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NDM-1-producing Enterobacteriaceae in a teaching hospital in Shanghai, China: IncX3-type plasmids may contribute to the dissemination of bla_{NDM-1}

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SUMMARY

Objectives: To provide the epidemiological dissemination and the genetic characteristics of bla_{NDM-1} in a teaching hospital in Shanghai, China.
Methods: Here, the carbapenemase genes of 114 CRE isolates were evaluated by polymerase chain reaction (PCR). Clonal relatedness was assessed by pulsed-field gel electrophoresis (PFGE). Conjugation experiments and Southern blot hybridization were performed to determine the transferability of plasmids. Then plasmids were completely sequenced by the shotgun method.
Results: Two *Klebsiella pneumoniae* strains (RJA1227 and RJF866) and one *Raoultella planticola* strain (RJA274) were identified as NDM-1 positive. The two *K. pneumoniae* isolates belonged to ST11 and exhibited highly similar PFGE patterns. Shotgun sequencing showed that plasmid pRJF866 (ca. 110 kb) contained genes associated with the IncFII-FIB group and was highly similar to plasmid pKOX_NDM1. RJA274 (ca. 50 kb) harbored bla_{NDM-1} on an IncX3 plasmid, which was nearly identical to plasmid pNDM-HN380 except that part of the IS*Aba125* element is missing.
Conclusion: This is the first report of NDM-1-positive Enterobacteriaceae from Shanghai, China. IncX3 plasmids, reported in various species in the United Arab Emirates and China, may contribute to the dissemination of bla_{NDM-1}. More attention should be devoted to monitoring the dissemination of the NDM-1 gene due to its potential horizontal transfer via mobile genetic elements.

1. Background

The emergence of β-lactamases with direct carbapenem-hydrolyzing activity has contributed to the dissemination of carbapenem-resistant Enterobacteriaceae.[1] New Delhi metallo-β-lactamase-1 (NDM-1), belonging to Ambler class B (zinc metallo-β-lactamase), has been reported to occur in various bacterial species all over the world since it was first reported in 2009.[2] Further analysis showed that, epidemiologically, most isolates originate from the Indian subcontinent.[3] In China, occasional clonal outbreaks and reports of NDM-1 carbapenemases in Enterobacteriaceae and non-fermenting bacteria, with an unknown epidemiological origin, have been reported.[4–10] NDM-1-producing bacteria are multidrug-resistant and are only susceptible to a few antimicrobials, such as tigecycline and colistin. Moreover, bla_{NDM-1}-encoding plasmids may co-exist with multiple other resistance mechanisms, including β-lactamase genes, leaving limited therapeutic options.[11,12] However, emergence and dissemination of these multidrug-resistance determinants have become a global concern and this indicates the need for dynamic screening.

The widespread dissemination of bla_{NDM-1} is mainly due to promiscuous plasmids and clonal outbreaks. This gene has been

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detected on different transferable plasmid types (IncL/M, IncA/C, IncN2, IncFII, IncH, IncHI1, and an untypeable plasmid). The insertion sequence, IS*Aba125*, first described in NDM-1-negative *Acinetobacter baumannii* isolates, serves as the promoter sequence for the gene, and has consistently been found upstream of bla_{NDM-1}.[13,14] In addition, bla_{NDM-1} has recently been shown to be chromosomally integrated in *A. baumannii*.[15]

In the present study, we isolated three bla_{NDM-1}-positive Enterobacteriaceae strains in a tertiary teaching hospital in Shanghai, between June 2011 and June 2013. In order to gain a better understanding of the epidemiological dissemination and evolutionary origin of NDM-1 plasmids in China, we compared the genetic characteristics of bla_{NDM-1} in these three isolates and other Enterobacteriaceae.

2. Materials and methods

2.1. Bacterial strains, identification, and antimicrobial susceptibility testing

Clinically relevant carbapenem-resistant Enterobacteriaceae (CRE) isolates were collected from June 2011 to June 2013 in a tertiary teaching hospital in Shanghai, China. Admission screening was performed for NDM-1-positive inpatients with recent hospitalization and a travel history.

The VITEK2 compact system (bioMérieux, Marcy l'Etoile, France) was used for bacterial identification and disc diffusion assays (for imipenem, ertapenem, and meropenem) were used to identify carbapenem-resistance. The minimum inhibitory concentrations (MICs) of imipenem, ertapenem, meropenem, cefepime, ceftazidime, cefotaxime, ceftriaxone, piperacillin-tazobactam, ciprofloxacin, gentamicin, amikacin, tigecycline, and aztreonam of the strains were determined by using the Etest (bioMérieux, France), and results were interpreted according to the CLSI guidelines, except for that of tigecycline.[16] *Escherichia coli* ATCC 25922 was used as a quality control reference strain.

2.2. Detection and sequencing of carbapenemase genes

The presence of key carbapenemase genes (bla_{KPC}, bla_{IMP}, bla_{NDM}, bla_{VIM}, and bla_{OXA-48}) were screened for by polymerase chain reaction (PCR), using previously described primers.[3] The PCR products were purified and sequenced twice, bidirectionally, at Sangon Biotech (Shanghai, China).

2.3. Multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE)

For bla_{NDM}-positive *Klebsiella pneumoniae* isolates, MLST was performed on seven housekeeping genes according to the guidelines given on the *K. pneumoniae* MLST website (http://www.pasteur.fr/-recherche/genopole/PF8/mlst/*Kpneumoniae*.html). Chromosomal DNA was prepared in agarose blocks and digested with *Xba*I. DNA fragments were separated by PFGE on a CHEF Mapper XA (Bio-Rad, Hercules, CA, USA) for 20 h at 14 °C, at 6 V/cm, a pulse angle of 120°, and pulse times ranging from 2.16 to 54.17 s.

2.4. Conjugation experiments, plasmid analysis, S1-PFGE, and Southern hybridization

The transferability of bla_{NDM} was assessed in broth culture using *E. coli*J53 Az^r (sodium azide-resistant) as the recipient.[17] Transconjugants were selected on MacConkey agar containing sodium azide (100 μg/ml) and meropenem (0.5 μg/ml), and were confirmed to have bla_{NDM-1} by PCR analysis. Plasmid DNA of the parental and transconjugant isolates were extracted using alkaline lysis, sized according to electrophoretic profiles and S1-PFGE, and were then transferred to a positively charged nylon membrane (Roche Applied Science, Penzberg, Germany). Membranes were hybridized with a digoxigenin-labeled bla_{NDM-1}-specific probe and detected using an NBT/BCIP color detection kit (Roche Applied Sciences, Penzberg, Germany).[7] Purified transconjugant plasmid DNA was digested with *Eco*RI and *Hind*III (Takara, Dalian, China), followed by electrophoresis in 0.8% agarose gels.

2.5. Plasmid sequencing

To identify the genetic characteristics of bla_{NDM-1}, Plasmids pRJF866 and pRJA274, harboring bla_{NDM-1} in transconjugants were extracted using QIAGEN Midi Kit (Qiagen,Hilden,Germany), sequenced with sanger dideoxy method by using the ABI3730XL platform, only contigs coverage larger than 800 bp were reserved for the subsequent finishing, and the reads were assembled using Phred/Phrad/Consed program, Gaps between contigs were closed by PCR.[18] Then plasmid sequencing results were annotated with GeneMarkS and each predicted open reading frame (ORF) was further blast-searched against the National Center for Biotechnology Information database for sequence comparison and analysis.[19] A schematic comparative analyses map was constructed using manual annotation.

2.6. Nucleotide sequence accession numbers

The pRJF866 and pRJA274 nucleotide sequences reported here have been assigned to the GenBank nucleotide database under the accession numbers KF732966 and KF877335, respectively.

3. Results

3.1. Carbapenemase gene detection, patient demographics, and strain characteristics

During the study period, a total of 114 nonduplicate clinical CRE isolates, including 67 *K. pneumoniae* strains and 20 *E. coli* strains, were collected and screened for carbapenemase genes. Among these were 78 bla_{KPC-2}-positive, 15 bla_{IMP-4}-positive, two bla_{IMP-8}-positive strains, and one bla_{KPC-2} and bla_{IMP-4} double-positive CRE isolate. Only three CRE isolates were bla_{NDM-1}-positive, including two *K. pneumoniae* (RJA1227 and RJF866) strains and one *Raoultella planticola* (RJA274) strain.

K. pneumoniae RJA1227 was isolated from the wound of a 21-year-old male patient who was admitted to a burn unit for extensive burns on his face, neck, chest, and abdomen on October 20, 2011. He had received treatment in Shandong province with unknown antibiotics and had travelled to Jiangxi province 1 month prior to hospitalization in Shanghai. *K. pneumoniae* RJF866 was subsequently isolated from the blood specimen of a 40-year-old male patient receiving treatment for burns in the same unit on October 26, 2011. This second patient had no travel history and had received imipenem treatment before infected with RJF866. MLST data showed that these two *K. pneumoniae* isolates belonged to the ST11 group and that their PFGE patterns were highly similar (Figure 1a). RJA274 was collected on December 5, 2012, from the drain of a rectal tumor patient who had been hospitalized in the same hospital twice before admission to surgery and who had no travel history over the previous year.

All patients received empirical antibiotic treatment during their hospitalization, improved clinically, and were discharged from the hospital thereafter. Antimicrobial susceptibility testing showed that all strains exhibited resistance to carbapenems, cephalosporins, and β-lactam/β-lactamase inhibitor combinations, but were

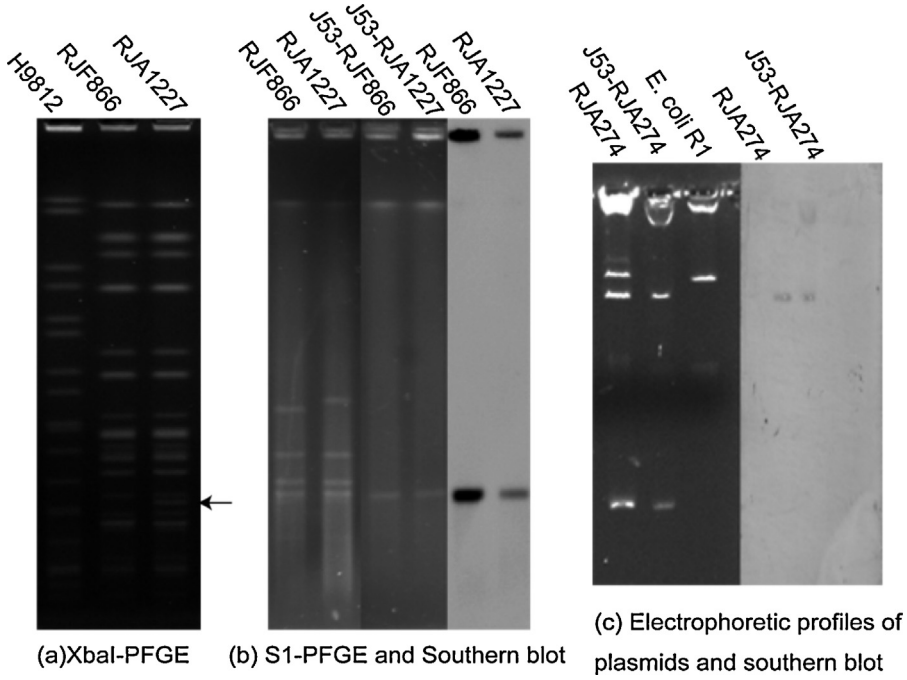

Figure 1. (a) Pulsed-field gel electrophoresis (PFGE) of *Xba*I-digested DNA of *Klebsiella pneumoniae* isolates. The arrow shows the difference between the two isolates. PFGE marker, *Salmonella enterica* H9812. **(b)** S1-PFGE of strains RJF866 and RJA1227, transconjugants J53-RJA1227 and J53-RJF866, and Southern blot hybridization of RJF866 and RJA1227with a bla_{NDM-1} probe. **(c)** Electrophoretic profiles of plasmids RJA274 and J53-RJA274, and the results of hybridization with a bla_{NDM-1}-specific probe. Marker, *Escherichia coli* R1 (ca. 90 kb).

susceptible to tigecycline (MIC = 0.38–1.0 μg/ml), as shown in Table 1.

3.2. Conjugation experiments and plasmid analysis

In conjugation experiments, the plasmids harboring bla_{NDM-1} in the three strains could all be transferred to *E. coli*J53 Az^r. PCR detection showed that all transconjugants were positive for bla_{NDM-1}. Phenotypic testing of transconjugants J53-RJA1227 and J53-RJF866 exhibited resistance to carbapenems, but were susceptible to ciprofloxacin and aztreonam; transconjugant J53-RJA274 showed susceptibility to gentamicin and amikacin, but was resistant to aztreonam (Table 1). RJA1227 and RJF866 harbored four plasmids according to S1-PFGE electrophoresis, and transconjugants J53-RJA1227 and J53-RJF866 each contained a single plasmid, the two plasmids showed identical patterns after *Eco*RI and *Hind*III digestion (data not shown). Southern hybridization analysis with a bla_{NDM-1}-specific probe revealed that bla_{NDM-1} was located on a 110-kb plasmid (Figure 1b). Plasmid analysis of the parental RJA274 and transconjugant J53-RJA274 isolates showed that they harbored four and two plasmids, respectively, and $bla_{NDM-1}$was found on the ~ 50 kb strip of transconjugant J53-RJA274, as shown in Figure 1c.

3.3. Characterization of the genetic environment of bla_{NDM-1}

Plasmid pRJF866 is 110,786 bp in size, with an average GC content of 54.8%; bla_{NDM-1} was carried on IncFII-FIB replication type plasmids, co-harboring the resistance genes *sul-1* (which encodes sulfonamide-resistant dihydropteroate synthase) and *rmtC* (which encodes 16S RNA methylase that blocks binding of aminoglycoside antibiotics). It shared 99% nucleotide identity with the published *K. oxytoca* NDM-1-encoding plasmid pKOX_NDM1 (GenBank no. **JQ314407**). The latter had been isolated from a strain obtained from a patient who underwent renal transplantation in Jangxi province, China, and who presented to a hospital in Taiwan 1 week later with abdominal pain and dysuria.[20] The backbone region of pRJF866 includes genes for conjugative transfer (*traM* to *traX*), regulation of replication (*repA1*, *repA2*, and *repA3*), partition (*parA* and *parB*), and maintenance (*ccdB* and *ccdA*) of the plasmid. Sequence alignment showed that the plasmid conjugative transfer system (nucleotide positions 18,016–51,046), which belongs to the type IV family of secretion systems, was highly similar to plasmid pG8786 of *Yersinia pestis* (GenBank no. **AJ698720**).[21] In the variable region, ISCR*3* and IS*Ehe3* insertion sequences (IS) flanked the bla_{NDM-1} composite transposon (Figure 2). It also harbored the following mobile elements: IS*26*, IS*Sen4*, IS*1*, and IS*5* in plasmid pRJF866.

Table 1
Antibiotic susceptibilities of bla_{NDM-1}-positive carbapenem-resistant Enterobacteriaceae and transconjugants. Minimal inhibitory concentrations are given in μg/ml.

Isolate	IPM	ETP	MEM	FEP	CAZ	CTX	TX	TZP	CIP	GEN	AMK	TGC	ATM
A1227	> 32	> 32	> 32	> 256	> 256	> 256	> 256	> 256	> 32	> 256	> 256	1.0	> 32
F866	> 32	> 32	> 32	> 256	> 256	> 256	> 256	> 256	> 32	> 256	> 256	1.0	> 32
A274	> 32	> 32	> 32	> 256	> 256	> 256	> 256	> 256	1.5	> 256	> 256	0.38	> 32
J53-RJA1227	> 32	> 32	> 32	96	> 256	> 256	> 256	> 256	0.012	> 256	> 256	0.125	0.064
J53-RJF866	> 32	> 32	> 32	96	> 256	> 256	> 256	> 256	0.023	> 256	> 256	0.094	0.064
J53-RJA274	> 32	> 32	32	192	> 256	> 256	> 256	> 256	0.023	0.25	2	0.125	> 32

IPM, imipenem; ETP, ertapenem; MEM, meropenem; FEP, cefepime; CAZ, ceftazidime; CTX, cefotaxime; TX, ceftriaxone; TZP, piperacillin/tazobactam; CIP, ciprofloxacin; GEN, gentamicin; AMK, amikacin; TGC, tigecycline; ATM, aztreonam.

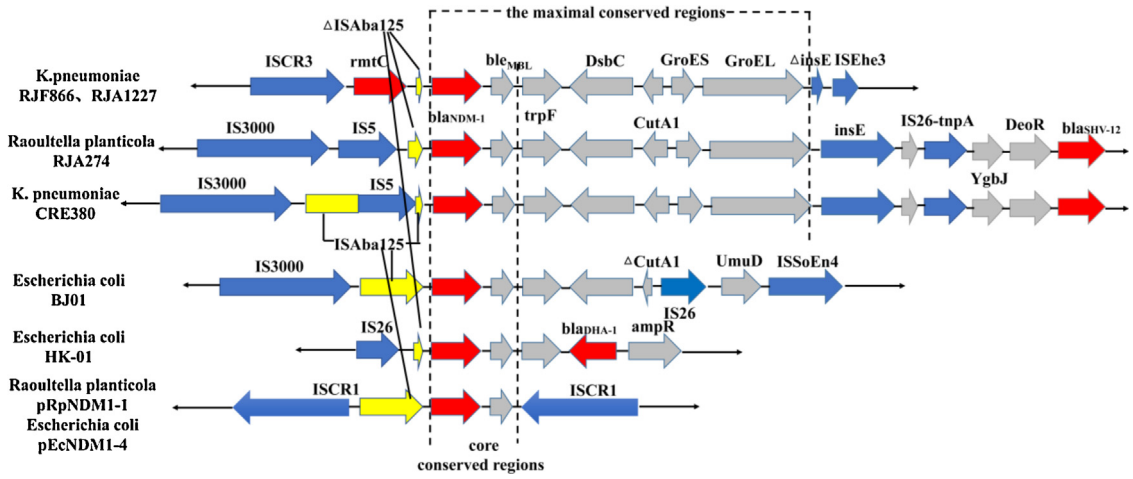

Figure 2. Schematic map of genetic structures surrounding bla_{NDM-1} identified among Enterobacteriaceae in China. Arrows indicate the predicted open reading frames with known function (gray), antimicrobial-resistance genes (red), IS*Aba125* (yellow), and other IS elements (blue). Dashed lines indicate the conserved sequences among these plasmids. *Klebsiella pneumoniae* CRE380 (GenBank no. **JX104760**), *Escherichia coli* BJ01 (GenBank no. **JX296013**), *Escherichia coli* HK-01 (GenBank no. **NC_019063**), *Raoultella planticola* pRpNDM1-1 (GenBank no. **JX515588**), *Escherichia coli* pEcNDM1-4 (GenBank no. **JX469383**).

pRJA274 is a 53,134-bp circular IncX3 plasmid that encodes a type IV secretion system protein and harbors bla_{NDM-1} and bla_{SHV-12}; it was nearly identical to the published *K. pneumoniae* NDM-1-encoding plasmid pNDM-HN380 (GenBank no. **JX104760**), except that part of the IS*Aba125* element (935-bp) between IS*3000* and IS*5*, at nucleotide position 40,753, was missing. This may constitute a recently arisen genetic variant.[22] Insertion sequences (IS*26*, truncated IS*Aba125*, and IS*5*) and transposases (IS*26-tnpA* and *tnpF*) are dispersed throughout the variable region (Figure 2). In addition, the backbone of pRJA274 markedly shared identity with *K. pneumoniae* plasmid pIncX-SHV (GenBank no. **JN247852**).

4. Discussion

Currently, the emergence of novel β-lactamases and intense selection pressure caused by inappropriate use of antibiotics have contributed to an increased prevalence of CRE worldwide. Clonal outbreaks and mobile genetic elements play vital roles in the widespread dissemination of bla_{NDM-1}.[11] In our study, clonal spread existed between the RJA1227 and RJF866 isolates, and we also found that the bla_{NDM-1} mobile genetic elements were more easily transferred to *E. coli*J53 Az^r than is bla_{KPC} (data not shown). Unlike in India, Pakistan, and the United Kingdom, where bla_{NDM-1} was mostly carried by Enterobacteriaceae species, this mobile gene was firstly and mainly detected in *Acinetobacter spp.* in China.[4,7] To the best of our knowledge, this is the first report of NDM-1-positive Enterobacteriaceae from Shanghai, China, and we comprehensive analyzed the prevalence and molecular characterization of NDM-1-producing Enterobacteriaceae in China.

Sequence types and plasmid replicon types may be related to the dissemination of resistance genes; the most frequently detected plasmid replicon type contributing to the dissemination of bla_{NDM-1} in India, Sweden, and the United Kingdom was IncA/C,[23] while we detected IncFII-FIBand IncX3-type plasmids in our study. The latter plasmids are thought to have a narrow host range and to be disseminated only among Enterobacteriaceae, and were

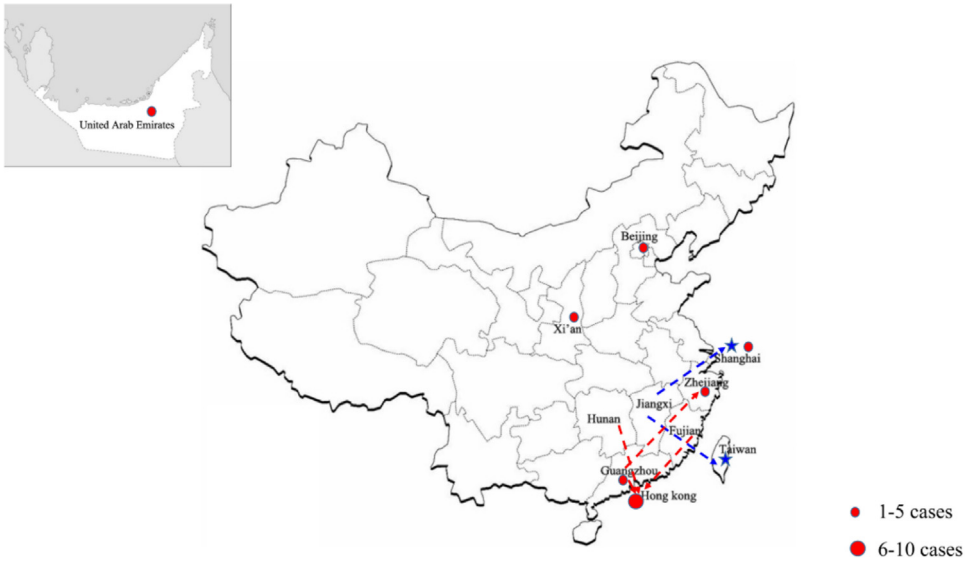

Figure 3. The distribution of NDM-1-producing Enterobacteriaceae in China. Blue stars show strains harboring the InFII-FIB plasmid; red circles denote those harboring the InX3 plasmid. The relative size of the circles corresponds to the number of reported cases in each place. IncX3 plasmids have been found to be widely distributed in various species: Beijing (*Klebsiella pneumoniae*), Xi'an (*K. pneumoniae*), Shanghai (*Raoultella planticola*), Zhejiang (*Citrobacter freundii*), Guangzhou (*Providencia rettgeri*), Hong Kong (*C. freundii*, *Enterobacter aerogenes*, *K. pneumoniae*, *Escherichia coli*, and *Enterobacter cloacae*), and the United Arab Emirates (*Enterobacter cloacae*, *Escherichia coli*, and *C. freundii*). The dashed lines show the treatment or travel history of patients.

firstly reported in Shanghai, China. Several epidemiological links were identified among NDM-1-positive Enterobacteriaceae in China. On the one hand, pRJF866 and pKOX_NDM1 were epidemiologically linked to Jiangxi province, where nine NDM-1-positive isolates were reported recently.[24] On the other hand, the incompatible IncX3 plasmid has been found to be widely distributed across various species in the United Arab Emirates and in different regions of China. Seven Enterobacteriaceae strains isolated in Hong Kong have been epidemiologically linked to Hunan and Guangdong province, one *Citrobacter freundii* strain was isolated in Zhejiang province from a patient who had received treatment in Guangzhou, and three *K. pneumoniae* strains were isolated in Xi'an (Figure 3).[8,25,26] Given that the Balkan states and the Middle East may act as secondary reservoirs for the spread of NDM-1, and the close distance or increased foreign travel and hospitalization transfer between different regions, it is possible that IncX3-type plasmids, originating from the same progenitor as the United Arab Emirates strains, could be transferred between different host species in China. It is noteworthy that the two *K. pneumoniae* isolates identified in our study, which belong to the ST11 group, the dominant clone and a frequent host of $bla_{\text{CTX-M}}$ and bla_{KPC} in China, are likely to be important carriers potentiating the spread of NDM-1.[27]

Comparison of the regions surrounding the NDM-1 gene shows that resistance genes were recruited into the variable genetic region by IS elements or transposons, while the backbone remained conserved; for instance, the truncated IS*Aba125* promoter and $bla_{\text{NDM-1}}$ composite determinant resistance genes were acquired by the pIncX-SHV plasmid. Comparison of the genetic environment of $bla_{\text{NDM-1}}$ in Enterobacteriaceae from China revealed that mobile genetic elements (IS*3000*, ISCR*3*, and IS*26*) reflect multiple modification events and play a vital role in intra- or interspecific transmission of $bla_{\text{NDM-1}}$. IS*Aba125*, found upstream of $bla_{\text{NDM-1}}$, serves as the promoter region for the gene; this was consistent with the recent assumption that $bla_{\text{NDM-1}}$ in Enterobacteriaceae may originate from *Acinetobacter spp.*[28] Although all strains harbored at least a remnant of IS*Aba125*, the length of the sequence differed (pRJF866: 103 bp; pRJA274: 174 bp; pNDM-HK: 256 bp), and IS*Aba125* was intact in *E. coli* BJ01, but was interrupted by IS*5* in pNDM-HN380. The core conserved regions contained $bla_{\text{NDM-1}}$, followed by *ble* (which mediates bleomycin resistance), while the highly conserved regions also included *trpF* (encoding the phosphoribosylanthranilate isomerase), *dsbC* (encoding oxidoreductase domain protein), *cutA1* (encoding periplasmic divalent ion tolerance protein), *groES/groEL* (encoding chaperonin), downstream of $bla_{\text{NDM-1}}$ (Figure 3). Moreover, several resistance genes (such as $bla_{\text{SHV-12}}$, $bla_{\text{DHA-1}}$) were located on the same plasmid as $bla_{\text{NDM-1}}$, which can explain the antimicrobial susceptibility profiles of transconjugant J53-RJA274.

5. Conclusions

In conclusion, the dissemination of $bla_{\text{NDM-1}}$ involves clonal spreads and mobile genetic elements; our study reported IncFII-FIB- (ca. 110 kb) and IncX3-type (ca. 50 kb) plasmids in NDM-1-positive Enterobacteriaceae strains from Shanghai, China, for the first time. IncX3 plasmids occur in various species, as reported from the United Arab Emirates and different regions of China, may contribute to the dissemination of $bla_{\text{NDM-1}}$. Co-expression of $bla_{\text{NDM-1}}$ mobile genetic elements with other resistance mechanisms will require early identification and effective infection control measures.

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