Infection and Drug Resistance

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ORIGINAL RESEARCH

Coexistence of bla_{NDM-1} and *rmtC* on a Transferrable Plasmid of a Novel ST192 *Klebsiella aerogenes* Clinical Isolate

This article was published in the following Dove Press journal:
Infection and Drug Resistance

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Introduction: The occurrence and development of antibiotic resistance are mainly caused by the spread of large plasmids carrying multiple antibiotic resistance genes. Recently, the association between 16S rRNA methyltransferase genes and β-lactamase genes carried by the same plasmid is of concern.

Methods: The *Klebsiella aerogenes* 1564 was isolated from the catheter tip of a patient in a tertiary hospital, Shanghai, China. The presence of the bla_{NDM-1} and *rmtC* genes were assessed by PCR. Complete sequence of plasmid p1564 was determined. The *K. aerogenes* 1564 was characterized by antimicrobial susceptibility testing, Carbapenemase phenotype confirmation testing, conjugation experiment, S1-PFGE and multilocus sequence typing (MLST).

Results: Herein, we found that a New Delhi Metallo-β-lactamase-1 gene (bla_{NDM-1}) and a 16S rRNA methyltransferase gene (*rmtC*) coexisted on a transferrable plasmid of a carbapenem-resistant *K. aerogenes* clinical isolate. The *K. aerogenes* clinical isolate was found to belong to a novel sequence type 192 (ST192) determined by MLST. The sequencing results of the plasmid p1564 carrying bla_{NDM-1} gene and *rmtC* gene showed that the size and guanine-cytosine content of the plasmid were 136, 902 bp and 51.8%, with 164 putative ORFs and two multidrug resistance gene islands. In addition to $bla_{NDM-1}$and *rmtC*, the plasmid contained bleomycin resistance gene (ble_{MBL}), CMY-6β-lactamase gene (bla_{CMY-6}) , quaternary ammonium compound resistance gene (*sugE*), truncated quaternary ammonium compound resistance gene (*qacEΔ1*), aminoglycoside resistance gene (*aacA4*) and sulfonamide resistance gene (*sul1*). By comparison, p1564 has high homology with pHS36-NDM from *Salmonella enterica* subsp. *enterica* serovar Stanley reported in China, with similar size and both belonging to plasmid incompatibility group A/C.

Conclusion: The present study demonstrated for the first time the co-existence of *rmtC* and bla_{NDM-1} in a novel ST192 *K. aerogenes*. The spread of plasmids harboring both bla_{NDM-1} and *rmtC* may occur among Enterobacteriaceae in China.

Keywords: *Klebsiella aerogenes*, plasmid, bla_{NDM-1}, *rmtC*

Introduction

Klebsiella aerogenes is a Gram-negative bacterium which is widely found in human gastrointestinal tract and various other in vivo environments, but is generally not pathogenic to healthy humans.[1] This organism was previously known as *Enterobacter aerogenes*. Since the early 1990s, *K. aerogenes* has become an important opportunistic pathogen, often leading to hospital-acquired infections such as pneumonia, urinary tract infections, bacteremia, intracranial infections and surgical wound

infections.[2–6] It has been considered an important new multidrug resistance (MDR) pathogen in the past two decades.[7]

With the increasing use of carbapenems in clinical practice, infections caused by carbapenem-resistant Enterobacteriaceae (CRE) pose a significant threat to human health.[8] The emergence and spread of antibiotic resistance have caused widespread concern around the world. New Delhi Metallo-β-lactamase-1 (NDM-1) was first identified in a clinical isolate of *Klebsiella pneumoniae* from a Swedish patient from India in 2009.[9] The $bla_{\text{NDM-1}}$ gene has the ability to spread widely in bacterial species through horizontal gene transfer.[10] In recent years, plasmid-mediated NDM-1 has spread rapidly among Enterobacteriaceae, mainly *Klebsiella pneumoniae* and *Escherichia coli* throughout the world.[11] It has recently been reported that 16S rRNA methyltransferase genes are associated with New Delhi Metallo-β-lactamase-1 (NDM-1) in Enterobacteriaceae.[12] The combination of beta-lactam and aminoglycoside plays an important role in antimicrobial therapy in severe infections. Gram-negative pathogens co-producing NDMs and 16S rRNA methylases have high levels of resistance to clinically important carbapenems and aminoglycosides, which may result in aminoglycoside and beta-lactam antibiotic combinations lose its clinical therapeutic significance. This study aimed to describe the first time that co-existence of *rmtC* and $bla_{\text{NDM-1}}$ genes in a novel ST192 *K. aerogenes*in China.

Materials and Methods

Bacterial Isolates

The *Klebsiella aerogenes* 1564 was isolated from the catheter tip of a patient in a tertiary hospital in Shanghai, China, and identified by Matrix-Assisted Laser Desorption/Ionisation - Time Of Flight (MALDI-TOF MS) according to the manufacturer's instructions. *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC25923 and *Pseudomonas aeruginosa* ATCC87253 were used as control strains for the identification of the species.

Antimicrobial Susceptibility Testing

The minimum inhibitory concentrations (MICs) of antimicrobial agents for the bacteria tested were determined using the broth microdilution method and interpreted according to CLSI standards.[13] A total of 17 antimicrobial agents were tested, including carbapenems (imipenem and meropenem), β-lactam /β-lactamase inhibitor complexes (piperacillin-tazobactam and ceftazidime-avibactam), monocyclic β-lactam (aztreonam), cephalosporin (cefoxitin, cefotaxime, cefepime and ceftazidime), aminoglycosides (gentamicin and amikacin), fluoroquinolones (ciprofloxacin), folate metabolic pathway inhibitors (sulfamethoxazole), tetracyclines (tetracycline, minocycline and tigecycline) and polymyxin B. *E. coli* ATCC25922 was used as a control strain for the antimicrobial susceptibility test.

Carbapenemase Phenotype Confirmation Testing

A modified carbapenem inactivation test (mCIM) was performed to detect carbapenemases according to CLSI 2018 standards.[13] The tested strains were incubated with meropenem disk (10 μg) in 2 mL TSB at 37°C for 4 hrs. *Escherichia coli* ATCC25922 was used as an indicator bacteria and its suspension was adjusted to 0.5 McFarland using sterile physiological saline solution. The *E. coli* ATCC25922 suspension was evenly coated on an MH agar plate. After the plate is dried for 3–10 mins, the meropenem disk (10 μg) was placed on the surface of the agar plate. The treated plate was incubated at 37°C for 18–24 hrs.

Detection of Resistance Genes

The carbapenemase genes responsible for carbapenem resistance (bla_{KPC}, bla_{VIM}, bla_{GES}, bla_{IMP}, bla_{SPM}, $bla_{\text{OXA-23}}$, $bla_{\text{OXA-48}}$, bla_{SME}, bla_{SIM} and bla_{NDM}) and 16S rRNA methyltransferase genes (*rmtA, rmtB, rmtC, rmtD, armA* and *nmpA*) were detected using PCR as described previously.[14–16] Following PCR, the DNA fragments were analyzed using gel electrophoresis on 1% agarose gels and were sequenced on both strands.

Conjugation Experiment

In order to determine whether the aminoglycoside antibiotic resistance gene *rmtC* and $bla_{\text{NDM-1}}$ carried by the plasmid on *K. aerogenes* 1564 can be transferred horizontally, conjugation experiment was carried out in LB broth medium using rifampicin-resistant *E. coli* EC600 (*E. coli* EC600Rif-R) as recipient. Cultures of donor and recipient cells in logarithmic phase (200 μL, 100 μL, respectively) were added to 4 mL of fresh LB broth and incubated overnight at 37°C without shaking. To screen for transconjugants, serial dilutions of mixed cultures were plated onto MH agar plates containing amikacin (128 mg/L) and rifampicin (600 mg/L). The donor cells alone and recipient cells alone were used as controls to ensure the effectiveness of the selective plates used. The transconjugant colonies were selected from the

selective plates and cultivated onto the selective plates again for purification of transconjugant strains. All transconjugants were confirmed by PCR for the presence of *rmtC* and bla_{NDM-1} genes. The antibiotic susceptibilities were also investigated as mentioned above.

Pulsed-Field Gel Electrophoresis (PFGE)

S1-PFGE was performed to obtain plasmid profiles in donor strains, recipient strains and transconjugants, as described previously.[17] The *Salmonella enterica* serotype Braenderup strain H9812 was used as a control standard strain and molecular size marker.

Multilocus Sequence Typing (MLST)

Multilocus Sequence Typing (MLST) was performed on *K. aerogenes* 1564 by amplifying internal fragments of the seven standard housekeeping loci, including*dnaA, fusA, gyrB, leuS, pryG, rplB* and *rpoB*. Sequence types (STs) were determined according to the *Klebsiella aerogenes* MLST Databases (https://pubmlst.org/kaerogenes/).

Plasmid Extraction and Sequencing

Plasmid DNA from *E. coli* EC600Rif-R transconjugants was extracted using Qiagen Plasmid Midi Kit (Qiagen, Valencia, CA, United States of America) according to the manufacturer's protocol. A library of different inserts was constructed using the Whole Genome Shotgun (WGS) strategy, and these libraries were Paired-end (PE) sequenced on the Illumina MiSeq sequencing platform. The sequencing reads were de novo assembled using the SPAdes v3.9.0.[18] The plasmid splicing results were co-linearly analyzed using mummer v3.1 software to determine the positional relationship between contigs. Gaps between contigs were closed through PCR and Sanger sequencing. The results were corrected using Pilon v1.18 software to obtain the final plasmid DNA sequence.[19] The circular representation of p1564 was generated with CGview (http://stothard.afns.ualberta.ca/cgview_server/).[20] Mauve 2.3.1 was used to perform comparative genome alignment for related plasmids.[21]

Result

Antimicrobial Susceptibility Testing

Antimicrobial sensitivity results are shown in Table 1. *K. aerogenes* 1564 was resistant to all beta-lactam antibiotics (cephalosporins, carbapenems, penicillins and monocyclic β-lactams) and aminoglycosides tested, but susceptible to ciprofloxacin, sulfamethoxazole, polymyxin B, tetracycline, minocycline and tigecycline.

Table 1 Antimicrobials MIC Values of for *K. Aerogenes*, Its Transconjugant and the Recipient Strain

Antimicrobials	MIC Values (mg/L)		
	1564	1564-EC600	EC600
Imipenem	8	4	≤0.5
Meropenem	16	8	≤0.5
Piperacillin/tazobactam	128/4	64/4	≤4/4
Ceftazidime/avibactam	>32/4	>32/4	≤0.25/4
Aztreonam	16	16	≤1
Cefoxitin	>32	>32	8
Cefotaxime	>64	>64	≤1
Cefepime	16	>16	≤0.5
Ceftazidime	>32	>32	≤1
Gentamicin	>16	>16	≤0.5
Amikacin	>64	>64	≤2
Ciprofloxacin	≤0.25	≤0.25	≤0.25
Sulfamethoxazole	2/38	≤0.5/9.5	≤0.5/9.5
Tetracycline	4	2	≤1
Minocycline	≤2	≤2	≤2
Tigecycline	0.5	0.5	≤0.25
Polymyxin B	≤0.5	≤0.5	≤0.5

Detection of Carbapenemases and Resistance Genes

K. aerogenes 1564 was positive for the mCIM assay, indicating that the isolate produced carbapenemases. Consequently, a carbapenemase gene (bla_{NDM-1}) was found among *K. aerogenes* strain 1564, which was determined by PCR and DNA sequencing. As the *K. aerogenes* strain 1564 was resistant to gentamicin and amikacin, 16S rRNA methyltransferase genes were detected by PCR and DNA sequencing and *rmtC* was identified.

Conjugation and S1-PFGE

The conjugation experiments result showed that the *rmtC* and bla_{NDM-1} genes were successfully transferred from *K. aerogenes* 1564 to the *E. coli* EC600Rif-R recipient, clearly demonstrates the potential for horizontal transfer of the *rmtC* and bla_{NDM-1} genes. The drug susceptibility spectrum of the transconjugant is consistent with its donor strain *K. aerogenes* 1564 (Table 1). The MIC of amikacin for transconjugant was >64 mg/L. In addition, the MIC value of imipenem for transconjugant is at least 8-fold higher than that of *E. coli* EC600Rif-R. S1-PFGE result showed that only one plasmid of approximately 136

Kb was found (Figure 1). It was confirmed that the plasmid harboring *rmtC* and bla_{NDM-1} genes were successfully transferred into recipient *E. coli* EC600Rif-R by conjugation experiment.

Multilocus Sequence Typing (MLST)

MLST result showed that *K. aerogenes* 1564 belonged to a novel ST (ST192). This new ST was arranged by the MLST database (Bacterial Isolate Genome Sequence Database) for ST number assignment. (https://pubmlst.org/kaerogenes/).

Analysis of Genetic Characteristics of p1564

p1564 is a circular molecule 136, 902 bp in length with an average G+C content of 51.8%, harbored 164 predicted ORFs and belonged to plasmid incompatibility

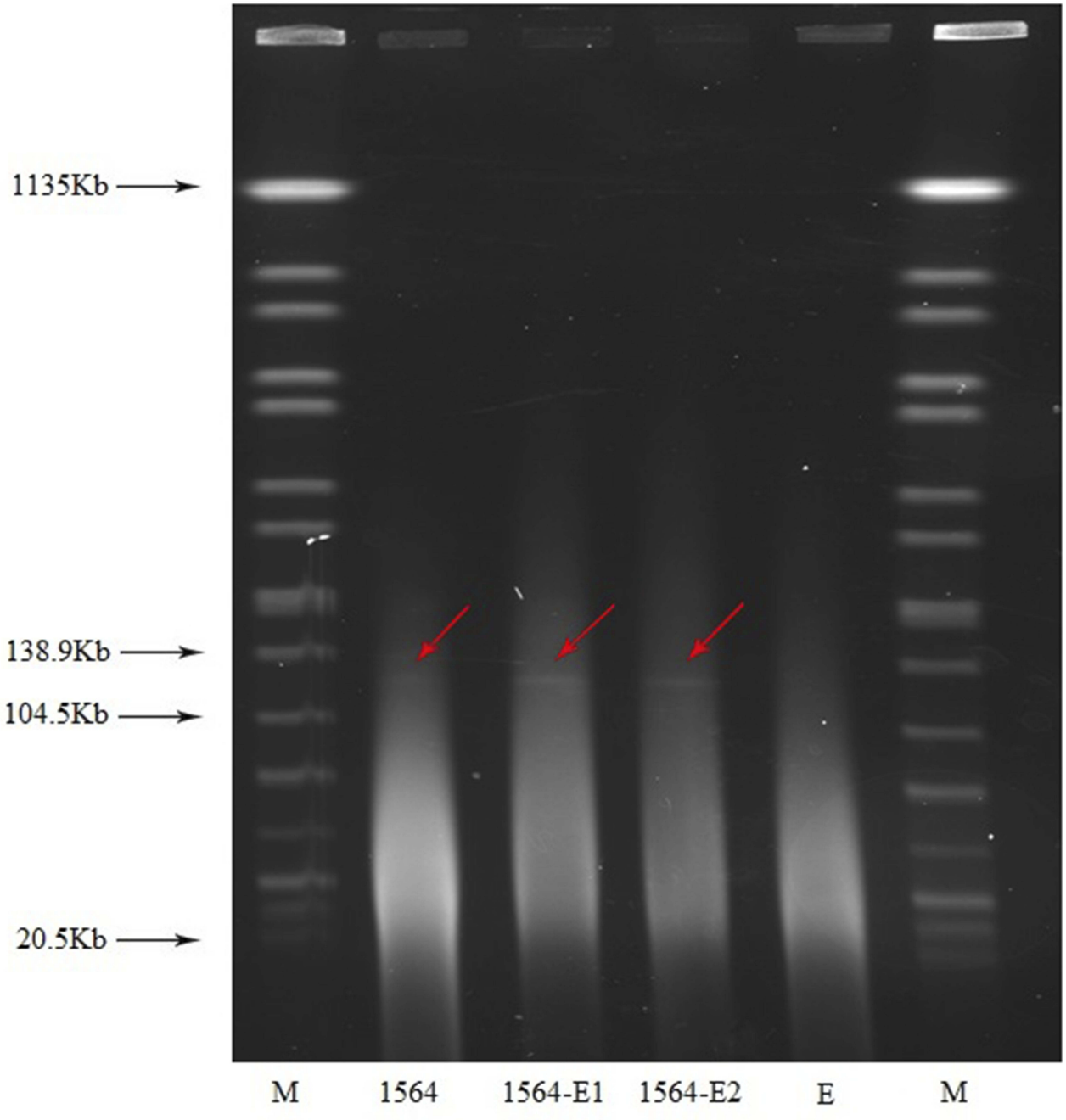

Figure 1 S1-nuclease pulsed-field gel electrophoresis profiles. M, *Salmonella enterica* serotype Braenderup strain H9812; E, *E. coli* EC600; 1564-E1, transconjugant1; 1564-E2, transconjugant2.

group A/C (Figure 2). Plasmid p1564 carries genes for plasmid replication (IncA/C repA), antibiotic resistance (*bla*$_{NDM-1}$, *rmtC, aacA4, ble*$_{MBL}$, *bla*$_{CMY-6}$ and *sul1*) and conjugation (*tra* clusters) (Figure 3). Two multidrug resistance gene islands were found on the plasmid p1564. The first was the IS*Ecp1-bla*$_{CMY-6}$ transposable unit containing the CMY-6β-lactamase gene *(bla*$_{CMY-6}$) and quaternary ammonium compound resistance gene (*sugE*). The other is the *intI1*-IS*CR21* module containing six resistance genes including two aminoglycoside resistance genes (*rmtC* and *aacA4*), truncated quaternary ammonium compound resistance genes (*qacEΔ1*), sulfonamide resistance gene (*sul1*), carbapenem resistance gene (*bla*$_{NDM-1}$) and bleomycin resistance gene (*ble*$_{MBL}$). Interestingly, the truncated fragments of the insertion sequences IS*Aba125* and IS*Ecp1* were found in the surrounding environment of*bla*$_{NDM-1}$ and *rmtC* genes, respectively, which promoted the expression and transposition of *bla*$_{NDM-1}$ and *rmtC* (Figure 3). The molecular chaperones *groEL* and *groES* genes and *rhs* gene were identified. The phage *integrase-rhs* regions were considered to be a hot spot for the integration of accessory genes in the IncA/C plasmid.[22] The Class 1 integron of p1564 is composed of integral gene *intl1* and the antibiotic resistance markers *aacA4, qacEΔ1* and *sul1*. A class 1 integron with a different gene cassettes arrangement was also found in pHS36-NDM from *Salmonella enterica* subsp. *enterica* serovar Stanley in China,[23] which is composed of *intl1, dfrA12, aadA2, qacEΔ1* and *sul1*. In addition, p1564 is highly similar to pHS36-NDM, and *bla*$_{NDM-1}$ and *rmtC* genes were identified in both plasmids.

Full plasmid sequence BLAST search against the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed an overall 99% query coverage with 100% sequence similarity to plasmid pNDM-PstGN576 from *Providencia stuartii*isolate GN576and 99% query coverage with 99. 98% sequence similarity to plasmid pNDM-US from *Klebsiella pneumoniae* strain ATCCBAA-2146.[24,25] Furthermore, pNDM-PstGN576 and pNDM-US were used as references for annotating p1564.

Discussion

Recently, 16S rRNA methyltransferases have emerged as an acquired high-level resistance mechanism to clinically relevant aminoglycosides such as gentamicin, tobramycin and amikacin.[26] Since 2003, ten 16S rRNA methyltransferase genes, including *armA, rmtA, rmtB, rmtC, rmtD, rmtE, rmtF, rmtG, rmtH* and *npmA*, have been identified in clinical isolates of Gram-negative bacilli from multiple geographic locations.[27] The genes encoding these enzymes are usually borne by mobile genetic elements and have been associated with other important mechanisms such as carbapenemases, extended-spectrum β-lactamases (ESBLs).[12] Despite having low prevalence in different types of bacteria, the 16S RMTase-encoding genes are globally spreading because of the plasmids that disseminate carbapenemase and ESBL genes among Gram-negative bacilli.[28] The recent appearance of multidrug-resistant bacteria with the 16S rRNA methyltransferases and carbapenemases such as NDM-1 is becoming an increasing clinical and public health threat.

This study revealed the complete nucleotide sequence of plasmid p1564 in *K. aerogenes*, harboring the *bla*$_{NDM-1}$ and *rmtC* genes, conferring high levels of resistance to carbapenems and aminoglycosides. Although it has been reported that coexistence of *bla*$_{NDM-1}$ and *rmtC* in *Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli* and *Salmonella enterica* subsp. *enterica* serovar Stanley, there has been no report in *K. aerogenes*.[10,29–31] In China, the *rmtC* gene has only been reported in *Salmonella enterica* subsp. *enterica* serovar Stanley.[23] To the best of our knowledge, this is the first report of co-existence in *K. aerogenes* isolate. Moreover, the MLST result showed that *K. aerogenes* 1564 belongs to a novel ST192 not reported before. High similarity of p1564 compared to *bla*$_{NDM}$-harboring plasmids pHS36-NDM from *Salmonella enterica* subsp. *enterica* serovar Stanley (KU726616), pMS6198A from *E. coli* (CP015835.1), pB577-NDM from *Enterobacter cloacae* (KX786648.1), pNDM-PstGN576 from *Providencia stuartii* (KJ802405.1) and pNDM-US from *Klebsiella pneumoniae* (CP006661.1) suggest lateral transfer of this plasmid among different members of the Enterobacteriaceae. The conserved type IV secretion system and the *stb*A gene were identified in p1564, indicating that this plasmid has the potential for horizontal transfer and stable passage, confirming the speculation obtained above. Previous studies have shown that multiple resistance transfer of plasmids can result from rare gene capture events mediated by different mobile genetic elements, clustering and combinatorial evolution of resistance genes and related mobile elements.[32] Upstream of the *bla*$_{NDM-1}$ gene, a truncated insertion sequence, IS*Aba125*, was identified, which provides a promoter for the expression of *bla*$_{NDM-1}$,[22] and indicates that the *bla*$_{NDM-1}$ gene may originally be derived from *Acinetobacter baumannii*.[33,34] Simultaneously, a truncated fragment of IS*Ecp1* was found in the downstream of *rmtC* gene, which promoted the

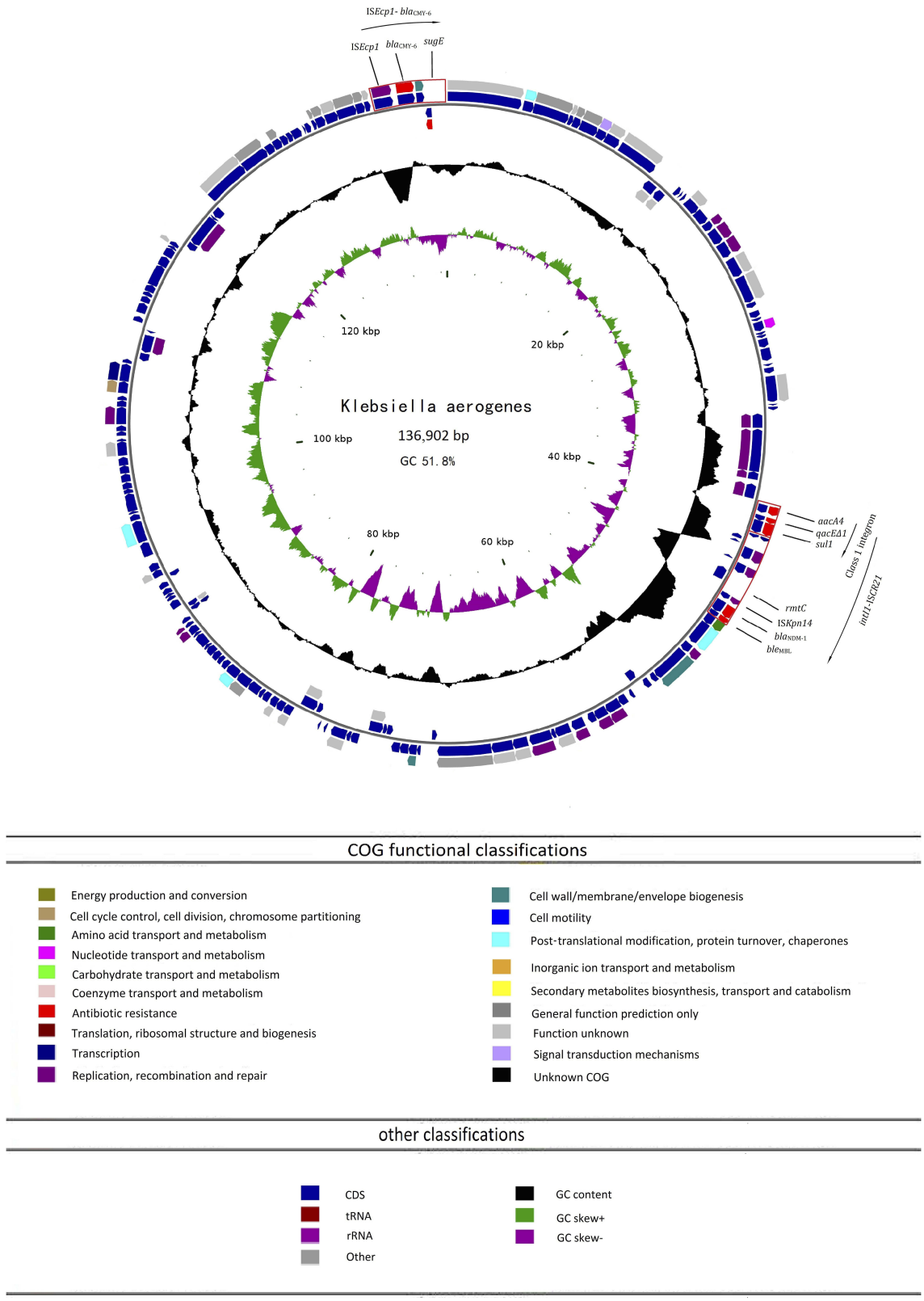

Figure 2 Ring diagram representation of plasmid p1564. From the inside to the outside, the first circle represents the scale; the second circle represents GC Skew; the third circle represents the GC content; the fourth and seventh circles represent the COG to which each CDS belongs; the fifth and sixth circles represent the CDS, tRNA, rRNA location on the plasmid. The loci for two multidrug resistance gene islands (IS*Ecp1*-bla_{CMY-6} and *intI1*-IS*CR21*) and class 1 integron are indicated in red boxes. GC, guanine + cytosine; *aacA4*, aminoglycoside resistance gene; *qacEΔ1*, truncated quaternary ammonium compound resistance gene; *sul1*, sulfonamide resistance gene; *rmtC*, 16S rRNA methyltransferase gene; bla_{NDM-1}, New Delhi Metallo-β-lactamase-1 gene; ble_{MBL}, bleomycin resistance gene; bla_{CMY-6}, CMY-6β-lactamase gene; *sugE*, quaternary ammonium compound resistance gene.

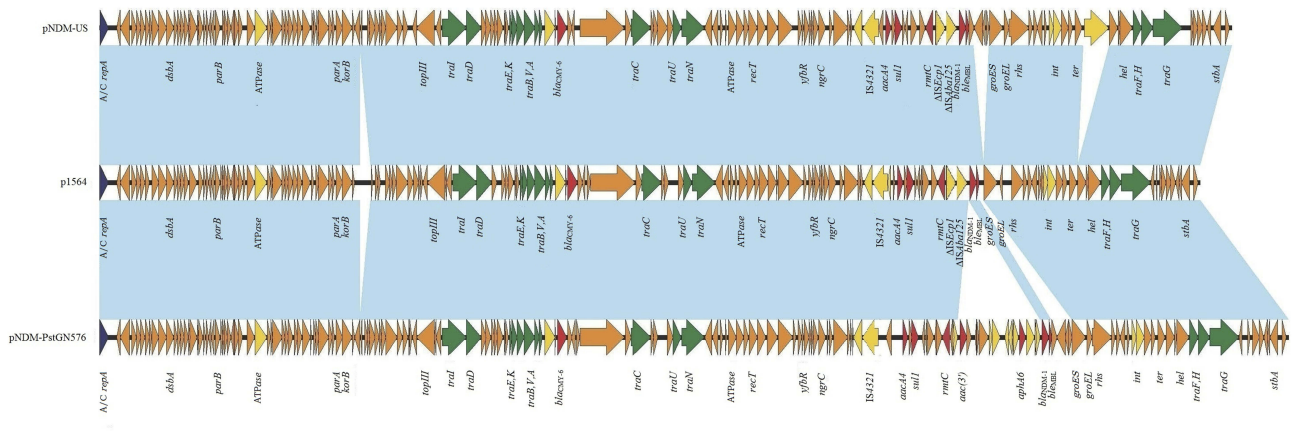

Figure 3 Comparative analysis of the bla_{NDM-1}-harboring plasmid characterized in this study with two closely related IncA/C plasmids, pNDM-PstGN576 and pNDM-US. Open reading frames (ORFs) are portrayed by arrows and are depicted in different colors based on their predicted gene functions. The genes associated with the *tra* clusters are indicated by green arrows, while the genes involved in replication are indicated by dark blue arrows. Resistance genes are indicated by red arrows and accessory genes are indicated by yellow arrows. Brown arrows represent the skeletal gene of the plasmid, and blue shading denotes shared regions of homology among different plasmids.

expression and transposition of *rmtC*.[35] The two above mentioned insertion elements (IS*Aba125* and IS*Ecp1*) may play acritical role in the widespread transmission of bla_{NDM-1} and *rmtC* genes in Enterobacteriaceae.

K. aerogenes 1564 was resistant to all beta-lactam and aminoglycoside antibiotics tested, but susceptible to ciprofloxacin, polymyxin B, tetracycline, minocycline and tigecycline. These antibiotic susceptibility results were consistent with the resistance genes carried in the two multidrug resistance gene islands IS*Ecp1*-bla_{CMY-6} and *intI1*-IS*CR21* identified in the plasmid. Comparing p1564 with other bla_{NDM-1}-bearing plasmids reported in China, it was found to be highly homologous to pHS36-NDM from *Salmonella enterica* subsp. *enterica* serovar Stanley strains, but the class 1 integron structures identified were not identical. Collectively, the variable region of these two plasmids was found to be composed of similar multiple antibiotic resistance markers and IS elements, suggesting that there may be some unknown association between the two plasmids. These results indicate that p1564 may be derived from the horizontal transfer of the harboring bla_{NDM-1} plasmid in Enterobacteriaceae in China, followed by a series of homologous recombination evolution events.

Conclusion

In conclusion, the present study demonstrated for the first time that co-existence of *rmtC* and bla_{NDM-1} in a novel ST192 *K. aerogenes*. The spread of plasmids harboring both bla_{NDM-1} and *rmtC* may occur among Enterobacteriaceae in China. Therefore, a prudent surveillance and therapeutic strategy should be implemented to restrict the widespread spread of plasmids containing bla_{NDM-1} and *rmtC*.

Nucleotide Sequence Accession Number

The complete nucleotide sequences of plasmid p1564 have been deposited in GenBank under accession no.MN603981.

Ethics Statement

As the *Klebsiella aerogenes* clinical isolate in this study was part of the routine hospital laboratory procedure, the Ethics Committee of Shanghai Pulmonary Hospital, Tongji University School of Medicine Academy of Sciences exempted this research for review.

Acknowledgments

The authors thank the excellent technical assistance provided by Liang Chen.

Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that they have no conflict of interest.

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