Dissemination of the $bla_{NDM-5}$ Gene via IncX3-Type Plasmid among Enterobacteriaceae in Children

Dongxing Tian, Bingjie Wang, Hong Zhang, Fen Pan, Chun Wang, Yingying Shi, Yan Sun

Department of Clinical Laboratory, Shanghai Children’s Hospital, Shanghai Jiaotong University, Shanghai, China

ABSTRACT

The continuous emergence of novel New Delhi metallo-$\beta$-lactamase-5 (NDM-5)-producing Enterobacteriaceae isolates is receiving more and more public attention. Twenty-two NDM-5-producing strains were identified from 146 carbapenemase-producing Enterobacteriaceae (CRE) strains isolated from pediatric patients between January and March 2017, indicating that the $bla_{NDM-5}$ gene has spread to children. All 22 isolates, including 16 Klebsiella pneumoniae strains, four Klebsiella aerogenes strains, and two Escherichia coli strains, showed significantly high resistance to $\beta$-lactam antibiotics (except aztreonam) but remained susceptible to tigecycline and colistin. K. pneumoniae and K. aerogenes strains were respectively defined as homologous clonal isolates by pulsed-field gel electrophoresis (PFGE). Multi-locus sequence typing (MLST) results confirmed the genetic relatedness with all K. pneumoniae strains belonging to sequence type (ST) 48. Two E. coli isolates (ST617 and ST1236) were considered genetically unrelated. Twenty-two $bla_{NDM-5}$ plasmids were positive for the IncX3 amplicon and showed almost identical profiles after digestion with HindIII and EcoRI. Four representative strains (K. pneumoniae K725, K. aerogenes CR33, E. coli Z214, and E. coli Z244) were selected for further study. Plasmids harboring $bla_{NDM-5}$ showed strong stability in both clinical isolates and transconjugants, without apparent plasmid loss after 100 serial generations. S1-PFGE followed by Southern blot analysis demonstrated that the $bla_{NDM-5}$ gene was located on an ~46-kb plasmid. Plasmid sequences of pNDM-K725, pNDM-CR33, and pNDM-Z214 were almost identical but were slightly different from that of pNDM-Z244. Compared with pNDM-Z244, ΔISAba125 and partial copies of IS3000 were missing. The genetic backgrounds of the $bla_{NDM-5}$ gene in four strains were slightly different from that of the typical pNDM-MGR194. This study comprehensively characterized the horizontal gene transfer of the $bla_{NDM-5}$ gene among different Enterobacteriaceae isolates in pediatric patients, and the IncX3-type plasmid was responsible for the spread.

IMPORTANCE

The emergence of CRE strains resistant to multiple antibiotics is considered a substantial threat to human health. Therefore, all the efforts to provide a detailed molecular transmission mechanism of specific drug resistance can contribute positively to prevent the further spread of multidrug-resistant bacteria. Although the new superbug harboring $bla_{NDM-5}$ has been reported in many countries, it was mostly identified among E. coli strains, and the gene transfer mechanism has not been fully recognized and studied. In this work, we identified 22 $bla_{NDM-5}$-positive strains in different species of Enterobacteriaceae, including 16 Klebsiella pneumoniae strains, four Klebsiella aerogenes strains, and two Escherichia coli strains, which indicated the horizontal gene transfer of $bla_{NDM-5}$ among Enterobacteriaceae strains in pediatric patients. Moreover, $bla_{NDM-5}$ was located on a 46-kb IncX3 plasmid, which is possibly responsible for this widespread horizontal gene transfer. The different genetic contexts of the $bla_{NDM-5}$ gene indicated some minor evolutions of the plasmid, based on the complete sequences of the $bla_{NDM-5}$ plasmids. These findings are of great significance to understand the transmission mechanism of drug resistance.
genes, develop anti-infection treatment, and take effective infection control measures.

**KEYWORDS** NDM-5, *Enterobacteriaceae*, ST48, IncX3-type plasmid, carbapenemase, children, *Enterobacteriales*

Carbapenemase-producing *Enterobacteriaceae* (CRE) have become a serious challenge to clinical therapy owing to the rapid worldwide dissemination of multidrug resistance (MDR) (1). New Delhi metallo-β-lactamase (NDM) is the main carbapenemase detected in children (2), which is capable of hydrolyzing almost all β-lactams and has the potential to cause a global health crisis. Since the first report of NDM-1, 21 variants of NDM enzymes (NDM-1 to NDM-21) have been identified worldwide (3).

New Delhi metallo-β-lactamase-5 (NDM-5) was first identified in a multidrug-resistant *Escherichia coli* ST648 isolate in the United Kingdom in 2011 (4). Since then, NDM-5 has been reported all over the world, including in Egypt (5), South Korea (6), China (7), the United States (8), Italy (9), and Spain (10). However, NDM-5 has mainly been identified in *E. coli* and a few other *Enterobacteriaceae* isolates (11). The NDM-5 enzyme differs from NDM-1 by only two amino acid substitutions (Val88Leu and Met154Leu) and shows increased resistance to carbapenems and broad-spectrum cephalosporins (4). It is a concern that *bla*<sub>NDM-5</sub> was detected in not only clinical specimens but also animals (12, 13) and environmental samples (14), indicating its potential to spread further in the community. The *bla*<sub>NDM-5</sub> gene was reported to be carried in different incompatibility typing plasmids to transfer genes such as IncFII, IncX3, IncN, and IncF (15). A fusion plasmid (IncX3 and IncFIB) bearing *bla*<sub>NDM-5</sub> in *E. coli* was also identified (16, 17). These plasmids can facilitate the spread of *bla*<sub>NDM-5</sub> in *Enterobacteriaceae* through horizontal gene transfer.

In this study, we screened NDM-5-producing *Enterobacteriaceae* strains in pediatric patients to elucidate the dissemination mechanism and provided the complete sequence of IncX3 plasmids to confirm the horizontal gene transfer of *bla*<sub>NDM-5</sub> among *Enterobacteriaceae*. In addition, to the best of our knowledge, this is the first time that clonal dissemination of NDM-5-producing ST48 *Klebsiella pneumoniae* and *Klebsiella aerogenes* has been reported in children.

**RESULTS**

**Bacterial strains and antimicrobial susceptibility testing.** Among 146 CRE isolates, 22 *bla*<sub>NDM-5</sub>-positive *Enterobacteriaceae* isolates were identified, including 16 *K. pneumoniae*, four *K. aerogenes*, and two *E. coli* isolates. The distributions of other carbapenemase genes are shown in Table S1 in the supplemental material and not discussed in this study. The *bla*<sub>NDM-5</sub>-positive isolates were all recovered from patients on the neonatal intensive care unit (NICU) or pediatric intensive care unit (PICU) wards and were mainly collected from blood and sputum samples. All isolates showed high resistance to β-lactam antibiotics and inhibitors (except aztreonam), including imipenem, meropenem, ertapenem, cefotaxime, cefepime, ceftazidime, cefmetazole, piperacillin-tazobactam, and ceftazidime-avibactam. Most strains showed resistance to sulfamethoxazole-trimethoprim but always remained susceptible to tigecycline and colistin; most were susceptible to amikacin, gentamicin, ciprofloxacin, levofloxacin, and aztreonam (Table 1).

**Genetic relatedness.** Multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) experiments were performed to analyze the clonal relatedness of NDM-5-producing *Enterobacteriaceae* isolates because *bla*<sub>NDM-5</sub>-positive isolates are not common in children. According to the MLST results, 16 *K. pneumoniae* isolates belonged to the same type, ST48, and two *E. coli* isolates belonged to ST617 and ST1236, respectively (Fig. 1). Sequence typing of *K. aerogenes* isolates was not performed because it has not been well established for this organism. In accordance with the MLST results, PFGE patterns confirmed the close genetic relatedness of 16 *K. pneumoniae*
isolation, and four *K. aerogenes* isolates also had similar PFGE profiles (Fig. 1). Two *E. coli* isolates had different PFGE patterns (Fig. 1).

**Characterization of the blaNDM-5 gene.** The plasmids carrying the blaNDM-5 gene of 22 *Enterobacteriaceae* isolates were successfully transferred into recipient *E. coli* J53 with a conjugation rate of $\sim 10^{-3}$ per recipient strain. Compared to *E. coli* J53, the transconjugants exhibited significantly increased resistance to carbapenems (Table S2). Twenty-two blaNDM-5 plasmids were positive for the IncX3 amplicon and negative for other plasmid types. Plasmids digested with EcoRI showed the same profiles, but the

### Table 1: Antimicrobial susceptibility of NDM-5-producing *Enterobacteriaceae* isolates

| Isolate | Species          | MIC (µg/ml) of drug |
|---------|------------------|---------------------|
|         |                  | ETP | IPM | MEM | AMK | GEN | SXT | LVX | CIP | CTX | FEP | CAZ | CMZ | TZP | CSL | ZCA | ATM | TGC | COL |
| K24     | *K. pneumoniae*  | 256 | 128 | 256 | 1   | 0.5 | >256/4,864 | 2   | 2   | >32 | >32 | >64 | >256 | >128 | >128 | >128 | >128 | <0.125 | 0.25 |
| K32     | *K. pneumoniae*  | 256 | 128 | 256 | 1   | 0.25 | >256/4,864 | 0.5 | 1   | >32 | >32 | >64 | >256 | >128 | >128 | >128 | >128 | <0.125 | 0.25 |
| K158    | *K. pneumoniae*  | 128 | 64  | 256 | 1   | 0.25 | >256/4,864 | 0.5 | 1   | >32 | >32 | >64 | >256 | >128 | >128 | >128 | >128 | <0.125 | 0.25 |
| K27     | *K. pneumoniae*  | 256 | 256 | 256 | 2   | 0.5 | >256/4,864 | 0.5 | 1   | >32 | >32 | >64 | >256 | >128 | >128 | >128 | >128 | <0.125 | 0.25 |
| K176    | *K. pneumoniae*  | 256 | 256 | 256 | 1   | 0.5 | >256/4,864 | 0.5 | 1   | >32 | >32 | >64 | >256 | >128 | >128 | >128 | >128 | <0.125 | 0.25 |
| K178    | *K. pneumoniae*  | 256 | 128 | 256 | 2   | 0.25 | >256/4,864 | 1   | 1   | >32 | >32 | >64 | >256 | >128 | >128 | >128 | >128 | <0.125 | 0.25 |
| K182    | *K. pneumoniae*  | 256 | 256 | 256 | 2   | 0.25 | >256/4,864 | 0.5 | 0.5 | >32 | >32 | >64 | >256 | >128 | >128 | >128 | >128 | <0.125 | 0.25 |
| K183    | *K. pneumoniae*  | 256 | 128 | 256 | 0.5 | ≤0.125 | >256/4,864 | 1   | 1   | >32 | >32 | >64 | >256 | >128 | >128 | >128 | >128 | 0.25 | 0.25 |
| K184    | *K. pneumoniae*  | 256 | 128 | 256 | 2   | 0.5 | >256/4,864 | 1   | 1   | >32 | >32 | >64 | >256 | >128 | >128 | >128 | >128 | 0.25 | 0.25 |
| K161    | *K. pneumoniae*  | 256 | 256 | 256 | >512 | >256/4,864 | 16  | 32  | >32 | >32 | >64 | >256 | >128 | >128 | >128 | >128 | <0.125 | 0.25 |
| K185    | *K. pneumoniae*  | 256 | 256 | 256 | 2   | 0.5 | >256/4,864 | ≤0.125 | ≤0.125 | >32 | >32 | >64 | >256 | >128 | >128 | >128 | >128 | <0.125 | 2   |
| K187    | *K. pneumoniae*  | 256 | 128 | 256 | 2   | 0.5 | >256/4,864 | 1   | 1   | >32 | >32 | >64 | >256 | >128 | >128 | >128 | >128 | <0.125 | 0.5 |
| K45     | *K. pneumoniae*  | 256 | 128 | 256 | 1   | 0.25 | >256/4,864 | 0.5 | 1   | >32 | >32 | >64 | >256 | >128 | >128 | >128 | >128 | <0.125 | 0.25 |
| K96     | *K. pneumoniae*  | >256 | 256 | 256 | 2   | 0.5 | >256/4,864 | 0.5 | 0.5 | >32 | >32 | >64 | >256 | >128 | >128 | >128 | >128 | <0.125 | 0.25 |
| K702    | *K. pneumoniae*  | 256 | 256 | 256 | 1   | 0.25 | >256/4,864 | 1   | 1   | >32 | >32 | >64 | >256 | >128 | >128 | >128 | >128 | <0.125 | 0.25 |
| K725    | *K. pneumoniae*  | 128 | 64  | 256 | 1   | 0.25 | >256/4,864 | 1   | 1   | >32 | >32 | >64 | >256 | >128 | >128 | >128 | >128 | <0.125 | 0.25 |
| Z214    | E. coli          | 16  | 16  | 32  | 2   | 0.5 | >256/4,864 | 0.5 | 0.5 | >32 | >32 | >64 | >256 | >128 | >128 | >128 | >128 | ≤0.125 | ≤0.125 |
| Z244    | E. coli          | 128 | 32  | 128 | 4   | 32  | ≤0.125/2.4 | 16  | 64  | >32 | >32 | >64 | >256 | >128 | >128 | >128 | >128 | ≤0.125 | ≤0.125 |
| CR33    | K. aerogenes     | 64  | 64  | 128 | 2   | 0.5 | 0.5/9.5 | 1   | 1   | >32 | >32 | >64 | >256 | >128 | >128 | >128 | ≥1   | ≤0.125 | ≤0.125 |
| CR39    | K. aerogenes     | 64  | 64  | 128 | 2   | 0.5 | 0.5/9.5 | 1   | 0.5 | >32 | >32 | >64 | >256 | >128 | >128 | >128 | >128 | <0.125 | 2   |
| CR50    | K. aerogenes     | 64  | 64  | 128 | 2   | 0.25 | ≤0.125/2.4 | 1   | 1   | >32 | >32 | >64 | >256 | >128 | >128 | >128 | >128 | ≤0.125 | ≤0.125 |

*Abbreviations: ETP, ertapenem; IPM, imipenem; MEM, meropenem; AMK, amikacin; GEN, gentamicin; SXT, sulfamethoxazole-trimethoprim; LVX, levofloxacin; CIP, ciprofloxacin; CTX, cefotaxime; FEP, cefepime; CAZ, ceftazidime; CMZ, cefmetazole; TZP, piperacillin-tazobactam; CSL, cefoperazone-sulbactam; CZA, ceftazidime-avibactam; ATM, aztreonam; TGC, tigecycline; COL, colistin.*

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**FIG 1** PFGE profiles and MLST results for NDM-5-producing *Enterobacteriaceae* isolates.
plasmid in Z244 isolates showed some differences when digested with HindIII (Fig. 2). Therefore, according to the restriction fragment length polymorphism (RFLP) of plasmids and genetic relatedness, K. pneumoniae K725, K. aerogenes CR33, E. coli Z214, and E. coli Z244 were selected for further study.

Plasmids harboring bla\textsubscript{NDM-5} showed strong stability in both clinical isolates and transconjugants, without apparent plasmid loss after 100 serial generations (data not shown). The results of S1-PFGE followed by Southern blot analysis demonstrated that K. pneumoniae K725 contains three plasmids (46, 70, and 320 kb), K. aerogenes CR33 contains two plasmids (46 and 70 kb), E. coli Z244 contains two plasmids (46 and 230 kb), and E. coli Z214 contains three plasmids (46, 90, and 115 kb). bla\textsubscript{NDM-5} genes are all located on plasmids of similar size (~46 kb) (Fig. 3).

Plasmid sequence and comparative analysis. The entire plasmid sequences were obtained to characterize the bla\textsubscript{NDM-5} plasmid better and enable comparative analyses in K. pneumoniae, K. aerogenes, and E. coli isolates. The plasmids of K725, CR33, Z214, and Z244 were 43,125, 43,252, 43,252, and 46,047bp in length, respectively, all belonging to the IncX3 incompatibility plasmid type. Comparative analysis showed almost identical sequences among pNDM-K725, pNDM-CR33, and pNDM-Z214 but a slight difference from pNDM-Z244 (Fig. 4A). Compared with pNDM-Z244, IS\textsubscript{Aba125} truncated by IS\textsubscript{5} was almost missing with only 73 bp remaining, and partial copies of IS\textsubscript{3000} were also deleted in pNDM-K725, pNDM-CR33, and pNDM-Z214 (Fig. 4B). The above deletions resulted in the smaller size of pNDM-K725, pNDM-CR33, and pNDM-Z214. The complete sequence of pNDM-Z244 was used as a reference to draw a circular map of the plasmids in four isolates. Using the approach of Norman and colleagues (35), pNDM-Z244 was determined to carry genes involved in replication (repB and copG), stability (taxA, cotH, parB, ftsH, topB, hns, mpr, trpF, dsbC, umuD, parA, and taxD), propagation (dnaB, virB1, virB2, virB3/virB4, virB5, virB6, virB8, virB9, virB10, virB11, virD4, and kikA), and adaptation (trpA-IS\textsubscript{3000}-IS\textsubscript{Aba125}-IS\textsubscript{5}-bla\textsubscript{NDM-5}-bla\textsubscript{MBL}-IS\textsubscript{26}-ΔumuD-IS\textsubscript{Kox3}). The plasmid harboring 67 predicted open reading frames (ORFs) contained only one resistance gene, bla\textsubscript{NDM-5} (Fig. 4A), indicating that antibiotic resistance genes in other plasmids may be responsible for the resistance to a variety of antibiotics.

The bla\textsubscript{NDM-5} gene was flanked in the upstream region by IS\textsubscript{3000}-ΔIS\textsubscript{Aba125}-IS\textsubscript{5}-ΔIS\textsubscript{Aba125} and downstream by ble\textsubscript{MBL}-trpF-dsbC-IS\textsubscript{26}-ΔumuD-IS\textsubscript{Kox3}, and this genetic background is the same as that of isolate pNDM_MGR194 in India (GenBank accession no. KF220657) (Fig. 4B) (18). Deletions of IS\textsubscript{Aba125} and IS\textsubscript{3100} in plasmids pNDM-K725, pNDM-CR33, and pNDM-Z214 suggest that additional gene deletions and rearrangements may occur in these plasmids. The bla\textsubscript{NDM-5} gene within pNDM-5-IT (GenBank

![FIG 2](msphere.asm.org) RFLP analysis of bla\textsubscript{NDM-5}-positive plasmids. (A) bla\textsubscript{NDM-5}-positive plasmids digested with EcoRI. (B) bla\textsubscript{NDM-5}-positive plasmids digested with HindIII. Lane M, 1 kb plus DNA ladder marker.
accession no. MG649062), which was detected in Italy, was located in a complex integron, bracketed by two IS26 sequences containing an ISCR1 element and a class 1 integron with the intI1 gene truncated by one of the IS26 copies and the aadA2-dfrA12 resistance gene cassettes (Fig. 4B).

**DISCUSSION**

To date, NDM-5 carbapenemase has been described mostly in *E. coli* and rarely in *K. pneumoniae* and other *Enterobacteriaceae* isolates (11). Furthermore, to the best of our knowledge, a neonatal outbreak of NDM-5-producing *Klebsiella quasipneumoniae* in Nigeria was recently reported, but other clonal dissemination of *bla*NDM-5 was very rarely found in children (19). In this study, we reported the dissemination of *bla*NDM-5 among different species of *Enterobacteriaceae* in children, including *E. coli*, *K. pneumoniae*, and *K. aerogenes*. Although NDM-5-producing strains are not as widespread as NDM-1-producing strains, they can also accompany multiple resistance gene determinants of resistance to different antimicrobials in the same strain, which makes them a potential public health threat. Furthermore, *bla*NDM-5 can occasionally occur simultaneously with *bla*OXA-181 (20–22), but *bla*OXA-181 was not found in our study. The NDM-5-producing strains described in our study showed high resistance to all β-lactams and inhibitors. Furthermore, most of them remained susceptible to aminoglycosides and fluoroquinolones, a finding which is not consistent with NDM producers usually also being resistant to aminoglycosides because they frequently harbor 16S rRNA methylases, such as armA and rmtB (23–25). The rare clinical usage of these drugs in children owing to their side effects may be the reason for the susceptibility to aminoglycosides and fluoroquinolones observed in our study. Fortunately, strains resistant to tigecycline and colistin were not found.
FIG 4  Sequence analysis of \textit{bla}_{NDM-5}-Positive plasmids. (A) Comparative analysis of pNDM-K725, pNDM-CR33, pNDM-Z214, and pNDM-Z244. The circular map was created by BRIG tools. Concentric rings represent the similarity between the reference sequence (pNDM-Z244) in the outer ring and other sequences in the inner rings. Color levels indicate the results of BLAST with a matched degree in the shared regions. Genes shown in purple, blue, green, (Continued on next page)
**K. pneumoniae** is one of the most important pathogens threatening children’s health. The emergence of the **bla**

\(\text{NDM-5}\) gene in **K. pneumoniae** increased the difficulty of clinical treatment of this pathogen. Sixteen **K. pneumoniae** isolates carrying **bla**

\(\text{NDM-5}\) in our study belonged to the same sequence type, ST48, and had similar PFGE profiles, strongly indicating that clonal dissemination of **K. pneumoniae** carrying **bla**

\(\text{NDM-5}\) had occurred in our hospital. To our knowledge, **bla**

\(\text{NDM-5}\)-positive **K. pneumoniae** isolates from clinical samples have been identified in ST2250 in China (7), ST2266 in New Zealand (26), ST147 in the United States (21), and ST231 in Singapore (22) and in untypeable isolates in India (18). This is very possibly the first report in the world of ST48 carbapenem-resistant **K. pneumoniae** carrying the **bla**

\(\text{NDM-5}\) gene.

Significantly, the **bla**

\(\text{NDM-5}\) gene was also found in two **E. coli** strains and four **K. aerogenes** strains. Four **K. aerogenes** strains with identical PFGE profiles were possibly caused by clonal dissemination, while two distantly related **E. coli** strains (ST617 and ST1236) may have acquired the **bla**

\(\text{NDM-5}\) gene by horizontal transfer. Previous studies suggested that the **bla**

\(\text{NDM-5}\) gene has been most frequently detected in **E. coli** of many sequence types, with the most common being ST167 (11), whereas **E. coli** carrying the **bla**

\(\text{NDM-5}\) gene detected in this study belonged to ST617. Interestingly, one study characterized ST167 and ST617 as sister clades with respect to ST10, with ST617 emerging as a nested clade from a single outlying ST167 genome (27). The study also indicated that lineage-specific alterations in intergenic regions were responsible for the emergence of the multidrug resistance (MDR) plasmid. Therefore, there is a need for a more thorough and detailed analysis of the genomic epidemiological investigation of bacteria carrying carbapenem resistance plasmids. Notably, most of the strains were collected from blood samples. Unlike other types of infection, bloodstream infections are always associated with high mortality. Therefore, we should be vigilant in preventing further spread of the **bla**

\(\text{NDM-5}\) gene in other **Enterobacteriaceae** isolates.

The **bla**

\(\text{NDM-5}\) gene has previously been reported to be carried on a 46-kb self-transmissible plasmid, which belongs to the IncX3 incompatible group. The results of plasmid sequencing in our study revealed that plasmid pNDM-Z244 in **E. coli** was mostly identical to pNDM-MGR194 reported in India, except for several mutations (18). Plasmids pNDM-K725, pNDM-CR33, and pNDM-Z214 were mostly identical to each other but were slightly different from pNDM-Z244. We speculated that **E. coli** Z244 possibly acquired the **bla**

\(\text{NDM-5}\) gene from commonly reported plasmids like pNDM-MGR194, which can also be found in strains isolated from environmental, animal, and human clinical samples (12, 14, 28). A study revealing that NDM-5-producing **E. coli** ST167 was simultaneously detected in a companion dog and his owners in a family in Finland (29) indicated that human-to-canine transmission is possible. Therefore, it may be logical to assume that some **Enterobacteriaceae** strains acquired the **bla**

\(\text{NDM-5}\)-positive plasmid by horizontal transfer, and it was further clonally disseminated, which resulted in this outbreak of the **bla**

\(\text{NDM-5}\) gene in our hospital.

Interaction with the host or the adaptation response during horizontal transfer possibly resulted in the loss of ISABA125 and part of IS3000 sequences. ISABA125 was always found in **Acinetobacter** spp. and was mainly embedded in the chromosome (30). Currently, it is widely accepted that the **bla**

\(\text{NDM-5}\) gene is transferred from **Acinetobacter** spp. to **Enterobacteriaceae** through ISABA125 and IS26 or other transposable elements (31). That the IncX3-type plasmid spreads easily in **Enterobacteriaceae** may be responsible for the dissemination of the **bla**

\(\text{NDM-5}\) gene. Transposable elements such as ISABA125 were not the main factor, nor were they essential for plasmid replication and proliferation or stability of host strains, so ISABA125 could be gradually deleted in the
process of transfer. Previous studies have reported a partial loss of ISAba125 around blaNDM-5 (7, 15), but the almost complete loss is reported for the first time. More experiments are needed to confirm whether this microevolution contributes to the plasmid transfer. The IncX3-type plasmid was also frequently reported to mediate the dissemination of other NDM variants, including blaNDM-3, blaNDM-4, blaNDM-13, blaNDM-17, blaNDM-19, blaNDM-20, and blaNDM-21 (3, 15), which indicated that the blaNDM-5-bearing IncX3-type plasmids might have evolved from the same ancestral plasmid through a series of mutations. Easy spread of the IncX3-type plasmid could be responsible for the dissemination of multiple NDM variants in Enterobacteriaceae isolates.

Plasmids in this study harbored only one resistance gene, blaNDM-5. Which had a similar genetic background except that part of IS3000 and ISAba125 remnants were deleted in pNDM-K725, pNDM-CR33, and pNDM-Z214. According to the complete sequences of the plasmids, the genetic background of blaNDM-5 in pNDM-Z244 was similar to that in the classical plasmid pNDM-MGR194, i.e., IS3000-IS5-ISAb125-blaNDM-5-blaMB, trpF-dsbC-IS26-umuD. In contrast, the blaNDM-5 gene in pNDM-5-IT was more complex and was found in the dfrA12-aza2-ISCRI-blaNDM-5 complex integron (9).

In conclusion, we characterized the IncX3-type plasmid carrying the blaNDM-5 gene of K. pneumoniae, E. coli, and K. aerogenes clinical isolates. Our results may serve as evidence of horizontal gene transfer of blaNDM-5 among different Enterobacteriaceae isolates. To our knowledge, this is the first report of blaNDM-5-carrying isolates in different species of Enterobacteriaceae in pediatric patients in China.

MATERIALS AND METHODS

Bacterial strains. A total of 146 carbapenem-resistant Enterobacteriaceae (CRE) strains were collected between January and March 2017 in a children’s hospital in Shanghai, China. They were mainly isolated from nasopharyngeal secretions, blood, pus secretions, urine, catheter, and ascites. The protocol was approved by the Ethics Committee of Shanghai Children’s Hospital, Shanghai Jiaotong University. Individual informed consent was waived because we used existing strains and did not pose any additional risks to the patients. Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (Bruker Daltonics GmbH, Bremen, Germany) was used for bacterial identification, and disc diffusion assays (for imipenem and meropenem) were used to identify carbapenem resistance. Common carbapenemase genes (blaOXA, blaNDM, blaIMP, blaVIM, blaAmpC, blagli, and blaTolC) were amplified for all strains using primers from the previous study, and the positive products were sequenced (2). Twenty-two blaNDM-5-positive strains were finally selected for further study.

Antimicrobial susceptibility testing. Antimicrobial susceptibility was determined using the broth microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (32). The antibiotics tested were ertapenem, imipenem, meropenem, ceftazidime, cefotaxime, cefmetazole, cefepime, pipercillin–tazobactam, ceferazone–sulbactam, ceftazidime–avibactam, amikacin, gentamicin, nitrofurantoin, sulfamethoxazole–trimethoprim, aztreonam, ciprofloxacin, levofloxacin, tigecycline, polymyxin, and colistin. The results were determined and interpreted as follows: colistin and tigecycline according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (36) and all others according to the CLSI M100-S28 criteria (32). E. coli ATCC 25922 was used for quality control.

Determination of genetic relatedness. MLST was determined using the platform for K. pneumoniae MLST maintained at the Institut Pasteur, Paris, France (https://bigdb.pasteur.fr/klebsiella/primer_used.html) and E. coli MLST maintained at the Achtman multilocus sequence typing scheme (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/documents/primersColi.html). Seven housekeeping genes of E. coli and K. pneumoniae were amplified by PCR, and the products were sequenced to analyze the ST. PFGE was further performed according to previously defined criteria (33). Briefly, the isolates were digested by XbaI endonuclease and analyzed using a CHEF- Mapper XP PFGE system (Bio-Rad, CA, USA) with a 2.16- to 54.17-s linear ramp for 19 h at 6 V/cm and 14°C. The PFGE profiles were analyzed with BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Salmonella enterica serotype Braenderup H9812 was used as a size marker.

Plasmid analysis and location of blaNDM-5. Filter-mating conjugation experiments were performed between 22 different isolates. E. coli J53 resistant to sodium azide was used as the recipient strain. Transconjugants that possessed the blaNDM-5-bearing plasmid were selected on Mueller-Hinton agar (MHA; Oxoid) plates that contained 180 μg/ml sodium azide with 1 μg/ml meropenem. Antimicrobial susceptibility testing and PCR amplification of the transconjugants were subsequently performed to confirm whether the plasmid was successfully transferred to the recipient. The PBRT 2.0 kit for PCR-based replicon typing was used for molecular typing of plasmids (Diathea, Fano, Italy). Plasmid relationships were tested by restriction fragment length polymorphism (RFLP) using HindIII and EcoRI. Digested plasmid DNA was electrophoresed in a 0.8% agarose gel for approximately 1 h. Four strains were selected for further study. Plasmid stability was tested by liquid experiments as previously described (34). S1-PFGE and Southern blotting were further performed to determine the plasmid location of the blaNDM-5 gene. Genomic DNA digested with S1 nuclease was subjected to PFGE as described above. The DNA fragments
DISSEMINATION OF THE blaNDM-5 GENE

were transferred to a positively charged nylon membrane (Millipore, USA) and then hybridized with a digoxigenin-labeled NDM-5-specific probe. S. enterica serotype Braenderup H9812 was used as the size marker.

**Plasmid sequencing and comparative analysis.** To obtain a comprehensive understanding of the plasmid carrying the blaNDM-5 gene, complete sequencing was further performed. The plasmid DNAs of transconjugants were extracted using a HiSpeed Plasmid Midi kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s recommendations. The plasmids were sequenced on an Illumina MiSeq 2000 (Illumina Inc., San Diego, CA, USA) platform with 2-by-300-bp paired-end reads. The raw data quality control was performed with FastQC software (v. 0.11.8, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The clean reads were assembled using SPAdes v3.9.0 and A.S-miseq v20150522. Prediction and annotation of the open reading frames (ORFs) were carried out using the RAST (Rapid Annotation using Subsystems Technology) website server (http://rast.nmpdr.org/). BRIG was used in comparative analysis and the generation of plasmid maps.

**Data availability.** The complete sequences of the plasmids were submitted to the National Center for Biotechnology Information (NCBI) database under the accession numbers in parentheses: pNDM-K725 (MK450348), pNDM-CR33 (MK450349), pNDM-Z214 (MK450347), and pNDM-Z244 (MK450346).

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**TABLE S1**, DOCX file, 0.02 MB.

**TABLE S2**, DOCX file, 0.02 MB.

**ACKNOWLEDGMENTS**

We extend our thanks to Fupin Hu and all members of the Huashan Institute of Antibiotics for their cooperation and technical help.

D.T., H.Z., B.W., and F.P. contributed conception and design of the study; C.W., F.P., Y.S., and Y.S. contributed materials; D.T. and H.Z. organized the database; B.W. and C.W. performed the statistical analysis; D.T. wrote the first draft of the manuscript; all authors contributed to manuscript revision and read and approved the submitted version.

This study was funded by the Youth Foundation of the Shanghai Municipal Commission of Health and Family Planning (2015ZB0203).

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