

Genomic Characterization of A Multidrug-resistant *Citrobacter freundii* Strain ZT01-0079 Co-Producing *bla*_{NDM-1} and *bla*_{SHV-12} using MiSeq and MinION

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Abstract

Background: The emergence of multi-drug resistant *Citrobacter freundii* poses daunting challenges to the treatment of clinical infections. The purpose of this study was to characterize the genome of a *C. freundii* strain with an IncX3 plasmid encoding both the $bla_{\text{NDM-1}}$ and $bla_{\text{SHV-12}}$ genes.

Methods: Strain ZT01-0079 was isolated from a clinical urine sample. The Vitek2 system was used for identification and antimicrobial susceptibility testing. The presence of $bla_{\text{NDM-1}}$ was detected by PCR and sequencing. Conjugation experiments and Southern blotting were performed to determine the transferability of the $bla_{\text{NDM-1}}$ -carrying plasmid. Nanopore and Illumina sequencing were performed to better understand the genomic characteristics of the strain.

Results: Strain ZT01-0079 was identified as *C. freundii*, and the coexistence of $bla_{\text{NDM-1}}$ and multiple drug resistance genes was confirmed. Electrophoresis and Southern blotting showed that $bla_{\text{NDM-1}}$ was located on a ~53kb IncX3 plasmid. The NDM-1-encoding plasmid was successfully transferred at a frequency of 1.68×10^{-3} . Both $bla_{\text{NDM-1}}$ and $bla_{\text{SHV-12}}$ were located on the self-transferable IncX3 plasmid.

Conclusion: The rapid spread of the IncX3 plasmid highlights the importance of continuous monitoring of the prevalence of NDM-1-encoding *Enterobacteriaceae*. Mutations of existing carbapenem resistance genes will bring formidable challenges to clinical treatment.

Background

New Delhi metallo- β -lactamase-1 (NDM-1) is an enzyme capable of hydrolyzing most β -lactam antibiotics. Since its first detection in 2009 in *Klebsiella pneumoniae* [1], 21 NDM variants have been reported from various *Enterobacteriaceae* species [2-15]. The increasing prevalence of NDM-producing bacteria is due primarily to intra- and inter-species exchanges of a variety of self-transferring plasmids. The coexistence of NDM and other resistance genes on a single plasmid is being reported with increasing frequency and confers high-level carbapenem resistance, which poses daunting challenges to clinical management [7,11,14-17].

Citrobacter freundii, a member of the *Enterobacteriaceae* family, is a constituent of the commensal intestinal microbiota of animal and humans. However, it can cause diarrhea, sepsis, meningitis, respiratory and urinary tract infections, and can serve as a reservoir of antibiotic resistance genes. In recent years, due to the abuse of antibiotics, *C. freundii* has acquired increasing resistance to common antibiotics. In addition, $bla_{\text{NDM-1}}$ -positive *C. freundii* has been reported in numerous countries that include but are not limited to China [2-5], India, Pakistan, France, Sweden, Australia, UK, USA [7], and South Africa [9]. We report a carbapenem-resistant strain of *C. freundii* with coexistent $bla_{\text{NDM-1}}$ and $bla_{\text{SHV-12}}$ genes on a transferrable IncX3 plasmid. Antimicrobial susceptibility tests, conjugation experiments, and whole-genome sequencing were performed to study the molecular characteristics of the multi-drug resistant

strain

Methods

Bacterial identification and isolation

Strain ZT01-0079 was isolated from a clinical urine sample in 2018, in Guangzhou, China. Species identification was conducted by using the VITEK2 compact system (Bio Merieux, France) and confirmed by 16S rDNA sequencing. The entire *bla*_{NDM} and *bla*_{SHV} genes were amplified with primers as described previously [18] and sequenced. All experiments were conducted in accordance with relevant regulations and approved by the Chinese PLA Center for Disease Control and Prevention.

Susceptibility testing

The VITEK2 system with the AST-GN09 card (bioMérieux, France) was used for minimum inhibitory concentration (MIC) determinations of strain ZT01-0079 and transconjugants. Results were interpreted in accordance with the Clinical and Laboratory Standards Association guidelines [19]. The following agents were tested: amikacin, aztreonam, nitrofurantoin, ciprofloxacin, piperacillin, gentamicin, cefepime, ceftriaxone, ceftazidime, tobramycin, imipenem and levofloxacin.

Southern blotting and Conjugation experiment

A DNA fragment of strain ZT01-0079 was prepared by electrophoresis and S1 endonuclease. Subsequent fragments were further separated by pulsed-field gel electrophoresis (PFGE) (Bio-Rad, USA). The transfer of plasmid DNA to a nylon membrane was evaluated using probe-NDM-R (5'-CGC AAC ACA GCC TGA CTT TC-3) and probe-NDM-F (5'-GGC GGA ATG GCT CAT CAC GA-3 ') specific for *bla*_{NDM-1}.

Strain ZT01-0079 and *Escherichia coli* strain J53 were used as donors and acceptors, respectively. Conjugation experiments were performed by filter mating and broth, and the mixture was then incubated at 37 °C for 18h. The transconjugant was selected on a MacConkey agar plate containing meropenem (4 µg/ml) and sodium azide (100 µg/ ml) at 37 °C for 12h. PCR amplification and sequencing were performed to determine the presence of *bla*_{NDM-1} and *bla*_{SHV-12} genes in the transconjugant.

Whole genome sequencing and comparative genome analysis.

Genomic DNA was extracted from strain ZT01-0079 using the QIAamp DNA Mini Kit (Qiagen, Inc, USA). Whole-genome sequencing was performed using the Illumina HiSeq 2500 sequencer in Novogene (Beijing, China), with a coverage of 109X. The nanopore sequencing library was prepared using the rapid sequencing kit SQK-RAD004 (Oxford Nanotechnology, UK). The mixed assembly of Illumina. and Nanopore (Oxford Nanotechnology, UK) reads was performed using Unicycler[22] (v0.4.7). PlasmidFinder[23] (<https://cge.cbs.dtu.dk/services/PlasmidFinder/>) was used to determine the plasmid replicon type. Genotyping was performed to query the 7 domesticated genes (*pyrG*, *gyrB*, *rplB*, *leuS*, *dnaA*, *rpoB* and *fusA*) via the multi-locus sequence typing (MLST) web service.

Genomic and plasmid sequences of strain ZT01-0079 have been deposited into GenBank under accessions CP055247 and CP022050.

Results

Microbiological features of strain ZT01-0079

Strain ZT01-0079 was identified as *C. freundii*. Susceptibility testing showed that *C. freundii* ZT01-0079 exhibited resistance to aztreonam, nitrofurantoin, ciprofloxacin, piperacillin, cefepime, ceftriaxone, ceftazidime, tobramycin, levofloxacin, and imipenem. S1-PFGE revealed that *C. freundii* ZT01-0079 contained three plasmids (~143kb, ~89kb and ~53kb) (Figure 1). Electrophoresis and Southern blotting showed that the bla_{NDM-1} gene was located on the ~53kb plasmid. The NDM-1-carrying plasmid was successfully transferred at a frequency of 1.68×10^{-3} . Transconjugants acquired resistance to ciprofloxacin, piperacillin, cefepime, ceftriaxone, ceftazidime, ceftazolin, and imipenem (Table 1). In silico MLST found that *C. freundii* ZT01-0079 belongs to sequence type (ST)19. In addition to bla_{NDM-1} , *C. freundii* ZT01-0079 carried multiple resistance genes including bla_{SHV-12} , $bla_{CTX-M-55}$, $bla_{CMY-152}$, *fosA3*, *aph(3'')-Ib*, *aph(6)-Id*, *aph(3'')-Ib*, *aph(6)-Id* and *sul2*. The $bla_{CMY-152}$ gene was located on the chromosome, while the other genes were located on plasmids. The 89kb plasmid carried *fosA3*, $bla_{CTX-M-55}$, *sul2*, *aph(3'')-Ib* and *aph(6)-Id* genes, whereas the 143kb plasmid carried *faph(3'')-Ib*, *aph(6)-Id* and *sul2* genes. Plasmid pZY-NDM1 had a length of 53kb, and carried both the bla_{NDM-1} and bla_{SHV-12} genes. All three plasmids belonged to type IncX3.

Genetic analysis of pZY-NDM1

Plasmid pZY-NDM1 is a 53573 bp circular plasmid with an average GC content of 49.24% and has 76 open reading frames. pZY-NDM1 belongs to type IncX3. BLAST comparison disclosed that the genome sequence of pZY-NDM1 shared >99% similarity with plasmids pNDM-HK3694 (Genbank accession JX104760.1) and p309074-NDM (Genbank accession MH909346.1). These three plasmids share a typical IncX plasmid backbone composed of replication (*repB*); plasmid stability (*parA*, *parB*); plasmid maintenance (*hns*, *topB*); and type IV secretion system (*taxA*, *taxB*, *taxC* and *pilx1-pilx11*) encoding regions. The 19.66 kb genetic load region of pZY-NDM1 located between *hns* and *resolvease* genes contained *Tn3*, *IS3000*, the transposon *Tn125* encoding bla_{NDM-1} , and a mobile element containing bla_{SHV-12} and *ISL3*. The transposon *Tn125* served as a mobile element for the transfer of bla_{NDM-1} and comprised *insE* (locus tag CS291_RS00115), *groEL* (locus tag CS291_RS00115), *tat* (locus tag CS291_RS00130), $\Delta trpF$ (locus tag CS291_RS00135), the bleomycin resistance gene *ble* (locus tag CS291_RS00140), $\Delta ISAbA125$, bla_{NDM-1} (locus tag CS291_RS00145), *dct* (locus tag CS291_RS00125), and *groES* (locus tag CS291_RS00120) as previously described [25]. Compared with the prototype *Tn125*, $\Delta ISAbA125$ was located upstream of *IS5* in the p309074-NDM plasmid, and had a length of 891bp, while $\Delta ISAbA125$ was deleted in plasmids pZY-NDM1 and pNDM-HK3694.

The *bla*_{SHV-12}-carrying transposon was located in two opposite IS26 elements and was composed of *ΔygbJ* (locus tag, CS291_RS00085), *ygbI* (locus tag, CS291_RS00080) and *bla*_{SHV-12} (locus tag, CS291_RS00075). Compared with pNDM-HK3694, the *bla*_{SHV-12}-carrying transposon of pZY-NDM1 was reversed. The *umuD* gene was split into two genes, *ΔumuD1* and *ΔumuD2*, which may serve as an insertion sites of regions containing the *bla*_{SHV-12}-carrying transposons *Tn125*-like transposon, IS3000 and *tnpA*.

Discussion

The prevalence of NDM-1-producing bacteria is receiving increasing attention as a threat to global health. Most *bla*_{NDM-1}-positive isolates of diverse species of *Enterobacteriaceae* show high-level resistance to β -lactam antibiotics. Concurrently, the *bla*_{NDM-1} gene has also spread in many environmental and animal reservoirs, such as sewage, rivers, soil, and many mammals and poultry in Asia and the Middle East [2,9,12,20]. However, reports on *C. freundii* are still rare. Therefore, we investigated the genomic characteristics of multidrug-resistant *C. freundii* (strain ZT01-0079) isolated from a clinical urine specimen. This strain has the IncX3 plasmid pZY-NDM1 that co-harbors *bla*_{NDM-1} and *bla*_{SHV-12}. Compared with other IncX3 plasmids [21], pZY-NDM1 has a higher transfer rate, which may be an important contributor to the rapid dissemination of the *bla*_{NDM-1} gene.

Coexistent *bla*_{NDM-1} and other β -lactamase genes in IncX3 plasmids, which have a broad host range, mediate resistance to broad-spectrum antibiotics such as carbapenems and cephalosporins, and can be transferred between *Enterobacteriaceae* [21]. Notably, there are few reports of such plasmids in *C. freundii*, which is emerging as an important opportunistic pathogen causing increasingly difficult to treat infections.

Conclusions

In summary, we characterized the genomic basis of multi-drug resistance in *C. freundii* strain ZT01-0079. MLST revealed that strain ZT01-0079 belongs to ST19. ZT01-0079 carried multiple *bla* genes, which increased the level of carbapenem resistance. pZY-NDM1 carries both the *bla*_{NDM-1} and *bla*_{SHV-12} genes; is transferable; and may thereby serve as a common vector for the rapid dissemination of carbapenemase-encoding genes. Our findings further underscore the threat of increased NDM-1 prevalence in *Enterobacteriaceae*, and emphasize that increased resources and effort should be dedicated to monitor the potentially rapid spread of NDM-1-encoding plasmids.

Declarations

Authors' contributions

TYZ, YFL and YKW performed genome analysis and experiment. ZHL and XL performed DNA extraction and performed bacterial culture. TYZ prepared

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the manuscript. HGW, PL, ZDL and LFL designed the study and revised the manuscript. All authors reviewed and approved the final manuscript.

Acknowledgements

Not applicable.

Ethics approval and consent to participate

The authors state that all experimental protocols were approved by the institutional ethics committees of Academy of Military Medical Sciences.

Availability of data and materials

Genomic and plasmid sequences of strain ZT01-0079 were deposited in GenBank under accession CP055247 and CP022050.

Competing interests

Not applicable.

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Consent for publication

The clinically separated samples used in this study are routine hospital procedures. We do not use patients' personal information, so written consent is not required.

Abbreviations

BLAST	Basic Local Alignment Tool
NDM-1	New Delhi metallo- β beta-lactamase-1
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
ST	Sequence type

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Table

Table 1 Antibiotic susceptibilities of ZT01-0079 and transconjugants

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Antimicrobial	MIC ($\mu\text{g/ml}$)	
	ZT01-0079	Transconjugant (<i>E.coli</i> . J53)
Amikacin	8	≤ 2
Aztreonam	≥ 64	≤ 1
Nitrofurantoin	128	≤ 16
Ciprofloxacin	≥ 4	1
Piperacillin	≥ 128	≥ 128
Gentamicin	≥ 16	≤ 1
Cefepime	≥ 64	≥ 64
Ceftriaxone	≥ 64	≥ 64
Ceftazidime	≥ 64	≥ 64
Tobramycin	≥ 16	≤ 1
Imipenem	≥ 16	≥ 16
Levofloxacin	≥ 8	≤ 1

Figures

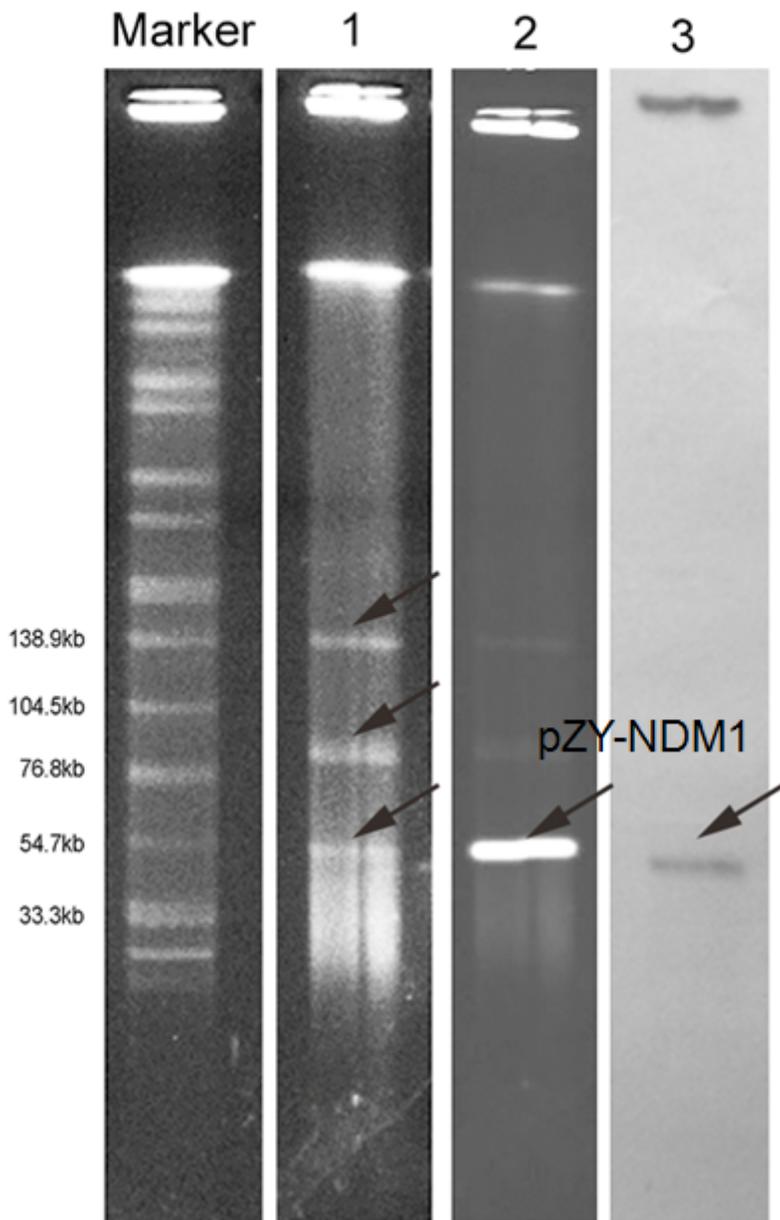


Figure 1

S1-PFGE pattern for strain ZT01-0079 and Southern blot analysis of blaNDM-1 genes. Lanes: Marker as a reference size standard; PFGE result of S1-digested plasmid DNA of strain ZT01-0079; Southern blot hybridization with probes specific to blaNDM-1, respectively.

