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ORIGINAL RESEARCH

An NDM-1-Producing *Acinetobacter towneri* Isolate from Hospital Sewage in China

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Background: The New Delhi metallo-β-lactamase-1 (NDM-1)-positive plasmid and its variants pose daunting threats to public health. Hospital sewage was considered as an important reservoir of antibiotic genes. Numerous and diverse taxa of multidrug-resistant (MDR) bacteria carrying NDM-1-positive plasmids have been identified during routine surveillance of hospital sewage. We herein report a carbapenem-resistant *Acinetobacter towneri* strain AeBJ009 with an NDM-1-positive plasmid isolated from hospital sewage.

Materials and Methods: Bacteria were isolated from cultures of hospital sewage and identified by using the Vitek 2 compact system and 16S rRNA sequencing. The bla_{NDM-1} gene was amplified and confirmed by sequencing. Antimicrobial susceptibility testing was performed using AST-GN14 on the Vitek2 compact system. In addition, the bla_{NDM-1} gene was located by Southern blotting. Conjugation experiment and whole-genome sequencing were performed for further analysis.

Results: Strain AeBJ009 was isolated from hospital sewage and identified as *A. towneri*. Antimicrobial susceptibility testing revealed an MDR phenotype. Pulsed-field gel electrophoresis and Southern blotting showed that strain AeBJ009 carries three plasmids and that bla_{NDM-1} is located on the 47kb plasmid pNDM-AeBJ009. However, the conjugation experiment to transfer pNDM-AeBJ009 to *Escherichia coli* strain J53 was unsuccessful. Whole-genome sequencing found that pNDM-AeBJ009 contains a Tn*125* element carrying bla_{NDM-1}. The *ble* gene downstream of bla_{NDM-1} displayed a single-nucleotide polymorphism compared to its homologue on plasmid pM131_NDM1. BLAST analysis using the Comprehensive Antibiotic Resistance Database identified no gene polymorphisms with 100% identity to our *ble* variant.

Conclusion: The *A. towneri* strain AeBJ009 exhibiting an extended spectrum of antibiotic resistance was isolated from hospital sewage and may potentially exacerbate the risk of MDR bacterial infections. The prevention of nosocomial infections due to drug-resistant bacteria will require enhanced monitoring and control of MDR pathogens in environmental reservoirs.

Keywords: NDM-1, *ble*, *Acinetobacter towneri*, drug resistance

Introduction

Multidrug-resistant (MDR) pathogens and residual antibiotics have been detected in hospital sewage consequent to their discharge into wastewater during the therapy of patients with infectious diseases.[1] Hospital sewage is an ideal environment for the selection of MDR pathogens,[2–4] for the interspecies exchange of drug-resistance genes on mobile genetic elements, and may serve as an environmental reservoir for their further spread.[5] Surveillance of hospital sewage has shown that many MDR bacteria belong to New Delhi metallo-β-lactamase-1 (NDM-1)-positive strains.[6]

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NDM-1 can hydrolyze nearly all beta-lactam antibiotics, including carbapenems.[7] The bla_{NDM-1} gene was widely distributed among gram-negative bacteria,[8,9] most of which are found on conjugative plasmids enhancing the spread of resistance.[10] Strains carrying bla_{NDM-1} are most often members of either the Enterobacteriaceae family[11,12] or the Acinetobacter genus.[13,14] Acinetobacter spp. can be isolated from multiple environmental reservoirs,[15] and are considered among the most formidable nosocomial pathogens.[16] NDM-1-positive plasmids have been detected with increasing frequency in Acinetobacter spp.[17] Studies from China have reported that *Acinetobacter towneri* isolates from both clinical specimens[18] and hospital sewage[19] carry multiple resistance genes.

We here reported an MDR *A. towneri* strain AeBJ009 with a bla_{NDM-1}-harboring plasmid. Further experiments and whole-genome sequencing revealed the plasmid shared nearly the same sequence with a transferable plasmid of a clinical sample but was non-conjugative, intriguing us to explore the genetic characteristics of the bla_{NDM-1} gene. Our results underscore the potential threat of the transmission of *bla* genes in environmental reservoirs, and highlight the roles of enhanced environmental surveillance of antimicrobial resistance and treatment of hospital sewage as risk reduction strategies.

Materials and Methods

Bacterial Isolation and Identification

Hospital sewage was collected and concentrated using centrifuge GR22G II (HITACHI, Japan) in Beijing in 2011. Bacteria was isolated by incubating the resuspended sediment on MacConkey agar plates containing 4μg/mL meropenem. Strain AeBJ009 was recovered and identified using the Vitek2 compact system (BioMérieux, France). The 16S rRNA gene was amplified with the universal primers (27F: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R: 5′-TACGGCTACCTTGTTACGACTT-3′).[20] The bla_{NDM-1} gene was detected by PCR and sequencing with primers NDM-F-38 (5′-GGCGGAATGGCTCATCACGA-3′) and NDM-R-344 (5′-CGCAACACAGCCTGACTTTC-3′).[21] An NDM-1-carrying *K. pneumoniae* strain KP14003[22] from our lab was used as positive control.

Antimicrobial Susceptibility Testing

The strain was cultured on LB agar plates. One hundred and forty-five microliter bacterial suspension of a 0.5-McFarland turbidity was mixed with 3mL 0.45% NaCl solution. The AST-GN14 card filled with the mixture was used. The minimum inhibitory concentrations (MICs) of ampicillin, amoxicillin/clavulanic acid, piperacillin, cefazolin, ceftazidime, ceftriaxone, cefepime, aztreonam, imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, levofloxacin, tetracycline, nitrofurantoin and sulfamethoxazole/trimethoprim were determined by the Vitek2 compact system (BioMérieux, France) following the manufacturer's instructions. Antimicrobial sensitivity results were interpreted according to the Clinical and Laboratory Standards Institute guidelines (M100-S24).[23] The *E. coli* ATCC25922 and *E. coli* J53 were used as quality control and negative control for antimicrobial testing, respectively.

Southern Blotting

Four hundred microliter bacterial suspension with turbidity of 3.7–4.2 McF was used for gel preparation. Genomic DNA was digested with S1 nuclease (Code No: 2410A, Takara). The linearized DNA fragments were separated by pulsed-field gel electrophoresis (PFGE) (Bio-Rad, Hercules, CA, USA). DNA on gel was dyed with ethidium bromide. Plasmid DNA was transferred to a Hybond $N^{+®}$ membrane (Sigma-Aldrich, St. Louis, MO, USA) and hybridized with a bla_{NDM-1} probe that was labeled with digoxin. The experiment was performed according to the manufacturer's manual of the DIG High Prime DNA Labeling and Detection Start Kit I (Cat. No: 11745832910, Roche).

Conjugation Experiment

Conjugation was carried out by broth and filter mating using azide-resistant *Escherichia coli* strain J53 as the recipient and the AeBJ009 strain as the donor. The donor/recipient LB suspensions in logarithmic phase were mixed at a 4:1 ratio. The mixture was incubated at 37°C for 18 hours. Transconjugants were selected on MacConkey agar plates containing 200μg/mL sodium azide and 4μg/mL meropenem.

Whole-Genome Sequencing and Analysis

Genomic DNA was extracted by TIANamp Bacteria DNA Kit (Cat. No: DP302, TIANGEN BIOTECH) from cultured bacterium. A total of 700ng genomic DNA was used for library preparation using NEBNext® Ultra™ II DNA Library Prep Kit (Cat. No: E7645S, NEB) according to the manufacturer's manual. Whole-genome sequencing was performed by Novogene Company (Beijing, China) on the Illumina HiSeq X platform using Dual Flow Cell with 150bp pair-end reads. Reads were assembled de novo by using Spades (v3.6.2) with k-mer sizes of 21,

33, 77, 99 and 127.[24] Gene annotation of plasmids was performed on the RAST webserver using the RASTtk pipeline with default parameters.[25] Plasmid replicon types were identified using PlasmidFinder 1.3.[26]

Results

Isolation, Identification, and Antimicrobial Susceptibility Testing of Strain AeBJ009

Strain AeBJ009 was identified as *A. towneri* by using the Vitek2 compact system, and confirmed by blasting the 16S rRNA sequence against the *nt* database of NCBI (Figure S1). *A. towneri* AeBJ009 showed resistance to cefazolin, ceftazidime, ceftriaxone, meropenem, ciprofloxacin, nitrofurantoin, and trimethoprim/sulfamethoxazole, but was sensitive to amoxicillin/clavulanic acid, piperacillin, cefepime, aztreonam, amikacin, levofloxacin and tetracycline (Table 1). The results of S1 PFGE revealed that AeBJ009 carried three plasmids (~47kb, ~76kb and ~300kb), and Southern blotting indicated that bla_{NDM-1} was located on the ~47kb plasmid (Figure S2). Conjugation using *E. coli* J53 as the recipient was unsuccessful.

Genetic Features and Plasmid Characteristics

Genome sequencing and analysis revealed that the *A. towneri* AeBJ009 chromosome contains approximately 3.02Mb. There were 18 resistance genes (Table 2) including $bla_{\text{PER-1}}$, $bla_{\text{NDM-1}}$, bla_{BRP}, bla_{OXA-58}, $bla_{CTX-M-55}$ and $bla_{\text{TEM-30}}$. Three resistant genes (bla_{NDM-1}, bla_{BRP} and *aph (3')-VI*) were located on the same plasmid, designated as pNDM-AeBJ009. This plasmid has a length of 47,271 bp and 40.8% guanine-cytosine content, and contains 55 open reading frames. The plasmid contains three sections that encode plasmid transfer and replication, a type IV secretion system, and Tn*125*, respectively (Figure 1).

pNDM-AeBJ009 could not be matched to any plasmid replicon type by PlasmidFinder. An NCBI BLAST analysis showed that the identity between plasmids pNDM-AeBJ009 and pM131_NDM1 (JX072963.1)[27] is over 99% (Figure 2). A composite transposon Tn*125* structure of pNDM-AeBJ009 is located downstream of the *aphA6* gene carrying bla_{NDM-1}. The second copy of IS*Aba125* in bla_{NDM-1} downstream is reversed from that of plasmid pNDM-JAVP01.[28] Compared with plasmid pNDM229,[29] there is no IS*Aba14* copy between the downstream Tn*125* element and *tnpR* in plasmid pNDM-AeBJ009 (Figure 2)

Plasmid pNDM-AeBJ009 has 41 single-nucleotide polymorphism (SNPs) compared to plasmid pM131_NDM1. Tn*125* has 14 SNPs, while the upstream and downstream IS have two and eleven SNPs, respectively. The only SNP in *ble* lead to the change of the 17th base from cytosine to thymidine. This SNP substitutes threonine for isoleucine during translation. Of the remaining SNP loci, four were in *orfA* and one in *VirB10*.

Table 1 Antibiotic Susceptibilities of *A. towneri* Strain AeBJ009

Antimicrobial Susceptibility	*A. towneri* Strain AeBJ009		*E. coli* J53	
	MIC (μg/mL)	S/I/R	MIC (μg/mL)	S/I/R
Ampicillin	16	I	≤2	S
Amoxicillin/Clavulanic acid	≤1	S	≤1	S
Piperacillin	≤16	S	≤4	S
Cefazolin	≥64	R	≤4	S
Ceftazidime	≥16	R	≤1	S
Ceftriaxone	8	R	≤1	S
Cefepime	8	S	≤1	S
Aztreonam	2	S	≤1	S
Imipenem	4	I	≤1	S
Meropenem	≥4	R	≤0.25	S
Amikacin	≤16	S	≤2	S
Gentamicin	8	I	≤1	S
Ciprofloxacin	4	R	≤0.25	S
Levofloxacin	≤2	S	≤0.25	S
Tetracycline	≤4	S	≤1	S
Nitrofurantoin	≥128	R	≤16	S
Sulfamethoxazole/Trimethoprim	≥512	R	≤0	S

Discussion

The bla_{NDM-1} gene has been identified in multiple strains since first reported in *Klebsiella pneumoniae*.[7] Many studies report that bla_{NDM-1} is not only found in clinical isolates, but also in environmental bacteria from sources such as hospital sewage.[3] In this study, we isolated an *A. towneri* strain with an NDM-1-carrying plasmid from

Table 2 Resistance Genes of *A. towneri* Strain AeBJ009

Resistance Against	Resistance Gene
Aminoglycoside	*aadA, aac(3), aph(3'), ant(3''), aac(6')*
Beta-lactam	bla_{OXY}, bla_{OXA}, bla_{CTX}, $bla_{\text{NDM-1}}$, bla_{CARB}, bla_{TEM}, bla_{PER}
Carbapenem	*adeJ*
Glycopeptide	*brp*
Phenicol	*cmlB*
Rifampicin	*arr*
Sulfonamides	*sul*
Macrolides	*msrE*

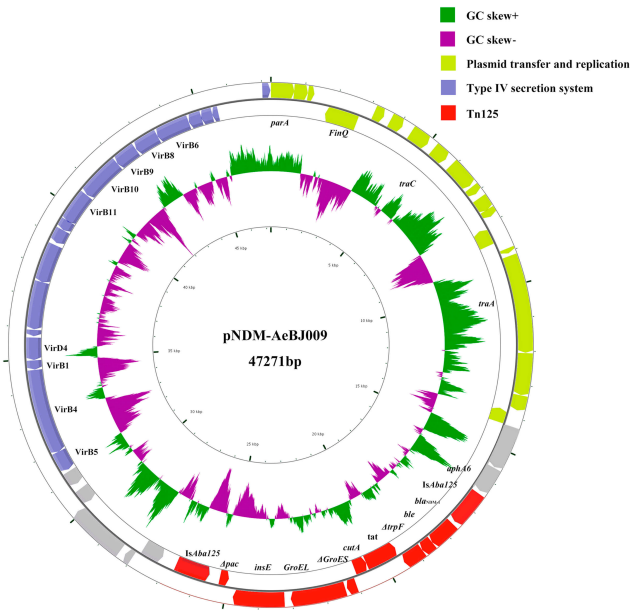

Figure 1 Circular maps of plasmid pNDM-AeBJ009. The outer circle contains the plasmid transfer and replication section (yellow arrows), type IV secretion system gene cluster section (blue arrows), and Tn*125* section (red arrows). In addition, the known gene names reported in the NCBI database are marked below the corresponding arrows. The inner-circle indicates the positive and negative GC skew of the plasmid in green and purple, respectively.

hospital sewage. The $bla_{\text{NDM-1}}$ gene was located on the common transposon Tn*125*, which is a primary carrier in the spread of $bla_{\text{NDM-1}}$ in Acinetobacter spp.[30,31]

Compared to other whole plasmid sequences in the NCBI database, pNDM-AeBJ009 was similar only to pM131_NDM1, and quite different from others. Similar to pM131_NDM, pNDM-AeBJ009 did not match any plasmid replicon type by PlasmidFinder. According to plasmid typing principles, the pNDM-AeBJ009 might lack a typing sequence, or its typing sequence is not yet in the database. Further studies are needed for the typing of pNDM-AeBJ009.

In the conjugation experiment, horizontal transfer of pNDM-AeBJ009 to *E. coli* strain J53 failed. However, the similar plasmid pM131_NDM1 can be effectively transferred to non-pathogenic Acinetobacter spp.[27] An attempt to transfer plasmid pNDM229[29] to different recipient strains also failed because of truncation or absence of conjugation-related genes. The plasmid pNDM-JAVP01[28] was transferred to the recipient strain *A. baumannii* ATCC 17978 but failed to transfer to *E. coli* MC1061. Compared to pM131_NDM1, pNDM-AeBJ009 has 14 SNPs in Tn*125* and one SNP in the conjugation-related gene *virB10*. These mutations may have caused the failed conjugation. Another possible explanation for failed conjugation may be that the *E. coli* J53 strain may not be a suitable recipient for pNDM-AeBJ009. For example, morphologic features of the strain may affect cell wall permeability and consequently impair conjugation. We cannot conclude that pNDM-AeBJ009 has no potential for horizontal transfer; further studies are needed to clarify this issue. The different transferability of these two highly similar plasmids

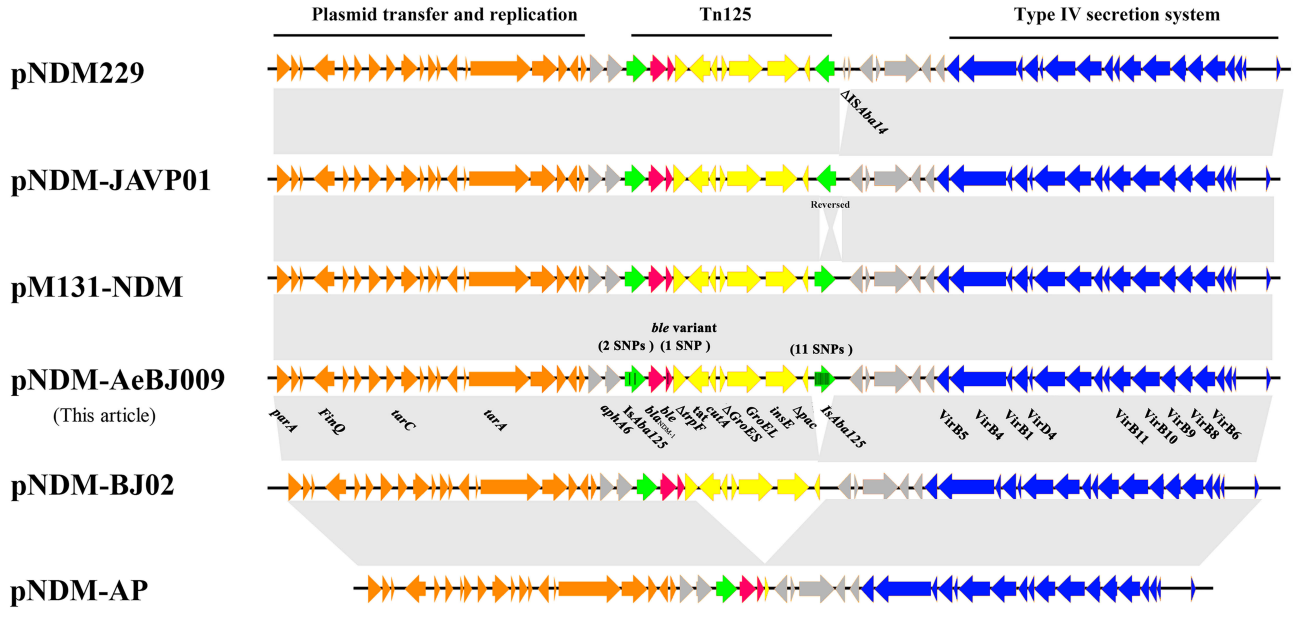

Figure 2 Comparative analysis of pNDM- AeBJ009 with other NDM-1-positive plasmids. The open reading frames are indicated by arrows. The $bla_{\text{NDM-1}}$ gene and *ble* gene variant are shown in red. The insertion sequences IS*Aba125* are shown in green. The black vertical line indicates the SNP in genes. Other genes of the backbone are shown in orange, gray, yellow and blue, respectively. Homology regions among different plasmids are denoted by light gray.

indicates that further understanding of the mechanisms promoting plasmid transfer is therefore of importance.

Our genotypic analysis disclosed a SNP in *ble* in which the 17th base is changed from cytosine to thymidine, causing an amino acid substitution during translation. This result suggests that the *ble* SNP may be a new drug-resistance mutation that increases the risk of plasmid-mediated high-level drug resistance. This novel *ble* variant may have emerged under selection pressure from biologically relevant concentrations of multiple antibiotics in hospital sewage. Though studies on the NDM variants showed that the amino acid changes were associated with different hydrolysis activity,[32] the effect of the amino acid substitution on the *ble* gene needs further study.

Moreover, other genes encoding OXA-58, CTX-M-55, and TEM-30 were also detected in our AeBJ009 strain. A previous study revealed that the prevalence of these beta-lactamase genes in hospital effluent was very high.[3] The challenge of resistance genes and novel mutants in hospital sewage is worthy of further study. As a reservoir of multiple resistance genes, hospital sewage may have an especially important clinical and epidemiologic relevance.

Conclusions

We report an *A. towneri* strain AeBJ009 that was isolated from hospital sewage and characterized by bioinformatic analysis. This strain carried multiple *bla* genes and a novel *ble* variant. The co-existence of these genes may confer increased carbapenem resistance. The findings of resistant bacteria and novel resistance genes in hospital sewage highlight the potential role of hospital sewage as an environmental reservoir of MDR pathogens that deserves further attention for surveillance and risk management.

Accession Number

The whole-genome sequence of *Acinetobacter towneri* strain AeBJ009 has been deposited at DDBJ/ENA/GenBank under accession NZ_SIST01000000. The complete sequences of plasmid pNDM-AeBJ009 and the new *ble* variant have been deposited in GenBank under accession numbers CM016430 and MN886241, respectively.

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Disclosure

The authors report no conflicts of interest in this work.

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