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SHORT REPORT

Characterization of an NDM-1-Producing *Citrobacter koseri* Isolate from China

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Purpose: The continuous rise in carbapenemase-producing *Enterobacteriaceae* infections is a major public health concern. However, there is limited information available on New Delhi metallo-β-lactamase-1 (NDM-1) producing *Citrobacter koseri*. In this study, we isolated a bla_{NDM-1}-carrying *C. koseri* from a stool sample of an inpatient. Our aim was to investigate the phenotypic and genomic features of this clinically derived carbapenem-resistant *C. koseri* isolate and to characterize the transmission pattern of the IncFII/IncN plasmid that carries the bla_{NDM-1} gene.

Methods and Results: S1-PFGE, Southern blot and conjugation assay confirmed the presence of bla_{NDM-1} gene in a conjugative plasmid. *C. koseri* L2395 and transconjugant L2395-EC600 strains showed similar resistance spectrum. Whole-genome analysis revealed that pL2395_NDM is an IncFII/IncN plasmid with a length of 67,839 bp. Moreover, bla_{NDM-1} gene was found encoded in the IS*Kpn19*-bla_{NDM-1}-*ble-tnpF-dsbD-cutA*-IS*Kpn19* cassette array. Phylogenetic analysis revealed that strain L2395 was close to an IMP-4-bearing *C. koseri* from Australia.

Conclusion: Ongoing surveillance will be essential to control and prevent the spread of carbapenem-resistant *Citrobacter* spp. in the future.

Keywords: *Citrobacter koseri*, carbapenem-resistant, NDM-1, whole-genome sequencing

Introduction

Citrobacter spp. are facultative anaerobic Gram-negative bacteria within the Enterobacteriaceae family.[1] They are frequently found in food, water, soil and intestines of humans and are considered as environmental contaminants.[2] Among the 11 species of *Citrobacter* spp., the most dominant are *Citrobacter freundii* and *Citrobacter koseri*, and regarded as commensal in humans.[3] *Citrobacter* spp. are now gaining importance as a clinical pathogen causing healthcare-associated infections such as urinary tract, liver, and biliary tract infections.[4] Moreover, *C. koseri* could cause central nervous system (CNS) infections in immunocompromised individuals.[5] *C. koseri* is intrinsically resistant to aminopenicillins and carboxypenicillins.[6] Infections caused by *C. koseri* are often treated with aminoglycosides, cephalosporins, chloramphenicol, quinolones and carbapenems.[6,7] However, the unceasing increase in infections with carbapenemase-producing Enterobacteriaceae is of significant public health concern.[8] Carbapenem-resistant *C. koseri* strains have been associated with a higher rate of in-hospital mortality compared to susceptible strains.[9] To date, several studies have reported the occurrence of infections involving *C. koseri* producing KPC, VIM and OXA.[3,7] However, reports of *C. koseri* carrying bla_{NDM} are rare compared to other carbapenemase genes, and there is a paucity of genomic analysis of NDM-producing *C. koseri*.[10,11] Yao et al reported that the detection rate of carbapenem-Resistant *Citrobacter* spp. has gradually increased.[2] Carbapenem-resistant *Citrobacter* spp. is fast gaining importance as a clinical multidrug-resistant pathogen.

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In this study, we identified a clinical isolate of *C. koseri* L2395 carrying bla_{NDM}, and described the complete sequence of a conjugative IncFII/IncN plasmid encoding NDM-1. S1-PFGE and southern blotting indicated the location of bla_{NDM-1} gene, and conjugation assay was performed to reveal the potential transmission mechanisms of bla_{NDM-1}. Whole-genome sequencing (WGS) revealed the genetic characterization of the isolate. Our study aims to evaluate the phenotypic characteristics of NDM-harboring *C. koseri* and emphasizes the importance of further monitoring of carbapenem-resistant *C. koseri* in the clinic.

Methods and Materials

Sample Collection

In the routine screening of the intestinal colonization carbapenems-resistant bacteria in a teaching hospital in Zhejiang Province since 2016, fecal samples were collected from patients with diarrhea. A female patient of 56-year-old was admitted to hospital for postoperative recurrence of cholangiocarcinoma in July 2020. Patients with pulmonary infections were treated with levofloxacin and piperacillin-tazobactam on admission. The patient developed diarrhea 6 days after admission. A fecal sample was collected from an inpatient on July 27, 2020. The sample was cultured in Brain Heart Infusion Broth (BD, Sparks, USA) and spread on MacConkey agar plate (OXOID, Hampshire, United Kingdom) with 2 mg/L meropenem (Meilunbio, Dalian, China) for preliminary screening. The isolate L2395 was detected and identified as *C. koseri* using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker, Bremen, Germany). The carbapenemase genes were identified using PCR as previously reported.[12]

Antibiotic Susceptibility Testing

Antimicrobial Susceptibility Testing (AST) was determined using VITEK 2 system with AST-GN16 panel.[13] Results were interpreted based on both the Clinical and Laboratory Standards Institute (CLSI) guidelines (https://clsi.org) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/clinical_breakpoints/).

S1-PFGE, Southern Blotting and Conjugation Assay

The plasmid characterization of *C. koseri* L2395 was determined by S1 Nuclease-Pulsed Field Gel Electrophoresis (S1-PFGE). Briefly, DNA plugs were digested using the S1 Nase restriction enzyme (Takara Bio Inc., Japan). Then, plugs undertaken on a CHEF-DR III (Bio-Rad, Hercules, CA, USA). *Salmonella enterica* serotype Braenderup H9812 was used as a size marker. The location of NDM was determined with Southern blotting and hybridization by a digoxigenin-labelled bla_{NDM-1} probe. Conjugation assays were conducted using *Escherichia coli* J53/EC600 as the recipient strains. Transconjugants were selected on plates contained 2 mg/L meropenem and 200 mg/L rifampicin/sodium azide (Meilunbio, Dalian, China) and verified with PCR and MALDI-TOF/MS identification.

Whole-Genome Sequencing (WGS) and in silico Analyses

Whole-genomic DNA of L2395 was extracted using Gentra Puregene Yeast/Bact. Kit (QIAGEN, Hilden, Germany) and subsequently sequenced on the Nanopore PromethION platform (Oxford Nanopore Technologies, Oxford, United Kingdom) and Illumina NovaSeq 6000 (Illumina, San Diego, CA, United States) by Novo Gene Co., Ltd. (Beijing, China). Assembly was performed using Unicycler v0.4.2. The acquired antimicrobial resistance genes (ARGs) and replicon types of plasmids were identified through the ResFinder (http://genepi.food.dtu.dk/resfinder) and PlasmidFinder (https://cge.food.dtu.dk/services/PlasmidFinder/).[14,15] Genomic sequences were annotated using Prokka v1.14.0, and the comparative maps of plasmids were drawn under the BLAST Ring Image Generator (BRIG) v0.95.[16,17]

Phylogenetic Analysis

A total of 220 publicly available *C. koseri* sequences were downloaded from the National Center for Biotechnology Information (NCBI) GenBank database. Genomes encoding carbapenemase genes were selected for phylogenetic analysis. The identification of ARGs was performed using ResFinder 4.0 database.[14] Finally, 43 genomes were identified carrying carbapenemase genes and selected for subsequent analysis (Table S1). The alignment file was generated by Roary v3.11.2, a rapid large-scale prokaryote pan genome analysis tool.[18] The multi-alignment of genomes was conducted with MAFFT.[19] A total of 3,507 core genes were identified. The substitution model was General Time Reversible (GTR) model, and the bootstrap replications was 100. Finally, the maximum likelihood tree was constructed and visualized by MEGA 11 and iTOL v6.8, respectively.[20,21]

Results and Discussion

A female patient of 56-year-old was admitted to hospital for postoperative recurrence of cholangiocarcinoma in July 2020. Patients with pulmonary infection were treated with levofloxacin and piperacillin-tazobactam on admission. The patient developed diarrhea 6 days after admission. Fecal sample was collected and spread on the surface of selected plates. *C. koseri* L2395 was recovered from the medium. NDM-1 was detected using PCR.

As shown in Table 1, *C. koseri* L2395 exhibited resistance to multiple antibiotics, including amoxicillin/clavulanate (MIC ≥ 32 μg/mL), piperacillin/tazobactam (MIC ≥ 128 μg/mL), cefuroxime (MIC ≥ 64 μg/mL), cefuroxime axetil (MIC ≥ 64 μg/mL), cefoxitin (MIC ≥ 64 μg/mL), ceftazidime (MIC ≥ 64 μg/mL), ceftriaxone (MIC ≥ 64 μg/mL), cefoperazone/sulbactam (MIC ≥ 64 μg/mL), cefepime (MIC = 16 μg/mL), ertapenem (MIC ≥ 8 μg/mL), imipenem (MIC ≥ 16 μg/mL), levofloxacin (MIC = 4 μg/mL), with the exception of amikacin (MIC ≤ 2 μg/mL), tigecycline (MIC = 2 μg/mL) and trimethoprim/sulfamethoxazole (MIC = 40 μg/mL).

According to the results of S1-PFGE and Southern-blot, the bla_{NDM-1} gene was located on a ~67 kb plasmid (designated as pL2395_NDM) (Figure 1). Whole-genome sequencing confirmed strain L2395 consist of a circular chromosome and two plasmids. And plasmid pL2395_NDM belonged to the IncFII/IncN type plasmid, with 67,839 bp in length and an average GC content of 51.7%. Conjugation assays revealed that the transmission of bla_{NDM-1} from *C. koseri* L2395 into *E. coli* 600 was successful. PCR and MALDI-TOF/MS identification confirmed pL2395_NDM was successfully transferred into recipient *E. coli* 600. In addition, the transconjugant L2395-EC600 showed similar resistance to antibiotics with L2395 (Table 1). This result indicated that the antimicrobial resistance phenotypes of

Table 1 Antimicrobial Susceptibilities of Strain *C. koseri* L2395 and Transconjugant L2395-EC600

Antimicrobials	MIC Values (μg/mL)	
	L2395	L2395-EC600
Amoxicillin/clavulanate	≥ 32	≥ 32
Piperacillin/tazobactam[a]	≥ 128	≥ 128
Cefuroxime	≥ 64	≥ 64
Cefuroxime Axetil	≥ 64	≥ 64
Cefoxitin	≥ 64	≥ 64
Ceftazidime	≥ 64	≥ 64
Ceftriaxone	≥ 64	≥ 64
Cefoperazone/Sulbactam	≥ 64	≥ 64
Cefepime	16	4
Ertapenem	≥ 8	≥ 8
Imipenem	≥ 16	8
Amikacin	≤ 2	≤ 2
Levofloxacin	4	≥ 8
Tigecycline	2	≤ 0.5
Trimethoprim/sulfamethoxazole	40	≥ 320

Notes: [a]tazobactam at a fixed concentration of 4mg/L.

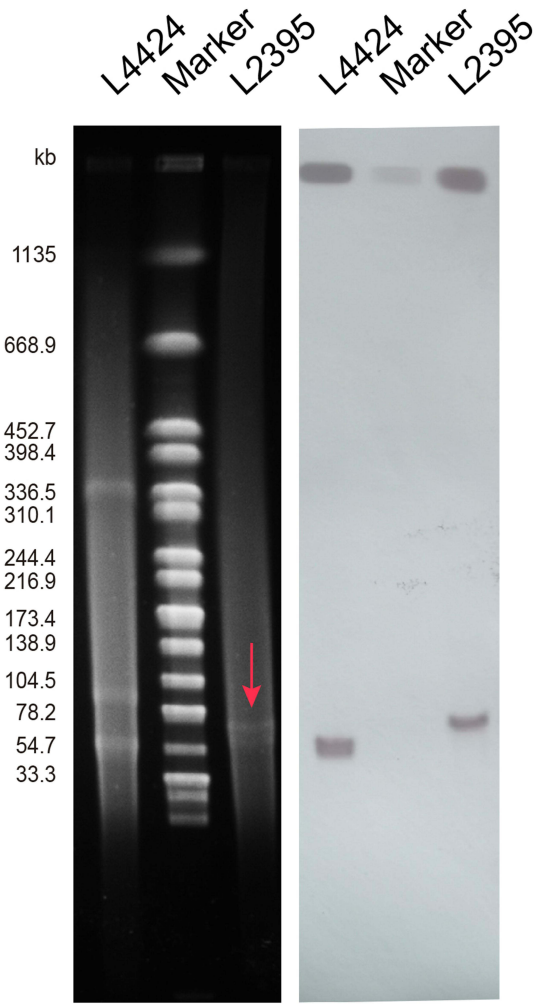

Figure 1 Plasmid profiles and Southern blot-hybridization of *C. koseri* L2395. Southern blot-hybridization of S1-nuclease digested DNA using a specific probe (bla_{NDM}). NDM-1-producing clinical isolates L4424 recovered from the same hospital were used as control. The red arrows indicated the plasmid carrying NDM-1. Marker: *Salmonella enterica* serotype Braenderup H9812.

transconjugant L2395-EC600 were acquired from pL2395_NDM. The transferability of plasmids increases the risk of transmission of drug-resistant bacteria and poses a great challenge to clinical treatment. In silico analysis identified strain L2395 also carry other antibiotic resistance genes (ARGs) including β-lactams (bla_{MAL-1}), trimethoprim (*dfrA14*) and quinolone (*qnrS1*). Multiple ARGs confer isolate multidrug-resistance, which increases the difficulty of clinical treatment.

A search of the nr/nt database found plasmid pL2395_NDM shared high similarity with plasmids unnamed2 (ON111450.1), pECC33-57 (CP098488.1) and pHKN-2 (MW191859.1) which identified from *Klebsiella pneumoniae, Enterobacter hormaechei* and *E. coli*, respectively (Figure 2).[22–24] Of note, plasmids unnamed2 and pECC33-57 were identified from clinic-originated isolates in China. The genetic environment of bla_{NDM-1} gene in L2395 was IS*Kpn19*-bla_{NDM-1}-*ble-tnpF-dsbD-cutA*-IS*Kpn19*. This genetic structure was also found in plasmids unnamed2 and pHKN-2 but was different with pECC33-57. The latter contained an incomplete *dsbD* and lacked of *cutA*. Restriction modification systems encoded *ecoRIIR* gene were found upstream of the bla_{NDM-1}. This restriction modification system could assist in the defense against phage infection and conduce to the spread and maintenance of plasmids.[25] Such a system can also be found in bla_{IMP-4}-carrying *C. freundii*.[26]

The genomes of 43 *C. koseri* isolates harboring the carbapenemase encoding genes were downloaded from the NCBI database and performed phylogenetic analysis together with *C. koseri* L2395. As shown in Figure 3 and Table S1,

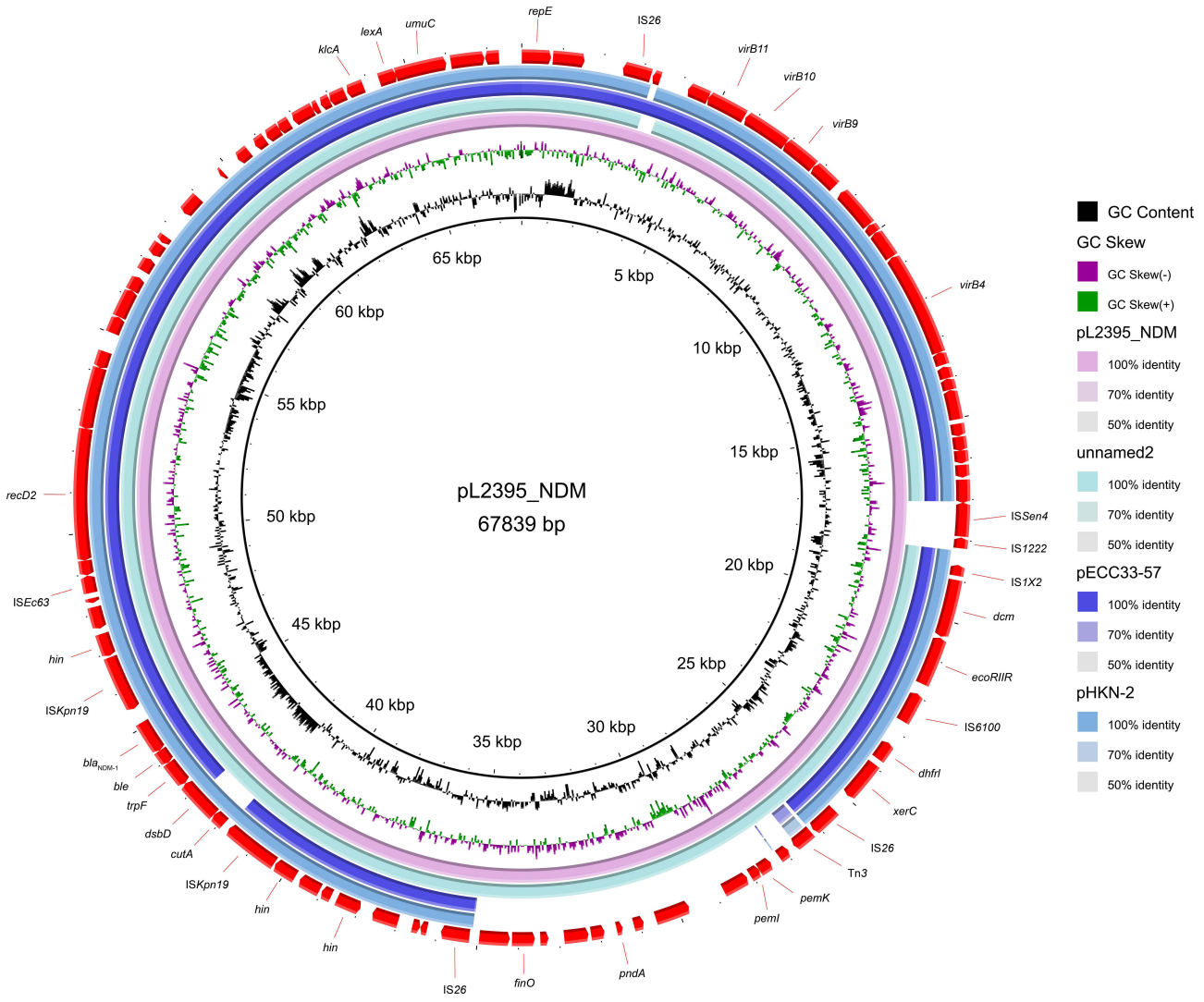

Figure 2 Major structural features and comparison of beta-lactamase-encoding plasmids. Genomic map of the bla_{NDM} producing IncFII/IncN pL2395_NDM plasmid with three closely related plasmids (ON111450.1, CP098488.1, MW191859.1). ORFs are portrayed by arrows and colored according to their putative functions. The alignment of the plasmids was performed and visualized by BLAST ring image generator (BRIG) software.

C. koseri isolates were detected across various countries, including Austria, China, France, Germany, Singapore, Spain, the United States, and the United Kingdom, suggesting the wide distribution. Strains isolated from different countries were not significantly clustered together, suggesting that carbapenemase-carrying *C. koseri* may be polyclonal at present. Multiple carbapenem resistance genes have been detected in *C. koseri* isolates, with bla_{KPC} genes being most popular. As one of the most common carbapenemase genes, bla_{KPC} has been found to be prevalent in the *Enterobacteriales*. However, reports of *Citrobacter spp*. carrying KPC are still limited. *C. koseri* L2395 is closely related to (GCA 023687185.1) from Australia carrying bla_{IMP-4}, but is far from the other three isolates from China (GCA 023156325.3, GCA 902387665.1, and GCA 003184045.1). These three isolates carrying different KPC variants, two of which carried KPC-2 (GCA 902387665.1, and GCA 003184045.1) showed close genetic relationships, and another isolate carries KPC-34 (GCA 023156325.3).

In conclusion, we isolated a multidrug resistant *C. koseri* strain L2395 carrying bla_{NDM-1} gene from a clinic in China. The genetic environment of NDM was elucidated, and the transferability of IncFII/IncN plasmid was confirmed. The emergence of carbapenem-resistant *C. koseri* highlights the importance of continuously monitored carbapenem-resistant Enterobacteriaceae in clinic.

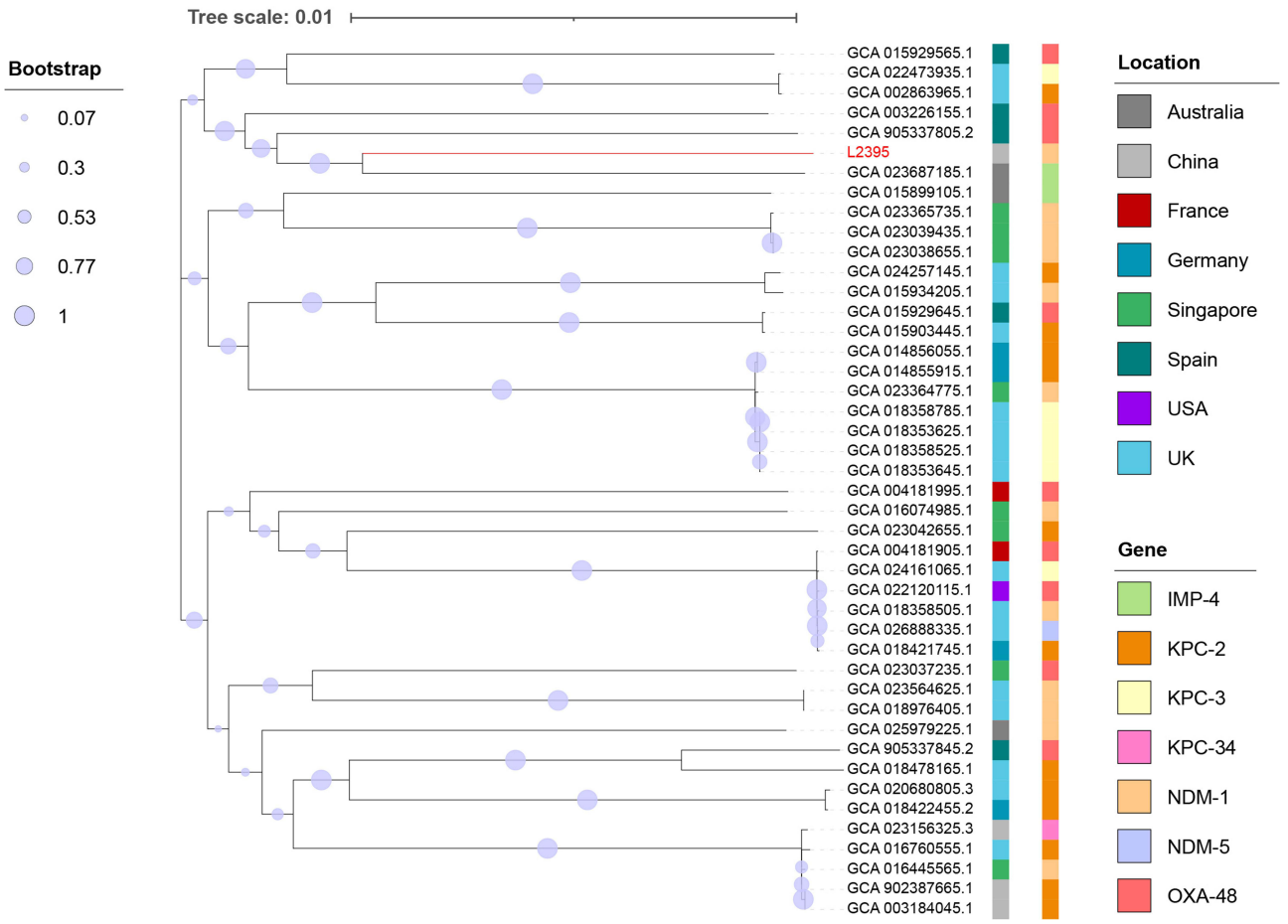

Figure 3 Construction of phylogenetic tree of NDM-1-producing *C. koseri* L2395 and other carbapenemase-producing *C. koseri* isolates. The locations and carbapenemase genes of isolates are shown. The *C. koseri* L2395 is indicated by red.

Ethics Approval and Consent to Participate

This study was conducted following the Declaration of Helsinki and obtained approval from the clinical research ethics committee of The First Affiliated Hospital of Zhengzhou University [no. 2023-KY-0264]. The patient provided written informed consent to allow the case details to be published.

Nucleotide Sequence Accession Numbers

The whole-genome sequence of the *C. koseri* L2395 was submitted to GenBank under the following BioSample number: SAMN33442942.

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Disclosure

The authors report no conflicts of interest in this work.

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