

Characterization of a *bla*_{NDM-1}-harboring plasmid from a *Salmonella enterica* clinical isolate in China

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Abstract. The plasmid-mediated transmission of antibiotic resistance genes has been reported to be involved in the development of antibiotic resistance in bacteria, and poses a serious threat for the success of bacterial infection treatment and human health worldwide. The present study used a 454 GS-FLX pyrosequencing system to determine the ~140 kb nucleotide sequence of plasmid pHS36-NDM, which was identified in a *Salmonella* Stanley isolate from the stool sample of an 11-month-old girl at Lishui Central Hospital, China, and which contains a New Delhi metallo-β-lactamase-1 (NDM-1) carbapenem resistance gene (*bla*_{NDM-1}). The 181 open reading frames encode proteins with functions including replication, stable inheritance, antibiotic resistance and mobile genetic elements. Both horizontal transfer and passage stability-related genes were identified in pHS36-NDM, including a conserved type 4 secretion system and *stbA* (stable plasmid inheritance protein A). Two multidrug resistance gene islands were identified: The *ISEcp1-bla*_{CMY} transposition unit which contains a CMY-6 β-lactamase gene (*bla*_{CMY-6}) and a quaternary ammonium compound resistance gene (*sugE*); and the *int11-ISC27* accessory region, which contained a trimethoprim resistance gene (*dfrA12*), two aminoglycoside resistance genes (*aadA2* and *rmtC*), a truncated quaternary ammonium compound resistance gene (*qacEΔ1*), a sulfonamide resistance gene (*sul1*), the *bla*_{NDM-1} carbapenemase and a bleomycin resistance gene (*ble*_{MBL}). pHS36-NDM shared high homology with other *bla*_{NDM-1}-containing plasmids reported in Sweden, Italy and Japan. However, no previous international travel history

was documented for the patient and her family, even to neighboring cities. Furthermore, pHS36-NDM is of a different incompatibility group to other published *bla*_{NDM-1}-carrying plasmids reported in China, with low homology in the surrounding structure of *bla*_{NDM-1}. The present study will facilitate the understanding of the underlying resistance and dispersal mechanism of pHS36-NDM, and will deepen our recognition of the ongoing spread of the *bla*_{NDM-1}-containing plasmids.

Introduction

The emergence of antibiotic resistance and the potential for transfer of resistance genes has raised great public concern (1). New Delhi metallo-β-lactamase-1 (NDM-1), encoded by *bla*_{NDM-1}, was originally identified in 2009 in a *Klebsiella pneumoniae* isolate from a Swedish patient transferred from India (2). NDM-1-producing bacteria, including clinical isolates of *Enterobacteriaceae* and *Acinetobacter spp.*, have since been reported across the Indian subcontinent and worldwide (2-9). NDM-1-producing bacteria are generally resistant to almost all β-lactam antibiotics, including carbapenems, which have brought great challenges to antibiotic therapy. The size of plasmids harboring *bla*_{NDM-1} varies considerably, ranging from 50 to >400 kb. In addition, the plasmids belong to different incompatibility (Inc) groups, including IncA/C, IncFI/FII, IncL/M and a non-typed group (10,11).

Our previous study identified an IncA/C plasmid, designated pHS36-NDM, that was identified in a carbapenem-resistant *Salmonella* Stanley strain isolated from the stool sample of an 11-month-old girl with community-acquired acute diarrhea, and could be transferred from *Salmonella* to *E. coli* C600 and *K. pneumoniae* (12). The plasmid was highly similar to NDM-1-carrying plasmids reported in Sweden, Italy and Japan, however the source of *bla*_{NDM-1} remains unclear due to the lack of relationship with the patient's social and travel history. In order to further understand the underlying mechanism of resistance, spread and passage stability, the present study sequenced pHS36-NDM and performed a comparison with reported NDM-1-harboring plasmids, and analyzed the phenotype-related genetic characteristics of pHS36-NDM.

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Materials and methods

Plasmid extraction and sequencing. A carbapenem-resistant *E. coli* C600 transconjugant was created by performing trans-conjugation between the *Salmonella* Stanley strain and *E. coli* C600 Rif^r, as previously described (12). pHS36-NDM plasmid DNA was purified from the carbapenem-resistant *E. coli* C600 transconjugant using a Qiagen Plasmid Midi kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Pyrosequencing was performed using the GS-FLX Titanium System (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's protocol, as previously described (13). Paired-end reads were collected at a single site and assembled using Newbler software version 2.3 (Roche Diagnostics). The assembly was further improved manually and with the aid of custom Perl scripts.

Bioinformatics analysis of pHS36-NDM DNA sequence. Open reading frames (ORFs) were predicted and annotated using the RAST server (<http://rast.nmpdr.org/>). Each predicted protein was compared with the National Centre for Biotechnology Information (NCBI) protein database using the protein basic local alignment sequence tool (BLASTP; National Institutes of Health, Bethesda, MD, USA; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>), with a minimum cutoff of 30% in identity and >80% in length coverage. Gene sequences were further compared and aligned with the GenBank database using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Proteins were assigned to clusters of orthologous groups (<http://www.ncbi.nlm.nih.gov/COG/>), and genes were initially annotated using In Silico Molecular Cloning (IMC) Genomics Edition (version 4.1.21D; In Silico Biology Inc., Yokohama, Japan). The circular representation of pHS36-NDM was generated with IMC. Linear comparative representations were based on results from Vector NTI 8 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Genetic contexts of pHS36-NDM. To investigate the homology of pHS36-NDM with previously reported NDM-1-containing plasmids, and the differences between flanking regions of the *bla*_{NDM-1} gene, pHS36-NDM was compared with the following 8 *bla*_{NDM-1}-harboring plasmids: pMR0211 from *Providencia stuartii* (GenBank accession no. JN687470.1), pNDM-1_Dok01 from *E. coli* (GenBank accession no. AP012208.1), pNDM-KN from *K. pneumoniae* (GenBank accession no. NC_019153.1), pNDM-HN380 from *K. pneumoniae* (GenBank accession no. JX104760.1), pNDM-BJ01 from *A. lwoffii* (GenBank accession no. JQ001791.1), pKpANDM-1 from *K. pneumoniae* (GenBank accession no. FN396876.1), pNDM-OM from *K. pneumoniae* (GenBank accession no. JX988621.1) and pGUE-NDM from *E. coli* (GenBank accession no. JQ364967.1). Furthermore, these plasmids were used as references for annotating pHS36-NDM.

Results

General features of plasmid pHS36-NDM. Sequencing of pHS36-NDM revealed it to be 138,001 bp in size with 52.0% guanine-cytosine content (Fig. 1). The plasmid contained 181

putative ORFs, of which 147 were on the same (plus) strand as the replication initiator gene, *repA*, and 34 were on the minus strand. BLASTN analysis revealed that the sequence of pHS36-NDM was well conserved with *E. coli* pNDM102337 (99% identity; GenBank accession no. JF714412.2), and *K. pneumoniae* pNDM10469 (99% identity; GenBank accession no. JN861072.1), indicating that pHS36-NDM may be frequently transmitted amongst virulent *Enterobacteriaceae*. Several common functional regions were predicted (Fig. 1), including: A replication region containing *repA* and plasmid-partitioning genes *parA* and *parB*, the *ISEcp1-bla*_{CMY} module region containing the type IV secretion system (T4SS) conjugative transfer genes and CMY-6 β -lactamase gene, *bla*_{CMY-6}; and a *bla*_{NDM-1}-containing transposon region flanked by a class 1 integron and insertion sequence common repeat 27 transposase (ISCR27).

The T4SS cluster and stable plasmid inheritance protein A (*stbA*) are responsible for horizontal transfer and passage stability. The transfer region of pHS36-NDM structurally belonged to a T4SS, comprising 15 transfer (*tra*) genes (*traI*, *traD*, *traL*, *traE*, *traK*, *traB*, *traV*, *traA*, *traC*, *traW*, *traU*, *traN*, *traF*, *traH* and *traG*), which were responsible for conjugation. The transfer region is conserved amongst pNDM-1_Dok01, pNDM102337, pNDM10469 and is conserved in gene order (14). The *stbA* gene identified in pHS36-NDM is necessary for the low copy number plasmid to be passed to the daughter cells (15,16).

Two multidrug resistance genes islands are responsible for the in vitro drug resistance phenotype. One or more copies of the *ISEcp1-bla*_{CMY} transposition unit genes have been reported within T4SSs in IncA/C plasmids (17,18). In pHS36-NDM, the *ISEcp1-bla*_{CMY} transposition unit contained two resistance genes, *bla*_{CMY-6} and a quaternary ammonium compound resistance gene, *sugE* (Fig. 2), flanked by *ISEcp1* transposases. Furthermore, *bla*_{CMY-6} and *sugE* of pHS36-NDM were embedded in the T4SS cluster at 60,922 bp, flanked by *traA* and a hypothetical protein 1,828 amino acids in size. This hypothetical protein had 100% identity with that found in pNDM102337, pNDM10469 and pNDM-1_Dok01, and high similarity with pMR0211; pMR0211 has 4 extra amino acids at its 5' terminus. In addition, a short sequence was identified at the 3' terminus of pHS36-NDM *bla*_{CMY-6} (ATTTCCCTA), which is almost identical to the 5' terminus (ATTTCCCTA), located adjacent to *traA*.

The second island was the *intI*-ISCR27 transposition unit (Fig. 3). Seven drug resistance genes were clustered together in a 14,802 bp accessory region bordered by *intI* [99,852-100,865 (-)] and ISCR27 [114,073-114,653 bp (+)]. The accessory region is 14,802 bp long and contains 24 genes, including genes conferring resistance to trimethoprim (*dfrA12*), aminoglycosides (*aadA2* and *rmtC*), quaternary ammonium compounds (*qacEΔ1*), sulfonamides (*sulI*), β -lactams including carbapenems (*bla*_{NDM-1}) and bleomycin (*ble*_{MBL}). One part of this accessory region, the class 1 integron, was composed of *intI* and the antibiotic resistance markers *dfrA12*, *aadA2*, *qacEΔ1* and *sulI*. Class 1 integrons have been found in several Gram-negative bacteria, such as pNDM-1_Dok01 (3).

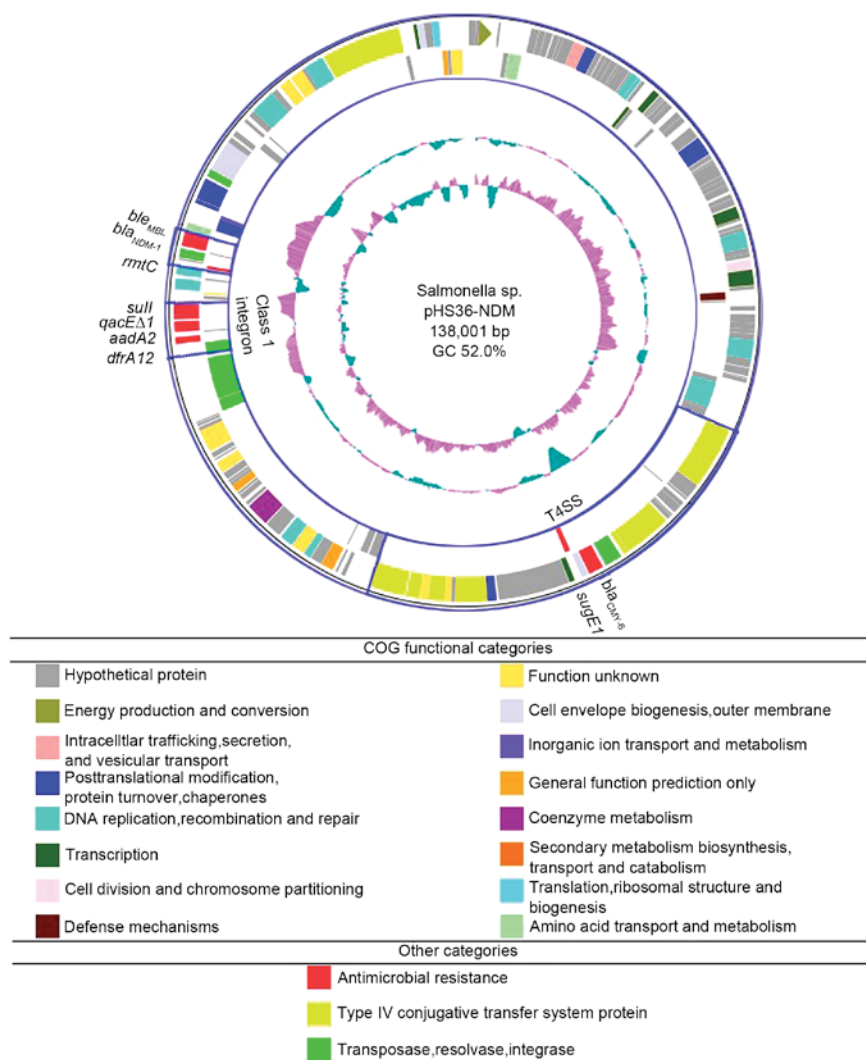


Figure 1. Circular representation of pHS36-NDM. The innermost circle represents GC skew; the second inner circle represents GC content (blue, GC+; purple, GC-). The outermost double circle depicts gene distribution relative to *repA* (represented as an arrowhead) with colored boxes depicting functional category and direction of transcription; the outer band is the plus strand; inner band is the minus strand. The loci for a T4SS operon and class 1 integron are indicated in blue boxes. NDM, New Delhi metallo- β -lactamase-1; GC, guanine + cytosine; *repA*, replication protein A; T4SS, type IV secretion system; *bla*_{CMY6}, CMY-6 β -lactamase gene; *sugE1*, quaternary ammonium compound resistance gene; *dfrA12*, trimethoprim resistance gene; *aadA2*, aminoglycoside resistance gene; *qacEΔ1*, truncated quaternary ammonium compound resistance gene; *sull*, sulfonamide resistance gene; *rmtC*, rRNA methyltransferase; *bla*_{NDM-1}, New Delhi metallo- β -lactamase-1; *ble*_{MBL}, bleomycin resistance gene.

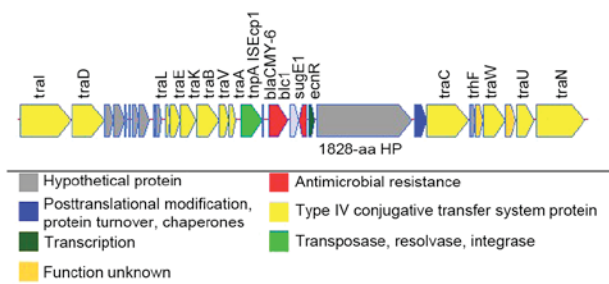


Figure 2. Schematic representation of the ISEcp1-*bla*_{CMY} transposition unit, with colored arrows depicting functional category and direction of transcription.

Sequence analysis of elements flanking *bla*_{NDM-1}. The pHS36-NDM *bla*_{NDM-1} gene was localized in a 7 kb region that was flanked by ISCR27 and the insertion sequence ISKpn14 (Fig. 4). Immediately downstream of the *bla*_{NDM-1}

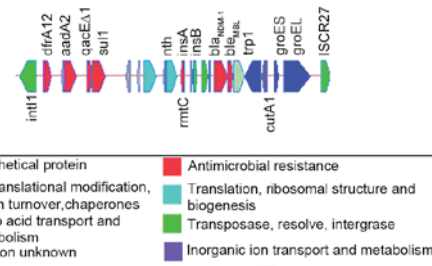


Figure 3. Schematic representation of the *intI*-ISC27 transposition unit, with colored arrows depicting functional category and direction of transcription.

gene, a *ble*_{MBL} gene was identified, encoding a putative protein conferring resistance to bleomycin. *ble*_{MBL} was followed by an N-(5'-phosphoribosyl) anthranilate isomerase (*trpF*) gene, found previously in other *bla*_{NDM-1}-bearing plasmids (Fig. 4).

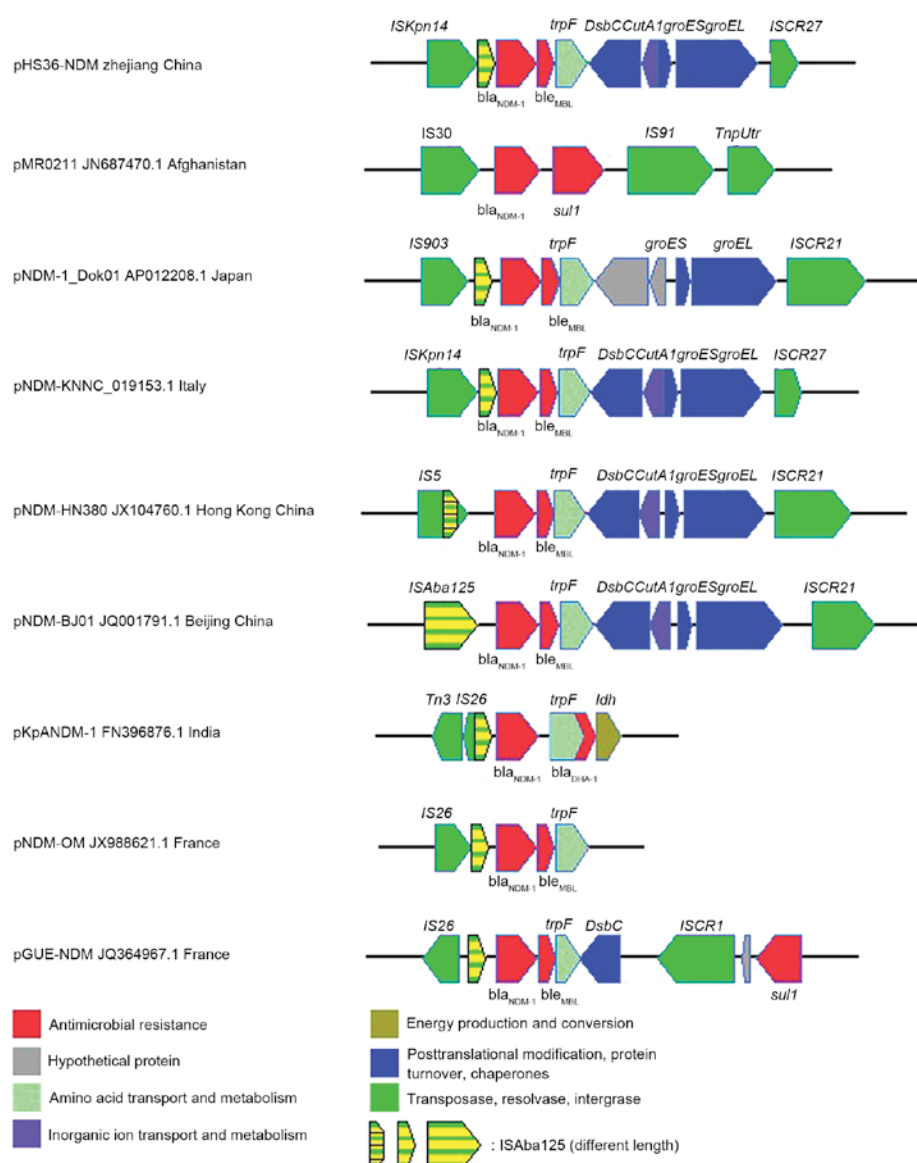


Figure 4. Comparison of the *pHS36-NDM bla*_{NDM-1} gene and its flanking elements, compared with those in *pMR0211*, *pNDM-1_Dok01*, *pNDM-KN*, *pNDM-HN380*, *pNDM-BJ01*, *pKpANDM-1*, *pNDM-OM* and *pGUE-NDM*. Comparisons performed using a nucleotide basic local alignment sequence tool search and visualized using *in silico* molecular cloning genomics edition software. The *bla*_{NDM-1} genes are identical among the aligned sequences. Annotated genes in these regions are depicted with colored arrows depicting functional category and direction of transcription. *bla*_{NDM-1}, New Delhi metallo- β -lactamase-1.

Furthermore, genes encoding a bifunctional protein-disulfide isomerase/oxidoreductase (*dsbC*) and CutA1 periplasmic divalent cation tolerance protein (*cutA1*) were commonly identified in other NDM-harboring plasmids (Fig. 4). Finally, chaperonins *groES* and *groEL*, which are involved in general stress responses, together with *ISCR27*, were also present in the regions flanking *bla*_{NDM-1} (Fig. 4). Although in many plasmids, the heat shock chaperone *groEL-groES* cluster is adjacent to an *rhs* gene, which belongs to the retrotransposon hot spot family that are known as hotspots for integration (19), no such *rhs* gene insertion was found in this region of *pHS36-NDM*.

Notably, a truncated *ISAbal25* insertion sequence adjacent to the *ISKpn14* insertion sequence was identified immediately upstream of the *bla*_{NDM-1} gene (Fig. 4). This truncated *ISAbal25* contained a 235-nucleotide promoter sequence, which drives *bla*_{NDM-1} gene expression. A truncated *ISAbal25* of varying

sizes containing this specific promoter was identified in almost all *bla*_{NDM-1}-containing plasmids (Fig. 4). Furthermore, the *ble*_{MBL} gene, followed by *trpF*, was observed to be consistently adjacent to the 3' end of *bla*_{NDM-1}. These results suggest that the *bla*_{NDM-1} gene may originally have been linked to *ISAbal25*.

Discussion

Salmonella is an important foodborne pathogen that it frequently causes infection and worldwide outbreaks (20). Although *Salmonella* is increasingly resistant to cephalosporins and quinolones, resistance to carbapenems is rare. The present study revealed the complete sequence of plasmid *pHS36-NDM*, which harbors a *bla*_{NDM-1} gene that was observed to confer carbapenem resistance to a *Salmonella* Stanley isolated from a child in China.

pHS36-NDM presented a well-conserved plasmid structure with *E. coli* pNDM102337, *K. pneumoniae* pNDM10469, *E. coli* pNDM10505 (GenBank accession no. JF503991.1) and *K. pneumoniae* pNDM-KN, indicating that the plasmid may frequently be transmitted amongst virulent *Enterobacteriaceae*. The presence of T4SS genes and *stbA* indicated the capability of horizontal transfer and passage stability of the pHS36-NDM plasmid. Previous studies have demonstrated that the T4SS is a double walled transmembrane structure, which macromolecular nanomachines utilize for the transport of proteins or DNA across the bacterial cell envelope of Gram-negative bacteria (21,22). The transfer of pHS36-NDM to daughter cells may be dependent on the presence of the *stbA* gene (16). Furthermore, the plasmid harbored several mobile genetic elements including *ISEcp1*, insertion sequence *IS4321*, transposon *Tn1696*, a *class 1 integron*, *ISKpn14*, *ISAb125* and *ISCR27*, which increased the plasticity of the plasmid. This feature of pHS36-NDM may contribute to its marked genetic stability in the donor and the transconjugant strains, and to the high conjugation frequency that has previously been reported between different types of bacteria (12). Further analysis indicated that, in the *ISEcp1-bla_{CMY}* transposition unit, the *bla_{CMY-6}* 3'ATTTCCTTA tandem repeat sequence and the 5'downstream sequence of *traA* are conserved across all IncA/C NDM-harboring plasmids investigated, such as pNDM-1_Dok01, pNDM102337 and pNDM10469, indicating that the short flanking repeat sequence is probably a transposon insertion site conserved in the IncA/C plasmid backbone.

Our previous study demonstrated that the *Salmonella* strain containing pHS36-NDM was resistant to all β -lactam antibiotics, including cephalosporins and carbapenems, but susceptible to chloramphenicol, ciprofloxacin, tetracycline, fosfomycin, and azithromycin (12). These antibiotic susceptibility results were consistent with the resistance genes identified in the two transposition units, *ISEcp1-bla_{CMY}* and *int11-ISC27*. pHS36-NDM was highly conserved with pNDM-KN from *K. pneumoniae* in Italy, pNDM10469 from *K. pneumoniae* and pNDM102337 from *E. coli* in Japan, however, the affected patient and her family had not traveled to any countries within the year, including 14 countries with a high prevalence of NDM-1 producers. Furthermore, the patient had been living in a small rural village in southern China without a special diet (12). Although it is important to investigate the origin and transmission of *bla_{NDM-1}*, there was no evidence to suggest that pHS36-NDM originated from Italy or Japan. Comparing pHS36-NDM with other *bla_{NDM-1}*-bearing plasmids reported in China, including pKPN5047 (GenBank accession no. KC311431.1) and pNDM-HN380 from *K. pneumoniae*, pNDM-BJ01 from *A. lwoffii* and pYE315203 (GenBank accession no. JX254913.2) from *C. freundii*, revealed that these plasmids belonged to a different Inc type and shared low homology in the surrounding structure of *bla_{NDM-1}*. These results implied that it is unlikely that the gene cluster in pHS36-NDM originated from other previously discovered *bla_{NDM-1}*-carrying plasmids in China. It is more likely that this occurred due to independent evolutionary events, resulting from the abuse of antibiotics on a global scale. Toleman *et al* (23) proposed two possible routes of *bla_{NDM-1}* construction, which involve a deletion event and a rolling-circle replication event. According to the present study,

it is postulated that *bla_{NDM-1}* and the surrounding region in pHS36-NDM may have been formed via multiple recombination events by genetic elements of different origins.

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