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ORIGINAL RESEARCH

Genetic Characterization of *bla*$_{NDM-1}$-Carrying *Citrobacter portucalensis* Sequence Type 328 and *Citrobacter freundii* Sequence Type 98

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Purpose: NDM-1-producing *Citrobacter portucalensis* and *Citrobacter freundii* simultaneously occurred in a hospital. This study aims to characterize the *bla*$_{NDM-1}$-carrying plasmids in these *Citrobacter* strains.

Methods: Cf7303, Cf7308, and Cf7313 were recovered from three patients in a teaching hospital from September 24 to October 1, 2021. Bacteria were identified by MALDI-TOF mass spectrometry, and antibiotics susceptibility tests were determined by VITEK® 2 compact system. Whole-genome sequencing (WGS) was performed using the HiSeq Illumina and QNome platform to characterize the genomes.

Results: Cf7303 was identified as *C. portucalensis* Sequence Type 328 by WGS, and harbored two plasmids, namely pCf7303 and a novel IncFIB pNDM-Cf7303 on which antibiotic-resistant genes (*bla*$_{TEM-1}$, *bla*$_{CTX-M-14}$, *bla*$_{NDM-1}$, *aac (3)-IId, aadA2, fosA3, sul1, sul2, catA2, tetD, dfrA12, qacEdelta1, mph(A)*, and *ble*$_{MBL}$) are located. *C. freundii* strain Cf7308 and Cf7313 belonged to the same Sequence Type 98. Cf7308 contained two plasmids, pCf7308, and an IncN1 pNDM-Cf7308 with homology to pNDM-BTR in *E. coli* and pNDM-CWH001 in *C. freundii*.

Conclusion: We characterized a putatively novel IncFIB plasmid carrying *bla*$_{NDM-1}$ in *C. portucalensis*. In addition, the closely related *bla*$_{NDM-1}$-carrying IncN1 plasmids in *E. coli* and *C. freundii* suggest that interspecies or intraspecies horizontal transfer occurs in China.

Keywords: NDM-1, *Citrobacter portucalensis*, *Citrobacter freundii*, whole-genome sequencing

Introduction

Carbapenem-resistant *Enterobacteriaceae* (CRE) have spread widely and emerged as a health concern worldwide. Since carbapenemase genes located on mobile genetic elements (MGEs) are easily transmissible within and across bacterial cells of the same or different species, carbapenemase production has become the predominant mechanism of CRE.

Carbapenemases include Ambler class A *Klebsiella pneumoniae* carbapenemase (KPC), class B metallo-β-lactamase (MBL), and some class D β-lactamases (OXA-48). Recently, a novel, non-β-lactam β-lactamase inhibitor avibactam performs excellent activity against KPC- and OXA-48-producing CRE,[1] albeit it cannot target MBL-producing strains, which remain worrisome to clinical practice. New Delhi metallo-β-lactamase-1 (NDM-1) is a kind of MBLs commonly reported in *Enterobacteriaceae*. Since *bla*$_{NDM-1}$-carrying *Escherichia coli* and *Klebsiella pneumoniae* were firstly described in 2009,[2] NDM has been reported in a wide variety of species, such as *Providencia rettgeri, Citrobacter freundii, Klebsiella oxytoca, Salmonella enterica*, and *Enterobacter cloacae*.[3–5]

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Here we report the simultaneous occurrence of NDM-1-producing *C. portucalensis and C. freundii* clinical strains in a tertiary hospital in Beijing, China and the characteristics of $bla_{\text{NDM-1}}$-carrying IncFIB and IncN1 plasmids.

Materials and Methods

Bacterial Isolation and Identification

Three carbapenem-resistant *C. freundii* strains Cf7303, Cf7308, and Cf7313 were isolated from postoperative intraabdominal drainage cultures in a teaching hospital from September 24 to October 1 in 2021. Cf7303 was from a surgical ward, while Cf7308 and Cf7313 were from an intensive care unit. These strains were identified as *C. freundii* using MALDI-TOF MS (Bruker Dalton GmbH, Leipzig, Germany).

Antimicrobial Susceptibility Testing

The minimal inhibitory concentrations (MICs) of piperacillin/tazobactam (TZP); ceftazidime (CAZ), cefepime (FEP), aztreonam (ATM), imipenem (IPM), meropenem (MEM), amikacin (AMK), ciprofloxacin (CIP), tobramycin (TOB), doxycycline (DOC), tigecycline (TGC), and sulfamethoxazole/trimethoprim (SXT) were determined by VITEK® 2 compact system (BioMérieux, France). The results were interpreted following the Clinical and Laboratory Standards Institute (CLSI) M100-Ed 31 in 2021. *E. coli* ATCC 25922 was used as quality control in antimicrobial susceptibility tests.

Carbapenemase enzymes, including KPC, NDM, OXA-48, imipenemase (IMP), and Verona integron-encoded metallo-β-lactamase (VIM), were detected by a lateral flow immunoassay NG-test CARBA5 (NG Biotech, France).

Whole-Genome Sequencing and Molecular Analysis

Genomic DNA was extracted from bacteria culture using Wizard® Genomic DNA Purification Kit (Promega). Sequencing was conducted using an Illumina HiSeq X Ten platform with a 500 bp insert size at Shanghai Majorbio Biopharm Technology Company (Shanghai, China). The genome was assembled *de novo* using SOAPdenovo2 and analyzed using the I-Sanger Cloud Platform (www.i-sanger.com) from Shanghai Majorbio. Assemblies were annotated using Prokka (https://github.com/tseemann/prokka).

Cf7303 and Cf7308 were selected for additional long-read sequencing. Libraries were prepared using a Qiagen-8 sequencing kit. Sequencing was performed using a Qcell-3841 sequencing chip on a QNome platform (QitanTech, China). Fast5 files were base-called using NiuTouGeng V3 (QitanTech, China). The raw reads were filtered using NanoPlot v1.38.1 and NanoFilt v2.8.0.[6] Hybrid assemblies were conducted using Unicycler v0.4.8 and Flye v2.8.[7,8] Furthermore, we used Pilon V1.24[9] to carry out genomes polishing and manually checking by remapping raw reads against the plasmids. Genomic sequences were annotated using Prokka V1.14.6.[10]

Multilocus sequence typing was determined by using the genomic sequence to query the multilocus sequence typing (MLST) database of *Citrobacter* spp. on the website of https://pubmlst.org/. Antimicrobial resistance genes and plasmid replicons were also predicted using the ResFinder tool (98%, minimum threshold for identity; 80%, minimum coverage) and the PlasmidFinder tool (90%, minimum threshold for identity; 80%, minimum coverage) from the Center for Genomic Epidemiology http://genomicepidemiology.org/, respectively.

Phylogenetic Analysis

Genome sequences of 34 available *C. freundii* isolates were downloaded from the NCBI database for phylogenetic analysis (accessed November 1, 2021). *C. freundii* strain B38 (GenBank accession number CP016762) was used as the reference genome for comparison. We used Snippy software (https://github.com/tseemann/snippy) to call SNPs for queried *C. freundii* isolates from the reference in order to produce an alignment of "core SNPs," and then the SNPs were concatenated and aligned to construct the Maximum-Likelihood phylogenetic tree using RAxML (v8.2.4). iTOL (https://itol.embl.de/) was used to graph the RAxML best-trees output.

Comparative Genomic Analysis of *bla*$_{NDM}$-Carrying Plasmids

In order to elucidate the evolution of *bla*$_{NDM-1}$-encoding plasmids, the similarity of sequences was analyzed using BLSATn and Minimap2 (95%, minimum threshold for identity). MCscan pipeline for synteny inference of JCVI utility libraries[11] was used for comparative analysis.

Nucleotide Sequence Accession Number

The genome sequences have been deposited to GenBank under the BioProject PRJNA792258. Sequences accession numbers: Cf7303 (CP092466-CP092468), Cf7308 (CP092463-CP092465), and Cf7313 (JAJUBD000000000).

Results

Antibiotic Resistance Profile

Strain Cf7303 was extensively drug-resistant (Table 1), resistant to piperacillin/tazobactam, ceftazidime, cefepime, imipenem, meropenem, ciprofloxacin, doxycycline, sulfamethoxazole/trimethoprim, but it was susceptible to aztreonam, amikacin, and tigecycline.

Meanwhile, strains Cf7308 and Cf7313 had identical antibiotic-resistant profiles, resistant to piperacillin/tazobactam, ceftazidime, cefepime, imipenem, meropenem, ciprofloxacin, but it was susceptible to aztreonam, amikacin, tobramycin, doxycycline, tigecycline, and sulfamethoxazole/trimethoprim.

NG-test CARBA5 results displayed that the three isolates produced the New Delhi metallo-β-lactamase.

Whole-Genome Sequencing and Molecular Analysis

Strain Cf7303 was identified as ST328 *C. portucalensis* by WGS analysis, consisting of a 5,120,606-bp chromosome and a 4187-bp plasmid pCf7303, and a 233,864-bp plasmid pNDM-Cf7303. Cf7303 contains multiple resistance determinants (Table 2). The resistance genes *bla*$_{CMY-77}$ and *qnrB6* were located on the chromosome, while other determinants including *sul2, catA2, aac (3)-IId, bla*$_{TEM-1}$, *tetD, dfrA12, aadA2, qacEdelta1, bla*$_{NDM-1}$, *ble*$_{MBL}$, *sul1, mph(A), fosA3*, and *bla*$_{CTX-M-14}$ located on pNDM-Cf7303 whose backbone genes were separated by multiple IS26 insertion sequences and other IS elements such as IS15, ISEc63. (Figure 1A).

The draft genome sequencing displayed *C. freundii* Cf7308 was identical to Cf7313, belonging to ST98 sequence typing. The representative Cf7308 consists of a 5,108,147-bp chromosome, a 319,832-bp plasmid pCf7308, and a 59,165-bp plasmid pNDM-Cf7308. Cf7308 contained six antibiotic resistance determinants (Table 2). The resistance genes *bla*$_{CMY-109}$ and *qnrB38* are located on the chromosome, while *dfrA14, bla*$_{NDM-1}$, *ble*$_{MBL}$, and *qnrS1* are located on pNDM-Cf7308.

To examine the phylogenetic relationship of Cf7308 and Cf7313 to other *C. freundii* strains producing less common beta-lactamase such as OXA beta-lactamases, KPC, VIM, and NDM, 34 available genomes of *C. freundii* were downloaded from the NCBI database to construct a whole-genome sequence phylogenetic tree (Figure 2). These multidrug-resistant isolates belonged to three lineages and diverse STs.

Characterization of *bla*$_{NDM-1}$-Carrying Plasmid pNDM-Cf7303

The complete nucleotide sequence of pNDM-Cf7303 was 233,864 bp in length, constituting a circular DNA with an average G + C content of 52.4%. 282 open reading frames were annotated. pNDM-Cf7303 was assigned to the IncFIB

Table 1 Antimicrobial Susceptibility Profiles for *Citrobacter* spp. Strains

Isolate	Minimum Inhibitory Concentrations (μg/mL)											
	ATM	TZP	CAZ	FEP	MEM	IPM	AMK	TOB	CIP	DOC	TGC	SXT
Cf7303	4	≥128	≥64	≥32	≥16	≥16	≤2	8	≥4	≥16	1	≥16/304
Cf7308	≤1	≥128	≥64	≥32	≥16	≥16	≤2	≤1	1	1	≤0.5	2/38
Cf7313	≤1	≥128	≥64	≥32	≥16	≥16	≤2	≤1	1	1	≤0.5	2/38

Abbreviations: ATM, aztreonam; TZP, piperacillin/tazobactam; CAZ, ceftazidime; FEP, cefepime; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; TOB, tobramycin; AMK, amikacin; DOC, doxycycline; TGC, tigecycline; SXT, sulfamethoxazole/trimethoprim.

Table 2 Overall Features of the *Citrobacter* spp. Genomes

Parameter	Cf7303	Cf7308 (Cf7313)
Species	*Citrobacter portucalensis*	*Citrobacter freundii*
Size (bp)	5,358,657	5,487,144
Number of contigs	3	3
G+C (%)	51.9	51.6
MLST[30]	ST328	ST98
Resistance determinants on Chromosome	bla_{CMY-77} *qnrB6*	$bla_{CMY-109}$ *qnrB38*
Resistance determinants on plasmid	bla_{NDM-1} ble_{MBL} $bla_{CTX-M-14}$ bla_{TEM-1} *aadA2* *aac(3')-IId* *catA2* *tetD* *dfrA12* *ΔqacE* *sul1* *sul2* *fosA3* *mph(A)*	bla_{NDM-1} ble_{MBL} *qnrS1* *dfrA14*
Plasmid types (Inc) of NDM-1 encoding plasmid[31]	IncFIB	IncN1

group due to containing an IncFIB-type repA (plasmid replication initiation) gene. Antibiotic-resistant genes of *sul2, catA2, aac(3)-IId,* bla_{TEM-1}, *tetD, dfrA12, aadA2, qacEdelta1, sul1,* bla_{NDM-1}, ble_{MBL}, *sul1, mph(A)* were clustered as the main 50,000 bp multi-drug resistance (MDR) region on pNDM-Cf7303, coupled with an additional 7,000 bp MDR region of IS26-*fosA3*-$bla_{CTX-M-14}$-IS26. IS26 frequently flanked the antibiotic-resistant genes. The genetic context of bla_{NDM-1} was IS26-*dfrA12-qacEdelta1-aadA2-sul1*-bla_{NDM-1}-ble_{MBL}-*sul1- mph(A)*-IS26.

No significant sequence homology to pNDM-Cf7303 was founded using BLASTn search and minimap2. According to the maximum identity of sequences, a pairwise comparison was conducted with pCFR17_1 (NC_ CP035277) and pHNTS45-1(NZ_MK167988). Figure 1A showed that the backbone of pNDM-Cf7303 was probably reconstituted by pCFR17_1 and pHNTS45-1 from *C. freundii*, and the bla_{NDM-1} gene could be acquired by IS26 elements.

Characterization of bla_{NDM-1}-Carrying Plasmid pNDM-Cf7308

The complete nucleotide sequence of pNDM-Cf7308 was 59,165 bp in length, constituting a circular DNA with an average G + C content of 52.1%. 75 open reading frames were annotated. pNDM-Cf7308 was assigned to the IncN1 group due to containing an IncN1-type repA (plasmid replication initiation) gene. The genetic context of bla_{NDM-1} was IS26-qnrS1-ble_{MBL}-bla_{NDM-1}-IS3000. A pairwise comparison showed it was homology to pNDM-BTR (KF534788) and pNDM-CWH001 (NZ_CM008471) which were harbored by *E. coli* BTR[12] and *C. freundii* CWH,[13] respectively. (Figure 1B).

Discussion

Being a member of the *Enterobacteriaceae* family, *Citrobacter* spp. are regarded as opportunistic pathogens owing to exiting ubiquitously in the environment, food, and intestine of humans or animals.[14] Carbapenem-resistance *Citrobacter* strains have been frequently collected from salad, urine, bloodstream, and rectal swabs.[14,15] Here, we reported three

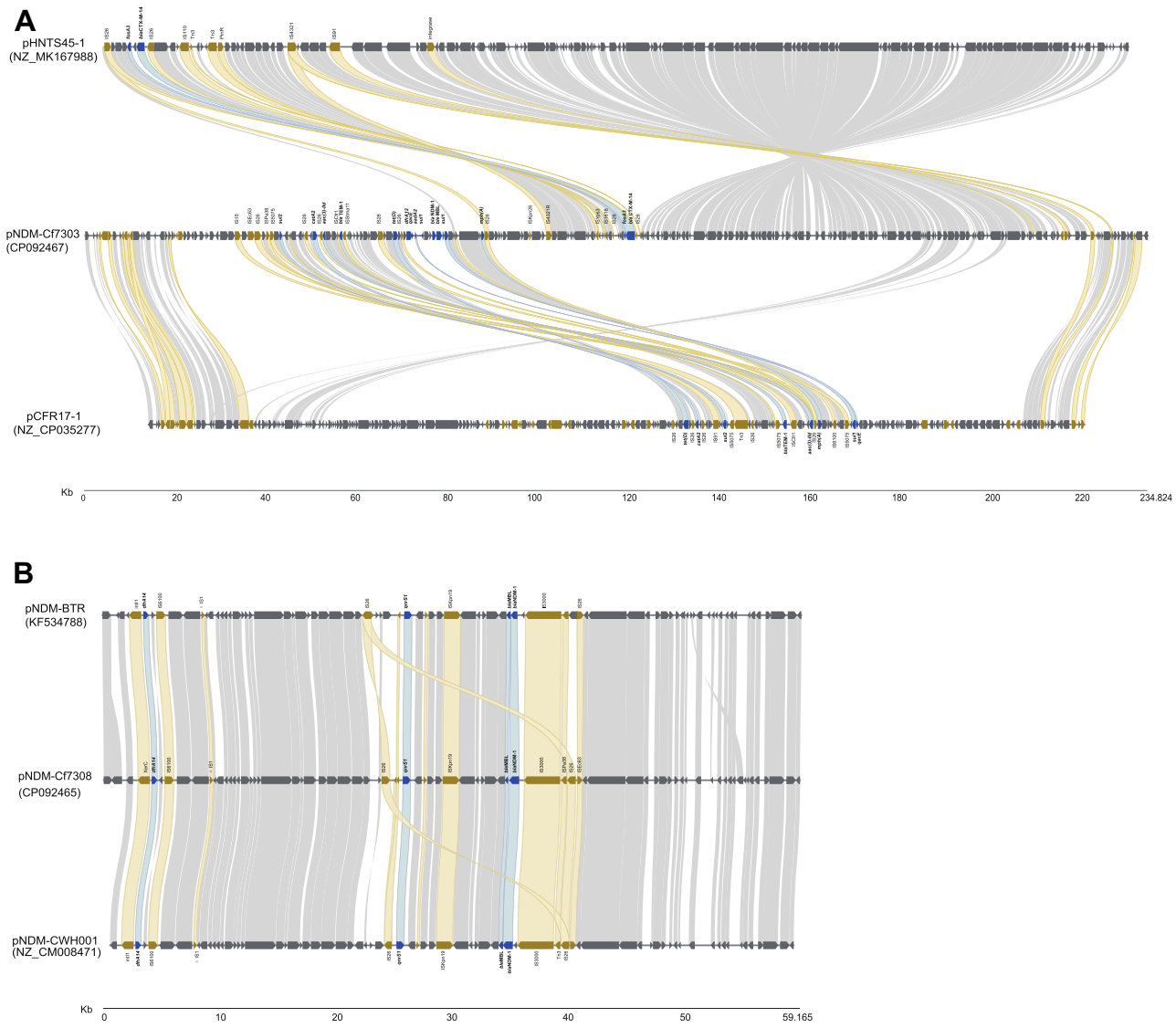

Figure 1 Comparison of *bla*$_{NDM-1}$-carrying plasmids. (**A**) Comparison of pNDM-Cf7303 (CP092467) with its maximum identity of sequences of pCFR17-1 (NC_CP035277) and pHNTS45-1(NZ_MK167988). (**B**) Comparison of pNDM-Cf7308 (CP092465) with pNDM-BTR (KF534788) and pNDM-CWH001 (NZ_CM008471). Open reading frames are denoted by block arrows and colored based on gene function. Insertion sequence (IS) elements are shown in yellow, antibiotics resistant genes in blue, and others in grey. Shading regions denote regions of homology (> 95% nucleotide identity).

clinical NDM-1-producing *Citrobacter* strains, including *C. portucalensis* strain Cf7303 and *C. freundii* strain Cf7308, Cf7313.

C. portucalensis was proposed as a novel species within the genus *Citrobacter* in 2017.[16] In 2019, a multidrug-resistant *C. portucalensis* strain NR-12 was isolated from poultry droppings.[17] Soon later, a carbapenem-resistant *C. portucalensis* 3839 ST165 was first isolated from the sputum of a patient with type 2 diabetes mellitus in China.[18] In this study, we isolated another NDM-producing *C. portucalensis* strain Cf7303 ST328 from a postoperative patient. In Cf7303, the *bla*$_{NDM-1}$ gene was carried by a novel IncFIB plasmid different from an IncX3 plasmid in *C. portucalensis* 3839.[19] IncF plasmids are the most prevalent narrow-host-range plasmids accounting for dissemination of about 40% of plasmid-borne carbapenemases.[5] For example, IncF plasmids, including pCRCB-101_1, pCB1_SE1_NDM, pKPX-1, are responsible for disseminating *bla*$_{NDM-1}$ in *C. freundii, Citrobacter werkmanii*, and *K. pneumoniae*.[20,21] As IncF plasmids have been mostly found to carry multiple antibiotic-resistant genes (ARGs), particularly *bla*$_{CTX-M-15}$, pNDM-Cf7303 harbored 14 ARGs for β-latam resistance (*bla*$_{TEM-1}$, *bla*$_{CTX-M-14}$, *bla*$_{NDM-1}$), aminoglycoside resistance (*aac(3)-IId,*

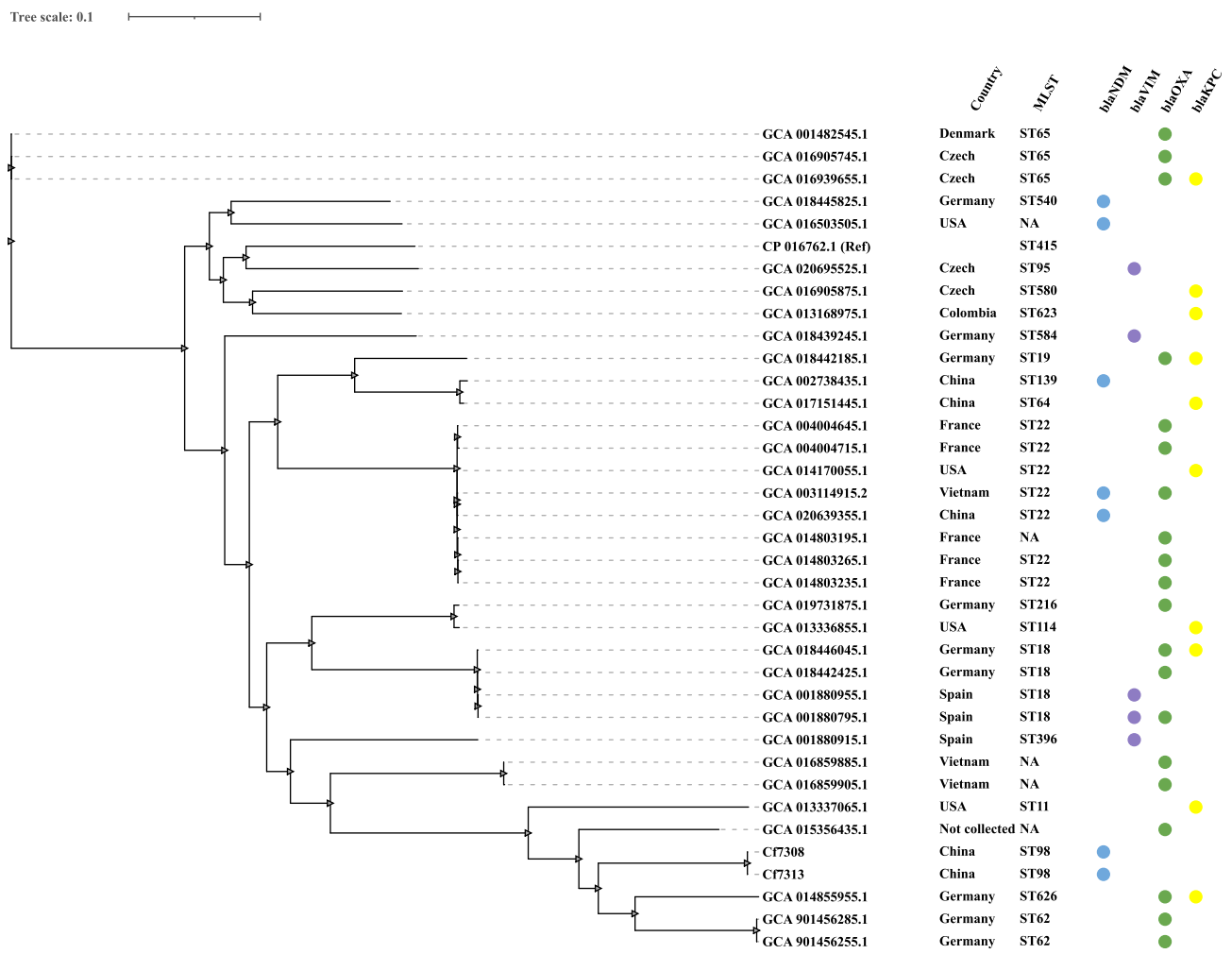

Figure 2 A whole-genome sequence phylogenetic tree calculated from Cf7308, Cf7313, and 34 available genomes of *C. freundii* producing less common beta-lactamase such as OXA beta-lactamases, KPC, VIM, and NDM. *C. freundii* strain B38 (GenBank accession number CP016762) was used as the reference genome for comparison. NA of MLST indicates not be assigned a matched ST when querying the multilocus sequence typing (MLST) database of *Citrobacter* spp.

aadA2), tetracycline resistance (*tetD*), macrolide resistance (*mphA*), fosfomycin resistance (*fosA3*), chloramphenicol resistance (*catA2*), glycopeptide resistance (ble_{MBL}), sulfonamide resistance (*sul1, sul2*), and trimethoprim resistance (*dfrA12*), resistance to quaternary ammonium (*qacEdelta1*). Given no sequence homology to pNDM-Cf7303, bla_{NDM-1} was carried by a novel IncF plasmid in Cf7303. We thus suppose *C. portucalensis* has the potential to disseminate multiple antibiotic resistance. Given the close relation to *C. freundii*, many *C. portucalensis* strains were previously misidentified as *C. freundii* in GenBank.[18] Thereby, the occurrence of carbapenem-resistant *C. portucalensis* may have been underestimated in the clinic.

Until recently, carbapenemase-producing *C. freundii* has been reported worldwide. The KPC-producing *C. freundii* isolates emerged early in 2005 in a hospital in China.[22] In addition to the sporadic spread, outbreaks of KPC-2, NDM-1, and IMP-4 producing *C. freundii* have been reported.[23–25] Here, a clone of ST98 *C. freundii* Cf7308 and Cf7313 was recovered from two patients within a week in an ICU, so bla_{NDM-1}-carrying *C. freundii* was spreading in this ward. To our knowledge, ST98 *C. freundii* has rarely been detected (www.pubMLST.org/cfreundii). Phylogenetic analysis of the WGS data showed that the OXA beta-lactamases, KPC, VIM, and NDM producing *C. freundii* strains belong to diverse STs.

In *C. freundii* strains, bla_{NDM-1} was found to be carried by diverse plasmids, including IncX3, IncA/C, IncHI1, IncL/M, IncFII, and IncN1.[5,13,19,26–29] In Cf7308, bla_{NDM-1} was carried on an IncN1 plasmid identical to pNDM-BTR from a local *E. coli* isolate and pNDM-CWH001 from a *C. freundii* isolate in Wuhan city. The closely related bla_{NDM-1}-carrying IncN1

plasmids in *E. coli* and *C. freundii* suggested interspecies or intraspecies horizontal transfer has occurred in China. Since the IncN plasmid is a broad-host-range type with high transmission efficiency, more intensive infection control measures should be employed.

Conclusions

Our study first disclosed a simultaneous occurrence of NDM-1-producing *C. portucalensis* and *C. freundii* in a hospital. In addition, our findings describe IncF and IncN1 plasmids are associated with the dissemination of $bla_{\text{NDM-1}}$ in *Citrobacter* spp. With the increasing number of carbapenem-resistant *Citrobacter* spp., we should underpin the active screening of multiple-drug resistance organisms.

Ethical Approval Statements

This retrospective study was approved by the Evaluation Committee and the Biomedical Ethics Committee of Beijing Tsinghua Changgung Hospital (22031-0-01). Because of the retrospective and anonymous nature of the study, the Ethics Committee did not require written informed consent provided by participants.

Disclosure

Yu He is affiliated with Qitan Technology Ltd. All authors declare no other conflicts of interest in this work.

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