IS26 Is Responsible for the Evolution and Transmission of $\text{bla}_{\text{NDM}}$-Harboring Plasmids in $\text{Escherichia coli}$ of Poultry Origin in China

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ABSTRACT Carbenem-resistant Enterobacteriaceae are some of the most important pathogens responsible for nosocomial infections, which can be challenging to treat. The $\text{bla}_{\text{NDM}}$ carbapenemase genes, which are expressed by New Delhi metallo-$\beta$-lactamase (NDM)-producing $\text{Escherichia coli}$ isolates, have been found in humans, environmental samples, and multiple other sources worldwide. Importantly, these genes have also been found in farm animals, which are considered an NDM reservoir and an important source of human infections. However, the dynamic evolution of $\text{bla}_{\text{NDM}}$ genetic contexts and $\text{bla}_{\text{NDM}}$-harboring plasmids has not been directly observed, making it difficult to assess the extent of horizontal dissemination of the $\text{bla}_{\text{NDM}}$ gene. In this study, we detected $\text{NDM-1} (n = 1)$, $\text{NDM-5} (n = 24)$, and $\text{NDM-9} (n = 8)$ variants expressed by $\text{E. coli}$ strains isolated from poultry in China from 2016 to 2017. By analyzing the immediate genetic environment of the $\text{bla}_{\text{NDM}}$ genes, we found that IS26 was associated with multiple types of $\text{bla}_{\text{NDM}}$ multidrug resistance regions, and we identified various IS26-derived circular intermediates. Importantly, in $\text{E. coli}$ strain GD33, we propose that IncHI2 and IncI1 plasmids can fuse when IS26 is present. Our analysis of the IS26 elements flanking $\text{bla}_{\text{NDM}}$ allowed us to propose an important role for IS26 elements in the evolution of multidrug-resistant regions (MRRs) and in the dissemination of $\text{bla}_{\text{NDM}}$. To the best of our knowledge, this is the first description of the dynamic evolution of $\text{bla}_{\text{NDM}}$ genetic contexts and $\text{bla}_{\text{NDM}}$-harboring plasmids. These findings could help proactively limit the transmission of these NDM-producing isolates from food animals to humans.

IMPORTANCE Carbenem resistance in members of the order Enterobacteriales is a growing public health problem that is associated with high mortality in developing and industrialized countries. Moreover, in the field of veterinary medicine, the occurrence of New Delhi metallo-$\beta$-lactamase-producing $\text{Escherichia coli}$ isolates in animals, especially food-producing animals, has become a growing concern in recent years. The wide dissemination of $\text{bla}_{\text{NDM}}$ is closely related to mobile genetic elements (MGEs) and plasmids. Although previous analyses have explored the association of many different MGEs with mobilization of $\text{bla}_{\text{NDM}}$, little is known about the evolution of various genetic contexts of $\text{bla}_{\text{NDM}}$ in $\text{E. coli}$. Here, we report the important role of IS26 in forming multiple types of $\text{bla}_{\text{NDM}}$ multidrug resistance cassettes and the dynamic recombination of plasmids bearing $\text{bla}_{\text{NDM}}$. These results suggest that significant attention should be paid to monitoring the transmission and further evolution of $\text{bla}_{\text{NDM}}$-harboring plasmids among $\text{E. coli}$ strains of food animal origin.

KEYWORDS $\text{bla}_{\text{NDM}}$, IS26, circular intermediate, recombination, evolution, plasmid, Escherichia coli

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Infection with carbapenemase-producing Enterobacteriaceae (CPE) strains is associated with high mortality rates because therapeutic options are limited. New Delhi metallo-\(\beta\)-lactamases (NDMs), a class of carbapenemases that hydrolyze virtually all \(\beta\)-lactams, are one of the most important resistance traits in *Escherichia coli* (1). Since *bla\(\text{NDM-1}\)* was initially identified in *Klebsiella pneumoniae* in 2009, *bla\(\text{NDM}\)* genes have been reported to be abundant worldwide, and the frequency of these genes in clinical isolates in Asia is increasing at an alarming rate (2–4). Although the rapid spread of NDM-encoding genes has gained global attention (5, 6), to date no study has focused on the dynamic evolution of the *bla\(\text{NDM}\)* genetic context.

The dissemination of NDM-1 mainly involves plasmids rather than clonal spread. In *E. coli* isolates, *bla\(\text{NDM}\)* has been identified on plasmids with a narrow (IncFIB and IncFII) or broad (IncX3, IncA/C, IncH, IncL/M, and IncN) host range (7, 8). Moreover, mobile genetic elements (MGEs) can facilitate the spread of *bla\(\text{NDM}\)* genes, and the similarity of the *bla\(\text{NDM}\)* flanking sequences in these plasmids suggests that horizontal mobilization of *bla\(\text{NDM}\)* via MGEs is responsible for its evolution and rapid transmission between plasmids and chromosomes (8–10). Furthermore, insertion sequences, such as IS\(\text{Ab}125\), IS\(\text{3000}\), IS\(\text{26}\), IS\(\text{55}\), IS\(\text{CR}\), Tn\(\text{3}\), Tn\(\text{125}\), Tn\(\text{3000}\), and Tn\(\text{1548}\), seem to play an important role in the dissemination of NDM-encoding genes (8, 11–13). Most of the *bla\(\text{NDM}\)* sequences available in GenBank are flanked upstream by a complete or truncated copy of IS\(\text{Ab}125\) and downstream by the *bleMBL* gene. Many different MGEs have been found bracketing these genes that could potentially mobilize them (14).

Despite the fact that carbapenems are rarely used in animals (9, 10, 15), NDM-producing *E. coli* strains have been isolated from food animals in Asia, suggesting that food animals are another important reservoir of NDM-producing strains (16–18). Farmed poultry are a particularly common source of NDM-positive *E. coli*, raising the possibility of food chain transmission, which further highlights the importance of controlling the spread of NDMS. Therefore, to assess the mechanism by which the *bla\(\text{NDM}\)* gene is transferred among *E. coli* strains isolated from poultry, the genomes of *bla\(\text{NDM}\)*-positive *E. coli* isolates from chickens and ducks were analyzed to determine how MGEs contribute to the generation of *bla\(\text{NDM}\)*-harboring plasmids. We found various IS\(\text{26}\)-flanked pseudo-compound transposons (PCTs) (19) and, furthermore, fusion and resolution plasmids that had formed when IS\(\text{26}\) is present in both IncHI2 and IncI1 plasmids. Our analysis allowed us to propose the contribution to evolution of MRRs and dissemination of *bla\(\text{NDM}\)* when mobile element IS\(\text{26}\) is present.

**RESULTS**

**NDM-producing *E. coli* isolates.** A total of 470 *E. coli* isolates were obtained from 470 samples collected from six poultry farms in Guangdong Province (four chicken farms and one duck farm) and Shandong Province (one chicken farm). In total, 33 (7.02%) of these *E. coli* isolates carried *bla\(\text{NDM}\)* genes (Fig. 1; see also Table S1 in the supplemental material). The prevalence of *bla\(\text{NDM}\)*-positive *E. coli* isolates in Shandong and Guangdong Provinces was 25.71% (9/35) and 5.52% (24/435), respectively. The *bla\(\text{NDM}\)*-positive *E. coli* strains were isolated from both chickens (5.34%, 21/393) and ducks (15.58%, 12/77). NDM-5 (72.7%, 24/33) was the predominant variant, followed by NDM-1 (3.0%, 1/33) (Fig. 1). All 33 of the *bla\(\text{NDM}\)*-positive *E. coli* isolates showed multidrug resistance (MDR), as determined by testing against a panel of 20 antimicrobial agents (Table S1). The 33 *bla\(\text{NDM}\)*-positive *E. coli* strains were grouped into 19 clusters based on XbaI pulsed-field gel electrophoresis (PFGE) sequence similarity (with a threshold of 85% similarity), suggesting that most of the strains were epidemiologically unrelated (Fig. 1). Each cluster carried the same NDM variant, while cluster H contained isolates GD11 and GD18, which appeared to be clonally related strains that were from different hosts (chicken and duck). Taken together, both horizontal transmission and clonal dissemination were responsible for the distribution of the *bla\(\text{NDM}\)* gene.

**Analysis of the localization of *bla\(\text{NDM}\)*.** To determine the location of *bla\(\text{NDM}\)* genes, we performed conjugation/transformation, PCR-based replicon typing (PBRT), S1-PFGE,
and Southern hybridization. There were four types of replicons carrying the \( {\text{bla}}_{\text{NDM}} \) gene, namely IncX3, IncB/O/K/Z, IncHI2, and IncY. \( {\text{bla}}_{\text{NDM-5}} \) was located on the IncX3 (23/33; 46-kb) and IncHI2 (1/33; 256-kb) plasmids, \( {\text{bla}}_{\text{NDM-9}} \) was located on the IncHI2 (4/33; 260-kb) and IncB/O/K/Z (4/33; 105 to 115-kb) plasmids, and \( {\text{bla}}_{\text{NDM-1}} \) was located on the IncY (1/33; 120-kb) plasmid (Fig. 1).

**Characteristics of \( {\text{bla}}_{\text{NDM-9}} \)-carrying IncB/O/K/Z plasmids.** Out of the four \( {\text{bla}}_{\text{NDM-9}} \)-carrying IncB/O/K/Z plasmids that we isolated from *E. coli* strains, we sequenced two, pNDM-T2 (105-kb) and pNDM-T6 (115-kb). The complete sequence of pNDM-T2 is a 106,300-bp circular molecule with a GC content of 54% and was predicted to harbor 215 open reading frames (ORFs). The plasmid of pNDM-T6 was 115,219 bp with a GC content of 55% and was predicted to harbor 240 ORFs. The plasmid backbones of both pNDM-T2 and pNDM-T6 were highly similar (99% coverage and 100% identity) to that of the \( {\text{bla}}_{\text{NDM-9}} \)-carrying IncB/O/K/Z plasmid pHNTH02-1 (GenBank accession number MG196294) isolated from chicken meat in China (see Fig. S1 in the supplemental material).

In pNDM-T6, the MRR bounded by \( \text{Tn1721} \) and \( \text{ΔTn5393} \) was interspersed with a number of different resistance genes, including \( \text{fosA3, dfrA12, aadA2, sul1, ble}_{\text{MBL}}, \text{mph} \) (A), and \( \text{mer} \), as well as several mobile elements, including IS26, ISCR1, \( \text{ΔISAb125}, \text{ΔIS5075}, \text{IS6100} \), and \( \text{ΔTn2} \). The \( {\text{bla}}_{\text{NDM-9}} \) gene was embedded in a 27.5-kb IS\( \text{CR1} \) complex class 1 integron (Fig. 2a). This region was bracketed by two IS26 elements in the same orientation. Similar structures have also been reported in *E. coli* plasmid pHNTH02-1 (GenBank accession number MG196294) and *Salmonella* plasmid pC629 (GenBank accession number CP015725) (20).

The pNDM-T6 hypervariable region in this MRR consisted of four IS26 elements flanking the following three different segments: (i) IS26-\( \text{int1-dfrA12-aadA2-qacED1-sul1-ISCR1-ΔISAb125-Δdct-tat-trpF-ble}_{\text{MBL}}-{\text{bla}}_{\text{NDM-9}}-\text{IS26} \), (ii) IS26-\( \text{merR-merT-merP-merC-merA-merD-merE-urf2-tmiA-IS26} \), and (iii) IS26-\( \text{mph(A)-mrx-mphR(A)-IS6100-IS26} \) (Fig. 2c).
FIG 2 Genomic and molecular analyses of the blaNDM-5-positive IncB/O/K/Z plasmids and the blaNDM-5-positive IncHI2 plasmids. (a) Genetic features of blaNDM-5 on IncB/O/K/Z. (b) Genetic features of blaNDM-5 on IncHI2. (c) Schematic

(Continued on next page)
In pNDM-T2, the MRR consisted of three IS26 elements flanking two different segments, as follows: (iv) IS26-hp-hp-fosA3-IS26 and (v) IS26-int1-dhA12-aadA2-qacED1-sul1-iscR1-adct-tat-tpf-blalmb-laNDM-5-IS26 (Fig. 2d). This MRR shared 100% identity with the corresponding segment in the pSCRE51-NDM9 (GenBank accession number CP021177) plasmid from an *E. coli* strain isolated from human urine (21).

To test and verify circular intermediates formed, reverse PCR using the primers shown in Table S2 in the supplemental material was performed with pNDM-T2 and pNDM-T6 as the template DNA. Five circular intermediates of the four IS26-flanked PCTs were identified (Fig. 2c and d). Sequence analysis showed that each circular intermediate contained only one copy of IS26, which suggests how IS26 facilitates construction of circular intermediates. These findings indicated that IS26-flanked PCTs are dynamic and that the circular intermediate could readily be excised from the plasmid, thereby facilitating its transposition into other plasmids, confirming how IS26 mediates transposition of antibiotic resistance genes (ARGs).

**Characteristics of IncX3 plasmids carrying blaNDM-5**. Out of the 23 *blaNDM-5*-harboring IncX3 plasmids (46 kb) isolated from *E. coli* strains, we randomly selected pNDM-T16 from GD16 for Illumina sequencing. The complete sequence of pNDM-T16 is a 46,161-bp circular molecule with a GC content of 47% and was predicted to harbor 100 open reading frames (ORFs). BLAST homology analysis showed that pNDM-T16 was highly homologous (100% coverage and 99.99% identity) to the *blaNDM-5*-harboring IncX3 plasmid pNDM5_IncX3 (GenBank accession number KU761328) from a *Klebsiella pneumoniae* clinical isolate from China (22).Taken together, these findings suggest that similar IncX3 plasmids were most likely responsible for spreading *blaNDM-5* in Enterobacteriaceae.

**Fusion of plasmid pNDM33-1 and pNDM33-2**. To determine why *blaNDM-5* was located on the 265-kb IncHI2 plasmid in parental strain GD33 but was located on a 366-kb plasmid in the transconjugant TJ33 when conjugated with the recipient strain *E. coli* J53, we performed whole-genome sequencing of GD33 and TJ33. Sequence analysis revealed that there were four plasmids of different sizes (266,777 bp, 113,068 bp, 90,896 bp, and 79,203 bp) in GD33, but only one plasmid (366,267 bp) in the transconjugant TJ33 (Fig. 3a). The pNDM33-1 plasmid was 266,777 bp with a GC content of 47.07%, predicted to harbor 100 open reading frames (ORFs). BLAST homology analysis showed that pNDM33-1 was highly homologous (100% coverage and 99.99% identity) to the *blaNDM-5*-harboring IncX3 plasmid pNDM5_IncX3 (GenBank accession number CP033636; 99% coverage and 99.98% identity), which was isolated from an *E. coli* strain isolated from chicken in China. On pNDM33-1, the 13,918-bp Tn3000 transposon unit (IS3000-ΔISaba125-IS5-ΔISaba125-blaNDM-5-phl-tpf-tat-dct-IS26-umuD-ISL3-IS3000) inserted between IS1 family transposon and IS4-like element genes was flanked by 5-bp direct repeats (DRs) (ACTTT), suggesting insertion of the Tn3000 transposon unit (see Fig. S2 in the supplemental material). This transposon unit was highly similar to that reported in the IncX3 plasmid pNDM5_IncX3 (GenBank accession number KU761328) isolated from a clinical *E. coli* strain (23), except for absence of IS3000 downstream of *blaNDM-5* (Fig. 2b). A similar Tn3000 transposon unit harboring *blaNDM-5* in an IncX3 plasmid pEc2A (GenBank accession number KR822247) was recently reported in a clinical *E. coli* strain from Brazil (13). This transposon unit was also identified in an IncX3 plasmid, pNDM-HN380 (GenBank accession number NC_019162) from *Klebsiella pneumoniae* of human origin in China. One circular form (F) was detected by reverse PCR and Sanger sequencing (see Table S2 in the supplemental material). This circular form contained only one copy of IS3000, which suggests where the circular form excised from the plasmid of pNDM33-1 (Fig. 2E). pNDM33-2 was
113,068 bp in size, had a GC content of 50%, was predicted to harbor 157 ORFs, belonged to the IncI1 incompatibility group, and carried \(\text{erm}(B)\), \(\text{mph}(A)\), \(\text{flr}\), and \(\text{aadA2}\) resistance genes. BLAST analysis showed that this type of plasmid has previously been reported in \(E.\) \(\text{coli}\) and \(\text{Salmonella}\); pS68 (GenBank accession number KU130396; 93% coverage and 99.78% identity) was isolated from an \(E.\) \(\text{coli}\) strain in China, and pUY_STM96 (GenBank accession number MN241905; 86% coverage and 99.9% identity) was isolated from a clinical \(\text{Salmonella enterica}\) subsp. \(\text{enterica}\) serovar Typhimurium strain in Uruguay.

The largest plasmid, pNDM-TJ33, that was obtained from transconjugant TJ33 was 366,267 bp in size with 485 ORFs, belonged to the IncI1 and IncHI2 plasmid families, and had a GC content of 47.67% (see Fig. S3 in the supplemental material). Detailed sequence analysis of these three plasmids (pNDM33-1, pNDM33-2, and pNDM-TJ33) enabled us to predict the possible mechanism of plasmid fusion. The IncHI2 and IncI1
plasmids both contain the same IS26, streptomycin 3'-adenylyltransferase, and truncated intI1 gene sequences (Fig. 3b). We named the fusion sites between pNDM33-1 and pNDM33-2 site A1 and site A2. The fusion plasmid, pNDM-TJ33, exhibits a significantly broadened resistance profile, covering several additional classes of antibiotics, including aminoglycosides, beta-lactams, quinolones, tetracyclines, and macrolides (see Table S3 in the supplemental material).

**Resolution of plasmid pNDM-TJ33.** To test whether the fusion plasmid could be mobilized, a conjugation assay was performed with TJ33 as the donor strain and streptomycin-resistant E. coli C600 as the recipient strain. This yielded a blaNDM-carrying plasmid, pNDM-TD33 (141,890 bp), which was IncI1 type, had a GC content of 50.32%, and was predicted to harbor 193 ORFs. Alignment of pNDM-TD33 with pNDM-TJ33 showed that pNDM-TD33 was derived from pNDM-TJ33, and pNDM-TD33 was excised at this homologous sequence, IS26 (named site B), as shown in Fig. 3b. The new plasmid, pNDM-TD33, belonged to the IncI1 replicon group and contained most of pNDM33-2, as well as two IS26 elements flanking the blaNDM-carrying fragment from pNDM33-1 (Fig. 3b).

Significantly, pNDM-TD33 seems to have obtained the carbapenem-resistance gene blaNDM from pNDM33-1 (Table S3).

**Dynamic balance of plasmid fusion and resolution.** A recently reported example shows that IS26 actively remodels resistance plasmids via both inter- and intramolecular replicative transposition (24). To determine whether the same mechanism was active among pNDM33-1, pNDM33-2, and pNDM-TJ33, we performed a conjugation assay using GD33 and TJ33 as the donor strains and azide-resistant E. coli J53 and streptomycin-resistant E. coli C600 as the recipient strains. A total of 120 carbapenem-resistant transconjugants from each conjugation were randomly selected and screened for the presence of sites A1, A2, and B using the primers shown in Table S2 (Fig. 3b). We also assessed the presence or absence of blaNDM as well that of as the IncHI2 and IncI1 replicons. Interestingly, 38 (31.7%) of the GD33 transconjugants (TJ33) contained all of the targets except for site B, 30 (25%) only carried blaNDM and the IncHI2 replicon, and 52 (43.3%) were negative for site A1, A2, or both (Fig. 3c). Twelve (10%) of the 120 TJ33 transconjugants (TD33) were positive for site B, blaNDM, and the IncI1 replicon, and 105 (87.5%) transconjugants were positive for sites A1 and A2, blaNDM, and the IncHI2 and IncI1 replicons. These results suggest that new plasmid formation could occur in the parent strains and that there was a dynamic balance of recombination in the host bacteria.

**Plasmid stability and fitness.** To explore the transmissibility of blaNDM-carrying plasmids, the conjugation frequency of blaNDM-carrying plasmids was assessed. The conjugation frequency of pNDM33-1 (recipient strain E. coli J53) and pNDM-TJ33 (recipient strain E. coli C600) at 37°C were 1.00 × 10^{-3} to 2.61 × 10^{-3} and 1.79 × 10^{-3} to 4.09 × 10^{-3}, respectively, while the conjugation frequency of pNDM-TD33 (recipient strain E. coli J53) was 1.09 × 10^{-2} to 1.74 × 10^{-2}. Additionally, stability experiments performed in the absence of antibiotics demonstrated that pNDM-TD33 was much more stable than pNDM33-1 and pNDM-TJ33 (see Fig. S4a in the supplemental material). Strains carrying pNDM33-1, pNDM-TJ33, or pNDM-TD33 had fitness values that ranged between 0.81 and 1.44 in the absence of antibiotic pressure. pNDM-TD33 had a fitness value greater than 1, representing a significant increase in E. coli fitness (Fig. S4b).

**DISCUSSION**

In this study, we investigated the dynamic evolution of the blaNDM genetic context in E. coli isolates from farmed poultry in China. Our results revealed that the genetic context of blaNDM contains multiple IS26-flanked PCTs and that a dynamic balance of fusion and resolution of plasmids containing blaNDM occurs when IS26 is present. Taken together, our findings suggest that IS26 contributes to formation of blaNDM multidrug resistance cassettes and actively remodels antibiotic resistance plasmids.

IS26, which is frequently associated with genes encoding antibiotic resistance factors, has been reported to flank PCTs (24, 25). A dynamic MRR containing various copy numbers of IS26-blaCTX-M-65 and IS26-fosA3 and corresponding circle intermediates has
been reported in the *E. coli* plasmid IncZ-7 (25). In our study, MRRs containing multiple IS26-flanked PCTs and various IS26-derived circular intermediates were detected in *bla*<sub>NDM</sub>-carrying IncB/O/K/Z plasmids. Harmer and Hall recently described the mobility of IS26 in detail and demonstrated that replicative transposition driven by this element generates circular molecules containing one copy of IS26 and an adjacent DNA segment carrying an antibiotic resistance (or other) gene, which they designated a "translocatable unit" (TU) (26). However, some reports suggest that a TU cannot be formed by replicative transposition when tandem arrays of a resistance gene are present with an IS26 between them (27) or when structures share an IS26 between two compound transposons (28). Furthermore, although in the absence of an active homologous recombination system, excision of TU was demonstrated in the context of Tn4352B when it was adjacent to the two G residues at the left end of the IS26; in other cases, this does not occur (29), and generation of a TU from a preexisting transposon would necessarily occur via homologous recombination (26). RecA-dependent simple homologous recombination from a IS26-based PCT could lead to excision for forming the TU (30, 31). In our study, the absence of GG at the left end of the IS26 and target site duplication (TDS) flanking IS26 suggest that the circular intermediates were most likely generated from preexisting tandem arrays of IS26-associated modules on MRR via homologous recombination in the *recA* -carrying (*recA*<sup>1</sup>) *E. coli* strain J53. Importantly, the different TU intermediates observed in a single strain in our study may reflect a dynamic process involving the insertion and deletion of a transposable unit by homologous recombination.

Fusion between plasmids during recombination or cointegration occurs frequently, further extending the resistance profiles of pathogens and broadening the host spectrum of the fusion plasmid (7). In this study, sequence analysis of pNDM33-1, pNDM33-2, and pNDM-TJ33 enabled us to shed light on IS26 mobility via homologous recombination. The sites on pNDM-TJ33 (A1 and A2), which was formed by fusion of the IncHI2 and IncI1 plasmids, suggest that the presence of homologous sequences IS26, streptomycin 3'-adenylyltransferase, and a truncated *IntI1* gene in both the IncI1 and IncHI2 plasmids could facilitate plasmid fusion (Fig. 3b). Based on analysis of site B on pNDM-TD33, we speculate that the MRR from pNDM-TJ33 transferred to pNDM33-2 during an intramolecular homologous recombination event, forming a new plasmid (pNDM-TD33) (Fig. 3b). It seems likely that the presence of homologous IS26 sequences may facilitate the formation of various plasmid types and accelerate the evolution of antibiotic resistance plasmids.

Intermolecular transposition can help plasmids acquire new genes, while intramolecular recombination accompanied by deletion can streamline resistance gene clusters by removing redundant or metabolically costly genes (24) and/or by creating new hybrid promoters to adjust the expression of remaining genes (32, 33). It has been reported that, if IS26 transposition remodels plasmid-borne genes involved in conjugal transfer and plasmid stability, the plasmid may then need to acquire new mechanisms to rectify these effects, resulting in new biological characteristics (24). In our study, we found that pNDM-TD33 had greater stability and a significant increase in fitness compared with pNDM33-1 and pNDM-TJ33, indicating that it is more capable of being transferred by conjugation and persisting within the host bacterium. The plasmid evolution process described in our study provides an example of how novel *bla*<sub>NDM</sub>-harboring plasmids with characteristics that help them spread widely can emerge.

In conclusion, we describe the generation of different MRRs by IS26-flanked PCTs that also give rise to various circular intermediates, thus providing important insight into contribution of IS26 to MRRs on plasmids which carry *bla*<sub>NDM</sub> and consequently into the rapid dissemination of NDM among *Enterobacteriaceae*. Furthermore, our study provides direct evidence of plasmid evolution and highlights the importance of homologous recombination in the evolution and diversity of carbapenem resistance plasmids when IS26 is present. While fusion between conjugative plasmids has been
reported previously, our work is the first to demonstrate the dynamic process by which IS26 facilitates fusion of \( bla_{\text{NDM-5}} \)-harboring plasmids. These data could be used proactively to assist the poultry industry in China in developing food safety measures designed to limit the transmission of these NDM-carrying isolates by food animals.

**MATERIALS AND METHODS**

**Strains and antimicrobial susceptibility testing.** From August 2016 to December 2017, a total of 470 nonduplicate samples were collected from five chicken and one duck farms in two provinces of China (Fig. 1; see also Table S1 in the supplemental material). Briefly, fecal samples were collected from randomly selected ducks \((n = 77)\) and chickens \((n = 393)\). Swabs of feces were inoculated into sterile selenite cystine broth and incubated for 24 h at 37°C, after which the culture was streaked to chromogenic medium selective for \( E. coli \) (CHROMagar Microbiology, France) and incubated for another 24 h at 37°C. One blue colony was selected from each plate and confirmed to be \( E. coli \) by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). The carbapenem-resistant isolates were screened for the \( bla_{\text{NDM}} \) gene by PCR using previously reported primers (34) and by sequencing (35).

The MICs of antibiotics were determined using the agar dilution method (36), and the results were interpreted following Clinical and Laboratory Standards Institution (CLSI) guidelines (M100-S25) (37) and veterinary CLSI guidelines (43). Mobile elements were identified using gradient temperature PCR, and amplicon identities were confirmed by Sanger sequencing.

**Transferability of \( bla_{\text{NDM}} \).** Conjugal transfers were performed using sodium azide-resistant \( E. coli \) J53 as the recipient (39). Transconjugants were selected on MacConkey agar containing 150 mg/liter sodium azide and 0.5 mg/liter meropenem. For isolates where no transconjugants were obtained, an alternative \( E. coli \) (DH5a) was used as a recipient for transformation experiments (40), and transformants were selected for on Luria-Bertani (LB) agar containing 0.5 mg/liter meropenem. The presence of \( bla_{\text{NDM}} \) in transconjugants/transformants was further confirmed by PCR and sequencing.

**Plasmids and genetic context of \( bla_{\text{NDM}} \).** The replicon types for all transconjugants and transformants with \( bla_{\text{NDM}} \)-carrying plasmids were determined by PCR-based replicon typing (PBRT) as previously described (41). All transconjugants and transformants were subjected to S1-PFGE and Southern blotting (42) using digoxigenin-labeled probes specific for the \( bla_{\text{NDM}} \) gene.

Plasmid DNA was purified using a Qiagen plasmid midi kit (Qiagen, Germany). The predominant plasmid was completely sequenced using Illumina HiSeq technology. Sequence reads were assembled into contigs using SOAPdenovo v.2.04. Whole-genome sequencing of strain GD33 was performed using an Illumina platform and a PacBio Sequel single-molecule real-time (SMRT) sequencing platform (Novogene, Beijing). De novo PacBio Sequel read assemblies were generated using SMRT Link v.5.0.1, and the sequences were optimized by Illumina reads using Burrows-Wheeler Aligner (BWA) v.0.7.8. Whole-genome sequencing of strains TJ33 and TD33 was performed using an Oxford Nanopore GridION platform and an Illumina platform (NextOmins, Wuhan, China). The Nanopore reads were assembled using Canu 1.7.11, and the sequences were optimized by Illumina reads using BWA V0.7.17 and Pilon v1.22.

The plasmid sequences were annotated using RAST (http://rast.nmpdr.org/rast.cgi) and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (43). ARGs and plasmid replicon types were identified using ResFinder (https://cge .cbs.dtu.dk/services/ResFinder/) and PlasmidFinder (https://cge .cbs.dtu.dk/services/PlasmidFinder/), respectively. Mobile elements were identified using ISfinder (https://www.is.biotoul.fr/). Specific primers used for further analysis of these plasmids were designed using Primer Premier v.5.0 (see Table S2 in the supplemental material). Target sequences were identified using gradient temperature PCR, and amplicon identities were confirmed by Sanger sequencing.

**Plasmid stability and fitness.** Two transconjugants (TJ33 and TD33) were chosen for competition and plasmid stability experiments, as previously described (44, 45). These strains were incubated for 8 days for stability assays and for 12 days for competition assays. Luria-Bertani agar plates containing meropenem \((1 \mu g/ml)\) were used for the stability experiment, and Luria-Bertani agar plates containing cefotaxime \((1 \mu g/ml)\) were used for the competition experiment. Plasmid stability was assessed by the percentage of strains that retained plasmids, and the fitness of the strains was assessed by measuring the relative fitness cost \((W)\), as follows: a \( W > 1 \) means the plasmid-harboring strains were more fit, while a \( W < 1 \) means the host strains used as a negative control were more fit.

**Data availability.** The sequencing data of the plasmids have been deposited in GenBank under the following accession numbers: MN353919 (pNDM-T2), MN335921 (pNDM-T6), MN335922 (pNDM-T16), MN915011 (pNDM3-1), MN915012 (pNDM3-2), CP076650 (pNDM3-3), CP076649 (pNDM3-4), CP076646 (GD33 chromosome), MN915010 (pNDM-T33), and MN915013 (pNDM-TD33).

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**FIG S1**, DOCX file, 0.1 MB.
FIG S2, DOCX file, 0.1 MB.
FIG S3, DOCX file, 0.3 MB.
FIG S4, DOCX file, 0.1 MB.
TABLE S1, DOCX file, 0.04 MB.
TABLE S2, DOCX file, 0.02 MB.
TABLE S3, DOCX file, 0.02 MB.

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We have no conflicts of interest to declare.

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