geographic distribution of all the *K. michiganensis* strains were conducted. An extensive comparison of the seven IncHI5 plasmids of *K. michiganensis* (one from this study, six from GenBank) was applied.

**Results:** This study revealed that all *K. michiganensis* strains carrying IncHI5 plasmids from different clonal groups were located in the southeast coastal area of China. The backbone regions of IncHI5 plasmids were composed of replicon (*repHI5B* and *repFIB*), partition (*parABC*), and conjugal transfer (*tra1*/*tra2*). The main accessory resistant regions of IncHI5 could be divided into two categories, *Tn1696*-related region and *Tn6535*-related region. These seven IncHI5 plasmids carried multiple drug-resistance genes which were all mediated by the mobile genetic elements (MGEs).

**Conclusion:** Data presented here help to provide an overall in-depth understanding of epidemiology and geographic distribution of IncHI5-carrying *K. michiganensis* and the structure and evolutionary history of IncHI5 plasmids.

**Keywords:** *Klebsiella michiganensis*, mobile genetic elements, *Tn1696*, *Tn6535*, antimicrobial resistance

**Introduction**

*Klebsiella michiganensis* is a potential emerging pathogen causing serious and difficult-to-treat nosocomial infections. In 2012, *K. michiganensis* was first recovered from a toothbrush holder and relatedness with *Klebsiella oxytoca* was originally recognized by a list of housekeeping genes, although it was finally proved by DNA-DNA hybridization (DDH) that it should be a new species.1 Since then, clinical cases caused by *K. michiganensis* have been reported worldwide. Some *K. michiganensis* isolates showed antibiotic resistance, and even multidrug-resistance (MDR) through the production of carbapenemases.2–4 In existing reports, there have been multiple genetic recombination and integration events of drug resistance genes through mobile genetic elements (MGEs) such as transposons and plasmids carried by *K. michiganensis*.5–8 The existence and properties of plasmids make *K. michiganensis* isolates a serious threat, and it is of interest to conduct in-depth studies of the structure and function of the plasmids carried by *K. michiganensis*.
IncHI5 is one of the five subgroups of plasmids subdivided under IncHI based on its replication genes repHI5B and repFIB. IncHI5 plasmids, like most IncHI plasmids, have a size usually greater than 200 kb and a wide host range. IncHI5 plasmids are significant vectors carrying genes encoding resistance to heavy metals and antibiotics. It is necessary to continuously monitor the prevalence of IncHI5 plasmids in different species. However, research on the structure and evolution of IncHI5 plasmids is still limited, and there is a lack of an overall study on IncHI5 plasmids within specific species.

IncHI5 plasmids carried by K. michiganensis were first reported in 2012 in a strain from Miaoli, Taiwan, China. There are limited reports on IncHI5 plasmids carried by K. michiganensis. The sequence of repHI5B (the key identified replicon of IncHI5 plasmids) was used to perform BLAST alignment against the GenBank of the National Center for Biotechnology Information (NCBI) to cover all known IncHI5 plasmids. From the alignment, a total of six IncHI5 plasmids whose host strain were K. michiganensis were obtained (last accessed on December 12, 2021): pKOX_R1 (accession number CP003684), p12084-HI5 (accession number MW810613), pCP024641 (accession number CP024641), pJNQH491-2 (accession number CP075883), p1 (accession number CP067094), and pKOX7525_1 (accession number CP065475).

In this work, we characterized IncHI5 plasmid pK92-qnrS (accession number OL828743; from Taizhou, Zhejiang, China) and presented further comprehensive genomic comparison of all IncHI5 plasmids carried by K. michiganensis (six plasmids mentioned above and pK92-qnrS), and discussed epidemiology and geographic distribution of K. michiganensis, to gain a deeper understanding of the genomic characterization of multidrug-resistant IncHI5-carrying K. michiganensis strains and detailed genomic dissection of the IncHI5 plasmids.

Materials and Methods
Identification of Bacterial Strain and Conjugal Transfer
K. michiganensis strain K92 was isolated from a patient’s sputum in Taizhou Municipal Hospital affiliated with Taizhou University of China in 2018. Strain K92 was initially identified as K. oxytoca by Vitek 2. Moreover, bacterial species identification was performed using chromosomal genome sequence-based average nucleotide identity (ANI) analysis and finally proved that K92 belongs to K. michiganensis. THO-011 (accession number AP022547), the standard strain of K. michiganensis, was used as reference. The OrthoANI value between THO-011 and K92 was 98.83% (> 95% cut-off), which was calculated by OAT 0.93.1.

Conjugal transfer experiments were carried out with sodium azide-resistant Escherichia coli J53 being used as a recipient, and strain K92 as a donor. The donor and recipient strains were grown in three milliliters (mL) brain heart infusion (BHI) broth overnight at 37°C. And then, 50 μL of donor strain culture was mixed with 500 μL of recipient strain culture (v:v = 1:10) and 4.5 mL of fresh BHI broth. In addition, 100 μL of the mixture was applied onto a cellulose filter membrane (pore size, 0.22 μm) already placed on a BHI agar plate. After incubation at 25 °C for 16–18 h, the filter membrane was taken out and vortexed in 1 mL of BHI broth. The vortex mixtures were plated on BHI agar plates containing 40 mg/L ciprofloxacin and 150 mg/L sodium azide for the selection of the E. coli J53 transconjugants. However, repeated conjugation experiments failed to transfer the qnrS marker from K92 to E. coli J53 (sodium azide resistance).

Sequencing and Sequence Assembly
Genomic DNA was extracted from strain K92 using a Gentra Puregene Yeast/Bact. Kit (Qiagen, Valencia, CA). Libraries were prepared separately using the TruePrepTM DNA Library Prep Kit V2 and the SQU-LSK109 Ligation Sequencing kit. After the preparation of the library was completed, it was separately sequenced on an Illumina HiSeq X Ten platform using the 2 × 150-base-pair paired-end mode (Illumina Inc., San Diego, CA, USA) and GridION X5 platform using the long reads (Oxford Nanopore, UK), the sequencing depth was 100x. To improve the reliability of data processing, raw data from HiSeq X Ten platform and GridION X5 platform were trimmed to obtain the high-quality clean reads (clean data) by Canu v1.8 (https://canu.readthedocs.io/en/latest/index.html). The paired-end short Illumina reads and the long Nanopore reads were assembled de novo utilizing Unicycler (v0.4.5) (https://github.com/rrwick/Unicycler).
Whole Genome Phylogeny and Genetic Background Analysis

A total of 32 publicly available complete genome sequences of *K. michiganensis* were downloaded from NCBI (last accessed on December 12, 2021), which were isolated from various sources in 2011–2021. Genomes were aligned against the reference genome (THO-011, the standard strain of *K. michiganensis*) using MUMmer v3.1, to identify the SNPs in the backbone regions. A total of 484,632 SNPs (from 3,011,423-bp length core genomes) were identified in the seven *K. michiganensis* strains chromosome sequences, and an ML phylogenetic tree was constructed using this SNPs dataset. Phylogenetic trees were drawn using the Interactive Tree of Life (iTOL) programs. The evolution analysis of the seven IncHI5 plasmids’ backbone regions was consistent with the above-mentioned phylogenetic analysis method.

Sequence Annotation and Comparison

Sequence annotation and prediction of ORFs and pseudogenes were performed using RAST2.0, and further manual annotation was done with BLASTP/BLASTN against the UniProtKB/Swiss-Prot and RefSeq databases. Annotation of drug resistance genes, MGEs, and other features was performed using online databases such as CARD, ResFinder, ISfinder, INTEGRALL, and the Tn Number Registry. MUSCLE 3.8.31 and BLASTN were used for multiple and pairwise sequence comparisons. The genome map was drawn using Inkscape 1.1 (https://inkscape.org/en). The heatmap of resistance genes carried by IncHI5 plasmids included in this study was drawn with iTOL.

Nucleotide Sequence Accession Number

The complete sequences of the chromosome of K92 and plasmid pK92-qnrS were submitted to GenBank database, under accession numbers CP089315 and OL828743, respectively.

Results

Genomic Characterization of *K. michiganensis* Genomes

A total of 32 complete genomes of *K. michiganensis* strains with geographic distribution and other background information were obtained from the Genome database of NCBI (last accessed on December 12, 2021) (Figure 1A and Supplementary Table S1). The chromosomal sequences of these 32 strains were aligned with the chromosomal sequence (accession number CP089315) of *K. michiganensis* strain K92 in this study, and strain HS11286 (accession number CP003200), the standard strain of *Klebsiella pneumoniae*, was used as the outer group, and a total of 484,632 SNPs were obtained. Among them, *K. michiganensis* strain RC10 (accession number CP011077) was excluded because of the low coverage value of the comparison with core genome of the 32 strains (14.8%). A maximum likelihood (ML) phylogenetic tree was constructed using these SNPs’ dataset (Figure 1B). The population structure showed that *K. michiganensis* strains carrying IncHI5 plasmids were scattered in different clades of the entire phylogenetic tree (orange dots and red star in Figure 1B), instead of being clustered and distributed in a certain branch of the tree, which may imply the acquisition of IncHI5 plasmids was an overall behavior of *K. michiganensis* and was not related to a specific branch of *K. michiganensis*. The geographic distribution showed that *K. michiganensis* strains were mainly distributed in North America, Europe, East Asia, and Oceania (Figure 1A). Although *K. michiganensis* had also been reported in South Africa before, the complete genome was not available in the NCBI Genome database, so *K. michiganensis* reported in South Africa was not included in this study. Sixteen of these strains originated in China. Coincidentally, all the *K. michiganensis* strains carrying IncHI5 plasmids were located in the southeast coastal area of China such as Zhejiang, Fujian, Taiwan, and Guangdong (orange dots and red star in Figure 1A–C). This geographic distribution indicated that *K. michiganensis* strains carrying IncHI5 plasmids were spread in a southeast coastal area of China, although the underlying mechanism is currently unclear.

Overview of All IncHI5 Plasmids Carried by *K. michiganensis*

The length of the seven plasmids mentioned above ranged from 274 kb to 397 kb, and the number of ORFs predicted on them ranged from 288 to 416 (Table 1 and Supplementary Figure S1). All the seven plasmids belonged to IncHI5...
plasmids, because they all contained the replicon combination repHI5B and repFIB unique to IncHI5 plasmids, and had a similar conserved backbone regions structure. Table 1 showed the accession number, host bacterium, total length, total number of ORFs, mean G+C content, length of backbone, mean G+C content of backbone, origin, and other information of these plasmids. With the exception of plasmid pCP024641, the difference in the length of the backbone region of these plasmids was small. The extra part of the backbone region of pCP024641 revealed in the comparative genomics that it may be derived from the integration of part of the backbone regions of the IncC plasmids. All these

Figure 1 Population distribution and geographic distribution of all known Klebsiella michiganensis strains with complete genomes. (A) Geographic locations of 32 K. michiganensis strains in the world. The dots in the figure are used to mark the geographic distribution of strains. If the number of strains distributed in countries except China was greater than one, one dot is used to represent. Strains located in China are represented by smaller dots due to their dense distribution. The black dots represent the K. michiganensis strains that did not carry IncHI5 plasmids, the orange dots represent the K. michiganensis strains carrying IncHI5 plasmids, and the red star represents the K. michiganensis strain carrying pK92-qnrS from this study. (B) Maximum-likelihood tree. The degree of support (percentage) for each cluster of associated taxa, as determined by bootstrap analysis, is shown with blue dots next to each branch. The bar corresponds to the scale of sequence divergence. The meaning of the symbols corresponds to that in (A). (C) Geographic locations of K. michiganensis strains in China. The meanings of the symbols correspond to those in (A).
Table 1 Major Features of IncH15 Plasmids Carried by Klebsiella michiganensis Included in This Study

| Plasmid          | Accession Number | Host Bacterium          | Total Length (bp) | Total Number of ORFs | Mean G+C Content (%) | Length of Backbone (bp) | Mean G+C Content of Backbone (%) | Origin          | Year | Specimen | Host            |
|------------------|------------------|-------------------------|-------------------|----------------------|----------------------|------------------------|-----------------------------------|----------------|------|-----------|----------------|
| pK92-qnrS        | OL828743         | Klebsiella michiganensis k92 | 312,620           | 318                  | 47.0                 | 215,280                | 44.7                              | Taizhou, Zhejiang China    | 2017 | Sputum    | Homo sapiens |
| pKOX_R1          | CP003684         | Klebsiella michiganensis E718 | 353,865           | 352                  | 47.5                 | 215,028                | 44.7                              | Miaoli, Taiwan China         | 2010 | –          | Homo sapiens |
| p12084-H15       | MW810613         | Klebsiella michiganensis 12084 | 273,923           | 288                  | 46.6                 | 202,404                | 45.0                              | Zhejiang, China             | 2021 | –          | Homo sapiens |
| pCP024641        | CP024641         | Klebsiella michiganensis F107 | 308,586           | 324                  | 47.1                 | 257,513                | 45.7                              | Fuzhou, Fujian China        | 2014 | Sputum    | Homo sapiens |
| pJNQH491-2       | CP075883         | Klebsiella michiganensis JNQH491 | 307,464           | 307                  | 47.9                 | 211,726                | 44.6                              | Xiamen, Fujian China        | 2020 | Blood     | Homo sapiens |
| p1               | CP067094         | K. michiganensis B106    | 284,262           | 298                  | 47.0                 | 203,453                | 45.1                              | Guangzhou, Guangdong China | 2021 | –          | Homo sapiens |
| pKOX72525_1      | CP065475         | Klebsiella michiganensis 7252 | 397,447           | 416                  | 49.1                 | 205,793                | 44.8                              | Hangzhou, Zhejiang China    | 2020 | Urine      | Homo sapiens |

Notes: Plasmid pK92-qnrS was fully sequenced in our laboratory while plasmids pKOX_R1, p12084-H15, pCP024641, pJNQH491-2, p1, and pKOX72525_1 were derived from GenBank. The comparison of the genome structure of all seven plasmids is explained in the main text.
seven IncHI5 plasmids had one or more relatively huge and complex MDR regions. Phylogenetic analysis, accurate annotation and detailed comparison were performed to gain an overall deeper evolution history and structural information of these plasmids.

**Comparison of the Seven IncHI5 Plasmids**

The genetic structure of all IncHI5 plasmids can be divided into backbone regions and accessory regions. The length of the backbone regions of these seven plasmids varies from 202kb to 257kb (Table 1). Pairwise sequence comparison using BLASTN showed that they all had 99% nucleotide identity and coverage greater than 73% (Supplementary Table S2). A linear comparison of the backbone regions of these seven plasmids revealed the following characteristics (Supplementary Figure S2): (1) The major IncHI5 backbone genes or gene loci such as repHI5B, repFIB, parABC, and tra1/tra2 were conserved. (2) Among the seven plasmids, all except pCP024641 contained two conjugal transfer regions (tra1/tra2). Although the structure of tra2 in different plasmids was not exactly the same, some lost a part of transfer genes, which may hinder their self-transferability. (3) pCP024641 had additional backbone regions compared with other plasmids, including a region of conjugative transfer. Comparing with IncC plasmid pR55, pCP024641 showed more than 95% identity, suggesting that this fragment may be derived from IncC plasmid. A total of 705 SNPs were identified from the backbone regions of these seven plasmids, and an ML phylogenetic tree was constructed using these SNPs dataset. Accordingly, these seven plasmids could be assigned into three clades: I (pKOX7525_1 and pJNQH491-2), II (pK92-qnrS and pKOX_R1), and III (p12084-HI5, p1, and pCP024641) (Figure 2).

Large accessory regions were inserted into the different loci of all the seven plasmid backbones, such as insertion sequences (ISs) and transposons. All the seven plasmids except p12084-HI5 had two main longer accessory resistance modules (Supplementary Figure S2). These accessory resistance modules could be divided into two main categories according to their structure and origin: Tn1696-related regions and Tn6335-related regions, and multiple drug-resistant genes were carried on these accessory resistance modules. (Figure 2 and Supplementary Table S3).

**Comparison of Tn1696-Related Regions**

There were nine Tn1696-related regions in these seven plasmids (Figure 3), including 81.1-kb MDR-1 region and 41.9-kb MDR-2 region from pKOX_R1, Tn7383 from pK92-qnrS, Tn7384 from p12084-HI5, Tn7385 from pCP024641, Tn7386 from pJNQH491-2, Tn7387 and 19.0-kb MDR region from p1, and 141.5-kb MDR-2 region from pKOX7525_1. Due to the similarity of structure, these regions can be regarded as derivatives of Tn1696. Tn1696 was a unit transposon of the Tn3 family, and its two ends were inverted repeat left (IRL) and inverted repeat right (IRR). The main backbone structure of Tn1696 was tnpA (transposase)–tnpR (resolvase)–res (resolution site)–mer (mercury resistance locus), and the concise class I integron In4 was inserted in the resolution site. Among the above nine Tn1696-related regions, five regions were identified as newly designated unit transposons, namely Tn7383, Tn7384, Tn7385, Tn7386,
and Tn7387, because these five regions contained paired terminal 38-bp IRL/IRR on both sides, and all (except Tn7387) contained 5-bp direct repeats (DRs, target site duplication signals for transposition). In the structure of each remaining four Tn1696-related regions, there was no pair of IRL/IRR, so the remaining four regions could not be defined as
transposons. Tn7383, 41.9-kb MDR-2 region from pKOX_R1, Tn7384, Tn7385, Tn7386, Tn7387, 19.0-kb MDR region from p1, and 141.5-kb MDR-2 region from pKOX7525_1 contained tnpA–tnpR fragments with high identity to Tn1696. 81.1-kb MDR region from pKOX_R1, Tn7383, Tn7384, Tn7385, Tn7386, and Tn7387 contained the mer locus–IRR fragments that were partially or completely identical to Tn1696.

Besides the similarity of Tn1696 backbones, all the nine Tn1696-related regions contained different integrons, transposons, and putative resistance units which were related to resistant genes. Five Tn1696-related regions had different derivatives of Tn1548, which was a composite transposon first reported in 2005 to be identified as a vector of armA, a worldwide disseminated aminoglycoside resistance methylase gene. The 81.1-kb MDR region from pKOX_R1 contained a total of 18 resistance genes, which were located at nine different resistance loci: truncated aacC2–tmrB region, truncated IS26–catA2–IS26 unit, ars region, ΔTn1548, In797 [gene cassette array (GCA): aadA5–gcu37–dfrA1], truncated IS26–bla\textsubscript{LAP-2}–qnrS1–IS26 unit, Tn5393c, IS26–\textit{bla}_{\text{SIM-12}}–IS26 unit, and IS26–fosA3–IS26 unit. The resistance markers from Tn7383 were truncated aacC2–tmrB region and In263 (GCA: aacA4–arr3). The 41.9-kb MDR region from pKOX_R1 included In263, ΔTn6339 (containing \textit{bla}_{\text{CTX-M-3}} and \textit{bla}_{\text{TEM-1B}}), and ΔTn1548. Tn7384 from p12084-HI5 contained three resistance loci: type A IS26–fosA3–IS26 unit, ΔTn1548, and In1630 (GCA: aadA17–catB3–arr3–\textit{bla}_{\text{SIM-1}}). Tn7385 from pCP024641 was most similar in structure to Tn1696, except that In4 inserted in Tn1696 was replaced by In37 (GCA: arr3–catB3–\textit{bla}_{\text{OXA-1}}–aacA4). Tn7386 from pJNQH491-2 included truncated IS26–\textit{bla}_{\text{LAP-2}}–qnrS1–IS26 unit, \textit{bla}_{\text{NDM-1}}, and \textit{bla}_{\text{MBL}} containing ΔTn725, In211 (GCA: dfrB4). IS26–fosA3–IS26 unit, IS26–\textit{mph}(A)–IS6100 unit, and In469 (GCA: arr3–dfrA27–aadA16) were distributed on the 19.0-kb MDR region from p1, and these two units shared the same IS26. 141.5-kb MDR-2 region from pKOX7525_1, a huge and complex Tn1696-related region, contained a total of 17 resistance genes associated with nine different resistance loci: \textit{ars} region, Tn6399b, In1021, truncated IS26–\textit{bla}_{\text{LAP-2}}–qnrS1–IS26 unit, ΔTn5393c, \textit{bla}_{\text{SHO-1}} region, ΔTn7053, ΔTn5563, and In1377 (GCA: \textit{bla}_{\text{IMP-4}}–qacG2–aacA4–\textit{dcatB3}) (Figure 3 and Supplementary Table S3).

Comparison of Tn6535-Related Regions
Tn6535 was a unit transposon of the Tn3 family, and its backbone structure was composed of tnpA–res–tnpR and the \textit{ars} locus. In the accessory regions of these seven plasmids, there were three Tn6535-related regions, which were 60.6-kb MDR region from pK92-qnrS, 34.8-kb MDR-1 region from pJNQH491-2, and 37.0-kb MDR-1 region from pKOX7525_1. Since there were no IRL/IRR pairs at both ends of these three regions, they could not be identified as intact transposons. The partial fragments of these three regions showed high identity to the tnpA–res–tnpR backbone region in Tn6535.

The 60.6-kb MDR region from pK92-qnrS had a lot of accessory resistant modules: ΔTn5393c, truncated IS26–\textit{bla}_{\text{LAP-2}}–qnrS1–IS26 unit, In263 (GCA: aacA4–arr3), ΔTn2 (containing \textit{bla}_{\text{TEM-1B}}), and ΔTn1548. Moreover, the 60.6-kb MDR region from pK92-qnrS had six copies of IS26, which indicated IS26 played a crucial role in the formation of MDR region through homologous recombination. Both of 34.8-kb MDR-1 region from pJNQH491-2 and 37.0-kb MDR-1 region from pKOX7525_1 contained \textit{chrA}–orf98 unit and IS26–\textit{mph}(A)–IS6100 unit. Besides, there were also \textit{msr}AB region (carrying \textit{msr}AB, confer resistance to erythromycin and streptogramin B) and ΔTn5393c (carrying \textit{str}A and \textit{str}B) distributed on the 37.0-kb MDR-1 region from pKOX7525_1. In2132 (GCA: aacA4–arr3), aacC2–tmrB region, and ΔTn21 were existed in 34.8-kb MDR-1 region from pJNQH491-2 (Figure 4).

18.4-Kb MDR Region from pCP024641
In addition, 18.4-kb MDR region from pCP024641 was different from other MDR regions and could not be divided into Tn1696-related regions or Tn6535-related regions according to the structure (Figure 5). It carried drug resistance genes such as \textit{str}AB and \textit{bla}_{\text{TEM-1}}, locating in ΔTn5393c and aacC2–tmrB region.

Discussion
Since first being discovered in 2012, \textit{K. michiganensis} has caused several serious and difficult-to-treat nosocomial infections. In IncHI5 plasmids, a subgroup of IncHI, usually carry a variety of MGEs and drug resistance genes according to previous reports. The \textit{K. michiganensis} carrying IncHI5 plasmids were identified frequently in the
Figure 4. Tn6535-related regions from pK92-qrnr1, plasmidH12, and pKOX2515. Genes are denoted by arrows. Genes, mobile genetic elements, and other features are colored based on their functional classification. Shading denoted regions of homology (nucleotide identity > 95%). The accession numbers of Tn6535 and Tn1545 are CP009706 and AF550415, respectively.
last two years. The similarities and differences of the genome structure of IncHI5 plasmids and the population and geographic distribution within the *K. michiganensis* species have urgently needed to be studied.

In this work, seven IncHI5 plasmids carried by *K. michiganensis* strains were accurately annotated and compared in detail, and their evolutionary history was analyzed (Table 1). Among the seven *K. michiganensis* strains carrying above-mentioned IncHI5 plasmids (Supplementary Table S1), strain E718 containing pKOX_R1 was the first reported case, and pKOX_R1 was reported as a multidrug resistant plasmid carrying three β-lactamase genes (blaCTX-M-3, blaTEM-1, and blaSHV-12). Strain E718 was obtained from urine and drainage from a pelvis cystic lesion of a patient in November 2010, and the original classification classified E718 as *K. oxytoca*, although it has now been reclassified as *K. michiganensis*. After that, IncHI5 plasmids and *K. michiganensis* have been reported worldwide, but there were no reports of their co-occurring in nearly eight years. Then, starting from 2020, *K. michiganensis* carrying IncHI5 plasmids have been reported several times. *K. michiganensis* carries IncHI5 plasmids at a low frequency, probably because some *K. michiganensis* was misclassified as *K. oxytoca* and thus not counted. Perhaps it is also because *K. michiganensis* have only recently attracted attention, and the rapid development of sequencing has also led to an increase in related sequencing samples in recent years.

There were five complete sequences of IncHI5 plasmids in *K. michiganensis* uploaded to NCBI since 2020 (p12084-H15, pCP024641, pJNQH491-2, p1, and pKOX7525_1). Among them pCP024641 was the first reported case, which was collected from Fujian Provincial Hospital, Fuzhou Fujian China. From a geographic view, the distribution of pCP024641 and pKOX_R1 is relatively close; from the perspective of a phylogenetic tree, branches of pCP024641 and pKOX_R1 are also close, which may indicate a transmission event from Taiwan to Fujian. Later, IncHI5 plasmids coexisting with *K. michiganensis* were reported in Guangzhou, Hangzhou and other places, and all these locations are geographically located in the southeast coastal area of China. This distinctive geographic distribution may imply that the acquisition of IncHI5 plasmids in *K. michiganensis* is related to some unique geographic-related conditions, although the relevant internal mechanisms are still unclear. Phylogenetic analysis showed that *K. michiganensis* strains carrying IncHI5 plasmids are located in different branches of the phylogenetic tree (Figure 1), and their distribution on the tree was relatively scattered. This may indicate that the prevalence of IncHI5 plasmids carried by *K. michiganensis* are not limited to a specific branch, but have spread in different clonal groups. In summary, *K. michiganensis* strains carrying IncHI5 plasmids have appeared densely since 2020, and have a potential trend of outbreak; at the same time, multiple clonal groups of *K. michiganensis* carrying IncHI5 plasmids have spread in the southeast coastal area of China. Therefore, cases of IncHI5 plasmids occurring in *K. michiganensis* should be focused on and studied, and the attention should be paid to their transmission mechanism.

These seven IncHI5 plasmids shared a similar structure with high nucleotide identity and coverage (Supplementary Table S2), and two main large accessory resistance modules (Tn1696-related regions and Tn6535-related regions) were contained in all these plasmids except p12084-H15. In the nine Tn1696-related regions (Figure 3), 81.1-kb MDR-1 region from pKOX_R1, Tn7383, Tn7384, Tn7385, Tn7386, and Tn7387 contained the mer locus–IRR fragments homologous to Tn1696. mer locus is a series of operons with mercury resistance. The upstream of these mer locus were all immediately adjacent to ISs such as IS26 and IS6100, indicating that the reason why these mer locus had different lengths was that mer locus were interrupted by the insertion of ISs.
Tn7384, Tn7385, Tn7386, Tn7387, 19.0-kb MDR region from p1, and 141.5-kb MDR-2 region from pKOK7525_1 contained the \textit{tnpA–tnpR} fragments that were almost identical to Tn1696. In Tn1696, the \textit{tnpA–tnpR} fragment is followed by concise class 1 integron In4, and after the \textit{tnpA–tnpR} fragments of these Tn1696 derivatives, In4 was replaced by In263 (in Tn7383 and 41.9-kb MDR-2 region from pKOKR1), In1630 (in Tn7384), In37 (in Tn7385), In211 (in Tn7386), In1706 (in Tn7387), In469 (in 19.0-kb MDR region from p1), and In1377 (in 141.5-kb MDR-2 from pKOK7525_1), respectively. This suggested integrations and replacements of integrons occurred downstream of \textit{tnpA–tnpR} fragments in the Tn1696-related regions. The \textit{mer} locus and \textit{tnpA–tnpR} fragments were located at the two sides of the Tn1696-related regions (the junction with backbone regions). Although the insertion of ISs and the integration of integrons occurred upstream and downstream respectively, main structure of \textit{mer} locus and \textit{tnpA–tnpR} fragments was partially or completely retained, which indicated the homology of these Tn1696-related regions with Tn1696. In the three Tn6535-related regions (Figure 4), each of their internal upstream contained the part of the \textit{tnpA–res–tnpR} backbone region of Tn6535, and they constituted the upstream boundary of each Tn6535-related regions respectively. All these three Tn6535-related regions did not have the complete \textit{tnpA–res–tnpR} backbone region of Tn6535. The \textit{tnpA–res–tnpR} regions were truncated due to the insertion of IS26 in the downstream, which suggested that these Tn6535-related regions were formed by Tn6535 through genetic recombination.

The evolutionary history of these large accessory regions can be inferred from their internal structure and the distribution of MGEs on it. The formation of these large accessory regions is mostly due to the insertion, transposition, and integration of MGEs. Some are the overall activities of several adjacent MGEs, and some are the individual behaviors of a single MGE such as the insertion of a single IS. In 34.8-kb MDR-1 region from pJNQH491-2 and 37.0-kb MDR-1 region from pKOK7525_1 (Figure 4), chrA–orf98 unit and IS26–mph (A)–IS6100 unit seemed to form an overall putative resistance unit, which was bounded by IS5075 and IS26. This unit was likely to mobilize as a whole element and cannot be classified as a transposon. However, not all modules in the immediate vicinity can be regarded as a single component. \textit{aacC2–tmrB} region and ISKpn19 were closely adjacent to each other in both 81.1-kb MDR-1 region from pKOKR1 and Tn7383 (Figure 3), but they cannot be regarded as a single MGE. This is because from their downstream structure, their downstream difference was only an insertion of IS10L. Therefore, their formation was more likely to be caused by the insertion of this extra single IS10L. The evolutionary history caused by the insertions of ISs also existed in Tn7384: ISKpn21 and ISKml1 were inserted upstream of \textit{orf543} and \textit{armA} in \textsl{ATn1548}, respectively, forming a new structure of accessory region. This view was also verified by comparison with the reference Tn1548.

There were a variety of resistance-related MGEs distributed on these plasmids, and various types of antibiotic resistance genes were discovered in these seven plasmids: a total of 14 drug-resistant genes phenotypes with 45 resistance markers, including β-lactam resistance, macrolide resistance, quinolone resistance, and so on (Figure 2 and \textbf{Supplementary Table S3}). Among them, the resistance genes for β-lactam resistance were \textit{bla\textsubscript{CTX-M-3}}, \textit{bla\textsubscript{CTX-M-14}}, \textit{bla\textsubscript{IMP-4}}, \textit{bla\textsubscript{NDM-1}}, \textit{bla\textsubscript{OXA-1}}, \textit{bla\textsubscript{OXA-16}}, \textit{bla\textsubscript{SFO-1}}, \textit{bla\textsubscript{SIM-1}}, and \textit{bla\textsubscript{TEM-1}}. Except for pK92-qnrS and p1, the other five plasmids all contained one or more β-lactam resistance genes, suggesting that the IncHI5 plasmids in \textit{K. michiganensis} were good vectors for antibiotic resistance genes.

\textbf{Conclusions}

In conclusion, IncHI5 plasmids have spread in different clonal groups of \textit{K. michiganensis} strains and have recently appeared intensively, suggesting that they have a potential for outbreak. Up to now, \textit{K. michiganensis} strains carrying IncHI5 plasmids have only appeared in the southeast coastal area of China, and their internal mechanism is urgently needed to be studied. The large accessory regions in the IncHI5 plasmids can be divided into two types: Tn6535-related regions and Tn1696-related regions. Their formation was related to the integration and insertion of MGEs, and the accumulation and expression of drug resistance genes carried on MGEs accelerated the spread of antimicrobial resistance in \textit{K. michiganensis} strains. We expect that the data in this work can further decipher the structural diversification, population distribution, and evolutionary history of IncHI5 plasmids in \textit{K. michiganensis}. 

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**Data Sharing Statement**

The data presented in this study are available on request from the corresponding author. The plasmid sequences analyzed in this study can be found in public NCBI GenBank database. The accession numbers were provided in this article when these plasmids were firstly indicated.

**Ethics Approval**

The specimens were obtained with the patient’s consent. The use of human specimens and all related experimental protocols was reviewed and approved by the Ethics Committee of Taizhou Municipal Hospital affiliated with Taizhou University, Zhejiang, China, in accordance with the medical research regulations of the Ministry of Health, China. Research and all related procedures involving biohazardous materials were approved by the Biosafety Committee of Taizhou Municipal Hospital affiliated with Taizhou University. This research was conducted in China.

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**Disclosure**

The authors report no conflicts of interest in this work.

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