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RESEARCH ARTICLE

# Genomic Epidemiology Insights on NDM-Producing Pathogens Revealed the Pivotal Role of Plasmids on *bla*$_{NDM}$ Transmission

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**ABSTRACT** Incidences of nosocomial infections mediated by New Delhi metallo-$\beta$-lactamase (NDM) enzyme-producing *Enterobacterales* are increasing globally, resulting in a great burden to public health. The carbapenem-resistant *Enterobacterales* (CRE) were collected from Henan, China during 2013–2016. The *bla*$_{NDM}$-positive strains were characterized using PCR, antimicrobial susceptibility testing, conjugation assay, S1 nuclease pulsed-field gel electrophoresis (S1-PFGE), Southern blot, whole-genome sequencing (WGS), and bioinformatics analysis. Eighty-one NDM-producing strains were identified among 391 nonduplicate CRE strains. Among them, four strains cocarried *mcr* and *bla*$_{NDM}$ genes, and two carried *bla*$_{IMP-4}$ and *bla*$_{NDM}$ genes. The coexistence of *bla*$_{NDM-5}$ and *mcr-9* in *Enterobacter hormaechei* was found for the first time. In total, four *bla*$_{NDM}$ subtypes were identified. Among them, *bla*$_{NDM-1}$ and *bla*$_{NDM-5}$ were predominant. There was an obvious increasing trend in *bla*$_{NDM-5}$ from 2013 to 2016. Thirteen different bacterial species were found among the 81 strains, and *Escherichia coli* was the dominant strain. *bla*$_{NDM}$ genes were located on nine different Inc-type plasmids, most of them on the IncX3 plasmids, except for the Pr-15-2-50 strain, which was located on the chromosome. We characterized two novel plasmids: the IncHI5-like plasmid carrying *bla*$_{NDM-9}$ found in *K. pneumonia*, and the IncI1 *bla*$_{NDM-5}$-positive plasmid. These findings provide the genomic basis for the widespread transmission of *bla*$_{NDM}$ and pave the way for the formulation of more effective monitoring and control methods.

**IMPORTANCE** To control the emergence and transmission of CRE, it is important to perform retrospective genomic investigations. It is important to evaluate the plasmid diversity, genetic environment, and evolutionary relationships of the *bla*$_{NDM}$-positive clinical strains in the early transmission stages. This study conducted an in-depth analysis of *bla*$_{NDM}$-positive pathogens during a 4-year period using different methods for observing the high prevalence and active transmission of *bla*$_{NDM}$-positive CRE. Moreover, we also explored the coexistence of the *bla*$_{NDM}$ and *mcr*, a clinically important mobile colistin resistance gene. This study shows that the prevalence of *bla*$_{NDM}$-positive pathogens in Henan is high and the isolation rates increase each year. Moreover, plasmid-mediated horizontal transfer plays an important role in *bla*$_{NDM}$ dissemination. The co-occurrence of multiple resistance genes highlighted a long-lasting evolutionary pathway. Therefore, we have suggested the long-term continuous surveillance of clinical pathogens carrying *bla*$_{NDM}$ to learn the future transmission trend and curb the public health risk caused by CRE.

**KEYWORDS** *Enterobacterales*, plasmid diversity, *bla*$_{NDM}$, molecular epidemiology, nanopore sequencing

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Carbapenem antibiotics are $\beta$-lactam antibiotics with a broad antibacterial spectrum and strong antibacterial activity. They are the most important antibiotics for the treatment of multidrug-resistant (MDR) Gram-negative bacterial infections (1). However, the clinical use of these drugs leads to the emergence of carbapenem-resistant *Enterobacterales* (CRE) (2) and makes clinical medication selection difficult. In 2013, the Centers for Diseases Control and Prevention in the U.S. reported that more than 9,000 health care-related infections were caused by CRE each year. It ranked CRE in the highest threat level. Moreover, the China CRE Monitoring Network showed that the hospital mortality rate of CRE was 33.5% (222/662) (3). It also showed that the mortality rate increased with the length of hospital stay.

Carbapenem-inactivating carbapenemases are predominantly divided into Classes A, B, and D according to the Ambler classification. Classes A and D belong to serine enzymes, and B belongs to metallo-$\beta$-lactamases (MBLs). NDM is a typical member of the B1 class of MBLs. It is capable of hydrolyzing all $\beta$-lactams, except monobactams (4). It recruits mobile genetic elements, such as plasmids belonging to different replicon or Inc types (IncFII, IncHI2, IncN, and IncX3), insertion sequences (IS*Aba125*, IS*CR1*), and transposons (Tn*125*) (5). $bla_{NDM}$ genes have already spread to various species of bacteria worldwide, including *Enterobacterales* and nonfermenting Gram-negative bacilli (6). The increasing prevalence of NDM-producing pathogens has seriously compromised the efficacy of carbapenems in clinical settings, and it poses a great threat to public health. According to current reports, 28 NDM variants have been identified in multiple species of *Enterobacterales*, *Acinetobacter*, and *Pseudomonas*. NDM-1 and NDM-5, which were encoded mainly by IncX3 plasmids, were the most frequently detected variants in *Enterobacterales*. However, NDM-5 was more prevalent compared to NDM-1 in *Escherichia coli*. Our previous study revealed only NDM-1, and no other variants were detected in NDM-producing *Enterobacterales* isolated from the Henan province between 2011 and 2012. Moreover, the IncA/C plasmids with broad-host-range were the predominant vehicles for $bla_{NDM}$ compared to the narrow-host range IncX3 plasmids (7). These differences indicate the changes in the prevalence and evolution of $bla_{NDM}$-bearing plasmids. Therefore, we continuously monitored the NDM-producing CRE strains in a teaching hospital in Zhengzhou University over a 4-year period (2013–2016). We tried to elucidate the molecular mechanisms for the $bla_{NDM}$ gene transfers, and study the evolution of the epidemic $bla_{NDM}$ plasmids and their clones.

## RESULTS

**Overview of NDMs-producing CRE isolates.** From 2013 to 2016, 391 nonduplicate CRE isolates belonging to 13 different species were collected from a teaching hospital in the Zhengzhou University for screening carbapenemase genes using PCR and Sanger sequencing. The result showed 291 *Klebsiella pneumoniae* strains (74.42%) carrying the $bla_{KPC-2}$ gene and another 81 (20.72%) belonging to various species carrying the $bla_{NDM}$ (Table 1). This illustrated that *K. pneumoniae* and *E. coli* were the main clinical CREs. KPC and NDM were the primary carbapenem-inactivating enzymes in CRE recovered from the Henan province. It was well recognized that $bla_{NDM}$ genes were mainly carried by Gram-negative *Enterobacterales*, including *E. coli*, *K. pneumoniae*, *Citrobacter freundii*, and *Enterobacter cloacae* (8–10). The prevalence of $bla_{NDM}$ in different *Enterobacterales* was 49.38% (40/81), 14.81% (12/81), 13.58% (11/81), 7.41% (6/81), and 4.94% (4/81) in *E. coli*, *K. pneumoniae*, *Enterobacter hormaechei*, *C. freundii*, and *Citrobacter portucalensis*, respectively. There was also 1.23% (1/81) in each *Citrobacter braakii*, *Klebsiella aerogenes*, *Klebsiella pasteurii*, *Klebsiella oxytoca*, *Raoultella ornithinolytica*, *Serratia marcescens*, *Proteus mirabilis*, and *Providencia rettgeri*. This indicates that *E. coli* was the most common host for $bla_{NDM,}$ followed by *K. pneumoniae* and *E. hormaechei*. Sanger sequencing of $bla_{NDM}$ genes identified four $bla_{NDM}$ subtypes, including $bla_{NDM-1}$ ($n$ = 41), $bla_{NDM-5}$ ($n$ = 38), $bla_{NDM-4}$ ($n$ = 2), and $bla_{NDM-9}$ ($n$ = 1) (Table 1). Among them, $bla_{NDM-5}$ was the most prevalent subtype in *E. coli* (33/40, 82.5%), and the

**TABLE 1** Basic information of the 81 $bla_{NDM}$-bearing strains

| Isolate | Species | MLST[a] | Collection date | Age/sex | Specimen type | Ward | Prognosis | Conjugation frequency | NDM-type | Plasmid type carrying $bla_{NDM}$ | NDM-positive plasmid size (kb) | Grouping of IncX3 $bla_{NDM}$-positive plasmids |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| KP-13-8 | *K. pneumoniae* | ST494 | 2013.01.06 | 61yr/female | blood | Gastroenterology dept | discharge | -[b] | NDM-5 | IncX3 | 46 | B |
| EC-13-1 | *E. coli* | ST40 | 2013.01.25 | 6days/male | blood | ICU | discharge | - | NDM-1 | IncX3 | 54 | B |
| KP-13-11 | *K. pneumoniae* | ST35 | 2013.04.25 | 2mo/female | sputum | ICU | death | $3.6 \times 10^{-4}$ | NDM-1 | IncX3 | 54 | C |
| CR-13-12 | *E. hormaechei* | ST419 | 2013.05.06 | 89yr/female | sputum | ICU | discharge | $3.3 \times 10^{-4}$ | NDM-1 | IncFII | 87 | - |
| EC-13-22 | *E. coli* | ST361 | 2013.08.05 | 41yr/female | drainage liquid | gynecology | discharge | $2.6 \times 10^{-6}$ | NDM-1 | IncC | 213 | - |
| EC-13-31 | *E. coli* | ST167 | 2013.09.04 | 68yr/male | blood | gynecology | discharge | - | NDM-5 | IncX3 | 46 | B |
| ECL-13-2 | *E. hormaechei* | ST177 | 2013.09.04 | 53yr/female | urine | urology | discharge | $1.8 \times 10^{-7}$ | NDM-1 | IncFII-IncFIB | 138 | - |
| EC-13-30 | *E. coli* | ST167 | 2013.09.18 | 35yr/male | secreta | endocrinology | discharge | - | NDM-5 | IncX3 | 46 | B |
| KP-13-7 | *K. pneumoniae* | ST1 | 2013.09.26 | 37yr/male | bile | hepatological surgery | discharge | $1.33 \times 10^{-4}$ | NDM-1 | IncX3 | 54 | C |
| EC-13-33 | *E. coli* | ST540 | 2013.10.06 | 68yr/male | blood | gynecology | discharge | $3 \times 10^{-4}$ | NDM-1 | IncFII-IncN | 78 | - |
| ECL-13-4 | *E. hormaechei* | ST88 | 2013.10.17 | 48yr/male | blood | ICU | death | $2.8*10^{-4}$ | NDM-5 | IncX3 | 46 | B |
| CF-13-34 | *C. portucalensis* | ST328 | 2013.10.19 | 23yr/male | secreta | hematology | death | $1.1 \times 10^{-4}$ | NDM-1 | IncX3 | 54 | E |
| EC-13-49 | *E. coli* | ST167 | 2013.11.07 | 78yr/female | urine | kidney internal | discharge | $3.5 \times 10^{-6}$ | NDM-1 | IncC | 215 | - |
| ECL-13-37 | *E. hormaechei* | ST231 | 2013.11.14 | 37yr/male | urine | urology | discharge | - | NDM-5 | IncX3 | 46 | B |
| KP-13-14 | *K. pneumoniae* | ST782 | 2013.11.23 | 21days/male | wound | pediatric surgery | discharge | - | NDM-9 | IncHI5 | 358 | - |
| CR-13-36 | *E. hormaechei* | ST419 | 2013.12.05 | 47yr/female | urine | kidney internal | discharge | $1.2 \times 10^{-5}$ | NDM-1 | IncFII | 87 | - |
| PM58 | *P. mirabilis* | NA | 2013.12.15 | 3yr/female | urine | rehabilitation medicine | discharge | $9.4 \times 10^{-6}$ | NDM-1 | - | 85 | - |
| KP-14-2-131 | *K. pneumoniae* | ST345 | 2014.01.23 | 44yr/male | urine tube tip | neurosurgery | discharge | $1.4 \times 10^{-6}$ | NDM-1 | IncHI5 | 358 | - |
| KOR-14-72 | *R. ornithinolytica* | NA | 2014.02.15 | 71yr/female | sputum | ICU | discharge | $3.9 \times 10^{-5}$ | NDM-1 | IncX3 | 46 | C |
| KO-14-71 | *K. pasteurii* | NA | 2014.02.20 | 67yr/female | sputum | ICU | discharge | $3.2 \times 10^{-3}$ | NDM-1 | IncX3 | 54 | C |
| EC-14-2-77 | *E. coli* | ST410 | 2014.03.30 | 66yr/male | drainage liquid | hepatological surgery | discharge | $2.5 \times 10^{-3}$ | NDM-4 | IncX3 | 54 | C |
| ECL-14-58 | *E. hormaechei* | ST177 | 2014.05.12 | 10yr/male | pus | pediatric surgery | discharge | $9.5 \times 10^{-5}$ | NDM-1 | IncX3 | 54 | C |
| ECL-14-60 | *E. hormaechei* | ST696 | 2014.06.05 | 62yr/male | blood | ICU | death | - | NDM-1 | IncC, IncX3 | 171-54 | D |
| EC-14-55 | *E. coli* | ST410 | 2014.06.06 | 14yr/female | blood | Pediatric medicine | death | - | NDM-4 | IncX3 | 46 | C |
| KA-14-61 | *K. aerogenes* | NA | 2014.08.30 | 33yr/male | secreta | Department of Burn Repair and Reconstruction | discharge | $2.2 \times 10^{-4}$ | NDM-1 | IncX3 | 46 | B |
| EC-14-54 | *E. coli* | ST167 | 2014.08.30 | 51yr/male | sanies | intestine surgery | discharge | - | NDM-5 | IncX3 | 46 | B |
| CF-14-50 | *C. freundii* | ST22 | 2014.09.20 | 44yr/male | urine | urology | discharge | $1.9 \times 10^{-4}$ | NDM-1 | IncX3 | 54 | D |
| ECL-14-56 | *E. hormaechei* | ST171 | 2014.11.02 | 45yr/male | blood | ICU | death | - | NDM-1 | IncX3 | 54 | C |
| KP-14-6 | *K. pneumoniae* | ST76 | 2014.11.13 | 10days/female | blood | Infectious disease | discharge | $2.9 \times 10^{-4}$ | NDM-1 | IncC | 200 | - |
| EC-14-2-134 | *E. coli* | ST101 | 2014.11.17 | 31yr/male | swab | Burn Repair and Reconstruction | discharge | $5.9 \times 10^{-5}$ | NDM-5 | IncX3 | 46 | B |
| EC-14-2-92 | *E. coli* | ST167 | 2014.11.27 | 50yr/male | blood | oncology | death | $4 \times 10^{-5}$ | NDM-5 | IncX3 | 46 | B |
| EC-14-2-94 | *E. coli* | ST167 | 2014.12.10 | 44yr/female | urine | urology | discharge | - | NDM-5 | IncX3 | 46 | B |
| EC-14-2-9 | *E. coli* | ST167 | 2014.12.19 | 51yr/female | sputum | Rheumatology | discharge | - | NDM-5 | IncX3 | 46 | B |
| EC-15-2-5 | *E. coli* | ST167 | 2015.01.16 | 26yr/male | sputum | ICU | death | - | NDM-5 | IncX3 | 54 | D |
| EC-15-2-14 | *E. coli* | ST2083 | 2015.01.25 | 23yr/female | sputum | Rheumatology | discharge | - | NDM-5 | IncX3 | 46 | B |
| CF-15-43 | *C. portucalensis* | ST17 | 2015.02.21 | 47yr/female | urine | urology | discharge | $3 \times 10^{-5}$ | NDM-1 | IncX3 | 54 | E |
| CF-15-2-98 | *C. portucalensis* | ST17 | 2015.02.21 | 47yr/female | urine | urology | discharge | $6.8 \times 10^{-5}$ | NDM-1 | IncX3 | 54 | E |
| KP-15-2-113 | *K. pneumoniae* | ST1083 | 2015.03.08 | 2mo/male | sputum | neonatology | discharge | $3.3 \times 10^{-5}$ | NDM-1 | IncX3 | 46 | C |
| EC-15-10 | *E. coli* | ST540 | 2015.03.14 | 74yr/male | sputum | ICU | discharge | - | NDM-5 | IncX3 | 54 | D |
| EC-15-3 | *E. coli* | ST6388 | 2015.03.23 | 53yr/female | urine | urology | discharge | - | NDM-1 | IncFII | 110 | - |
| CF-15-61 | *C. freundii* | ST22 | 2015.04.08 | 75yr/female | drainage liquid | gastrointestinal surgery | discharge | $1.7 \times 10^{-4}$ | NDM-1 | IncX3 | 54 | C |
| CF-15-33 | *C. freundii* | NA | 2015.05.20 | 7yr/male | blood | Pediatric medicine | discharge | $1.7 \times 10^{-5}$ | NDM-1 | IncX3 | 54 | C |
| EC-15-34 | *E. coli* | ST746 | 2015.05.22 | 60yr/male | blood | urology | death | - | NDM-5 | IncX3 | 46 | B |
| KP-15-35 | *K. pneumoniae* | ST17 | 2015.05.22 | 10days/male | blood | neonatology | death | $1.4 \times 10^{-5}$ | NDM-1 | IncX3 | 54 | C |
| EC-15-2-35 | *E. coli* | ST540 | 2015.06.27 | 75yr/female | urine | urology | discharge | - | NDM-5 | IncX3 | 54 | D |
| EC-15-2-56 | *E. coli* | ST167 | 2015.06.27 | 52yr/male | urine | urology | discharge | - | NDM-5 | IncX3 | 46 | A |
| EC-15-2-24 | *E. coli* | ST540 | 2015.06.29 | 75yr/female | urine | urology | discharge | $2.5 \times 10^{-5}$ | NDM-5 | IncX3 | 54 | D |

(Continued on next page)

**TABLE 1** (Continued)

| Isolate | Species | MLST[a] | Collection date | Age/sex | Specimen type | Ward | Prognosis | Conjugation frequency | NDM-type | Plasmid type carrying $bla_{NDM}$ | NDM-positive plasmid size (kb) | Grouping of IncX3 $bla_{NDM}$-positive plasmids |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EC-15-2-47 | *E. coli* | ST540 | 2015.06.29 | 67yr/male | urine | urology | discharge | - | NDM-5 | IncX3 | 54 | D |
| SM-15-2-16 | *S. marcescens* | NA | 2015.07.01 | 27yr/female | sputum | respiratory medicine | discharge | $2.5 \times 10^{-5}$ | NDM-1 | IncX3 | 46 | B |
| EC-15-2-132 | *E. coli* | ST410 | 2015.07.15 | 38ye/female | blood | Hematology dept | discharge | - | NDM-5 | IncX3 | 46 | D |
| EC-15-2-51 | *E. coli* | ST617 | 2015.07.21 | 9mo/male | urine | ICU | discharge | - | NDM-5 | IncX3 | 46 | B |
| EC-15-2-65 | *E. coli* | ST6388 | 2015.07.30 | 65yr/male | urine | ICU | discharge | $6.8 \times 10^{-5}$ | NDM-5 | IncX3 | 46 | C |
| KP-15-2-62 | *K. pneumoniae* | ST490 | 2015.07.30 | 2yr/female | blood | ICU | death | - | NDM-5 | IncX3 | 46 | D |
| KP-15-2-52 | *K. pneumoniae* | ST1440 | 2015.08.03 | 66yr/male | urine | urology | death | $5.5 \times 10^{-6}$ | NDM-5 | IncX3 | 46 | B |
| CF-15-2-29 | *C. freundii* | ST22 | 2015.08.04 | 1mo/female | sputum | ICU | discharge | $8.8 \times 10^{-4}$ | NDM-1 | IncX3 | 54 | C |
| EC-15-2-26 | *E. coli* | ST167 | 2015.08.08 | 49yr/female | urine | gynecology | discharge | - | NDM-5 | IncX3 | 46 | B |
| Pr-15-2-50 | *P. rettgeri* | NA | 2015.08.13 | 19yr/female | joint fluid | internal medicine | discharge | - | NDM-1 | - | - | - |
| EC-15-2-1 | *E. coli* | ST167 | 2015.10.28 | 67yr/female | urine | cardiac surgery | discharge | - | NDM-5 | IncX3 | 54 | B |
| EC-15-2-2 | *E. coli* | ST617 | 2015.10.28 | 56yr/male | drainage liquid | hepatological surgery | discharge | $3.3 \times 10^{-5}$ | NDM-5 | IncX3 | 54 | D |
| KP-15-2-6 | *K. pneumoniae* | ST11 | 2015.11.05 | 78yr/male | sputum | respiratory medicine | discharge | $1.1 \times 10^{-5}$ | NDM-1 | IncX3 | 54 | C |
| EC-15-2-152 | *E. coli* | ST405 | 2015.12.02 | 59yr/female | blood | ICU | death | - | NDM-5 | IncX3 | 46 | B |
| EC-15-2-153 | *E. coli* | ST405 | 2015.12.04 | 61yr/male | drainage liquid | gastrointestinal surgery | discharge | - | NDM-1 | IncX3 | 46 | B |
| EC-15-2-159 | *E. coli* | ST167 | 2015.12.08 | 23yr/male | urine | urology | discharge | - | NDM-5 | IncX3 | 46 | B |
| CF-15-2-165 | *C. portucalensis* | NA | 2015.12.11 | 79yr/male | urine | urinary surgery | discharge | $3.3 \times 10^{-4}$ | NDM-1 | IncX3 | 54 | E |
| EC-16-7 | *E. coli* | ST167 | 2016.01.08 | 52yr/female | urine | kidney internal | discharge | $4.3 \times 10^{-4}$ | NDM-1 | IncX3 | 54 | B |
| ECL-16-5 | *E. hormaechei* | ST51 | 2016.01.08 | 82yr/male | sputum | ICU | death | $3.3 \times 10^{-4}$ | NDM-1 | IncX3 | 54 | B |
| EC-16-10 | *E. coli* | ST1193 | 2016.03.03 | 79yr/male | blood | ICU | death | - | NDM-5 | IncI1 | 93 | - |
| CF-16-17 | *C. freundii* | ST18 | 2016.07.08 | 70yr/male | secreta | endocrinology | discharge | $1.8 \times 10^{-5}$ | NDM-1 | IncX3 | 54 | E |
| KO-16-21 | *K. oxytoca* | NA | 2016.07.10 | 83yr/male | sputum | ICU | discharge | $3.8 \times 10^{-5}$ | NDM-1 | IncHI5 | 370 | - |
| EC-16-20 | *E. coli* | ST617 | 2016.07.10 | 48yr/male | ascites | Infectious disease | discharge | - | NDM-5 | IncX3 | 46 | B |
| EC-16-35 | *E. coli* | ST167 | 2016.07.16 | 10yr/female | ascites | pediatric surgery | discharge | $3.2 \times 10^{-5}$ | NDM-5 | IncX3 | 46 | B |
| EC-16-37 | *E. coli* | ST46 | 2016.07.18 | 51yr/female | urine | urology | discharge | $1.5 \times 10^{-6}$ | NDM-5 | IncFII-IncFIA-IncFIB | 159 | - |
| EC-16-52 | *E. coli* | ST410 | 2016.07.21 | 63yr/female | urine | pediatric surgery | discharge | - | NDM-5 | IncX3 | 46 | B |
| KP-16-57 | *K. pneumoniae* | ST716 | 2016.07.26 | 10yr/male | sputum | ICU | discharge | $7.3 \times 10^{-4}$ | NDM-1 | IncC | 180 | - |
| CF-16-58 | *C. braakii* | NA | 2016.07.27 | 57yr/male | urine | respiratory medicine | discharge | $5.9 \times 10^{-4}$ | NDM-1 | IncX3 | 54 | C |
| EC-16-59 | *E. coli* | ST167 | 2016.07.29 | 45yr/male | tissue | kidney internal | discharge | - | NDM-5 | IncX3 | 46 | B |
| EC-16-60 | *E. coli* | ST167 | 2016.07.29 | 2mo/female | sputum | ICU | discharge | $6.3 \times 10^{-4}$ | NDM-5 | IncX3 | 54 | C |
| CF-16-61 | *C. freundii* | ST22 | 2016.07.30 | 50yr/male | blood | ICU | discharge | $8.8 \times 10^{-5}$ | NDM-1 | IncX3 | 54 | C |
| ECL-16-74 | *E. hormaechei* | ST93 | 2016.08.03 | 45yr/male | drainage liquid | Liver transplantation | discharge | - | NDM-5 | IncX3 | 46 | C |
| EC-16-76 | *E. coli* | ST2172 | 2016.08.06 | 58yr/male | urine | emergency internal medicine | discharge | - | NDM-5 | IncX3 | 54 | C |
| ECL-16-79 | *E. hormaechei* | ST51 | 2016.10.20 | 54yr/female | bile | intervention department | discharge | $8.1 \times 10^{-4}$ | NDM-5 | IncX3 | 46 | B |

[a]MLST, multilocus sequence typing; NA, not available.
[b]-, not detected.

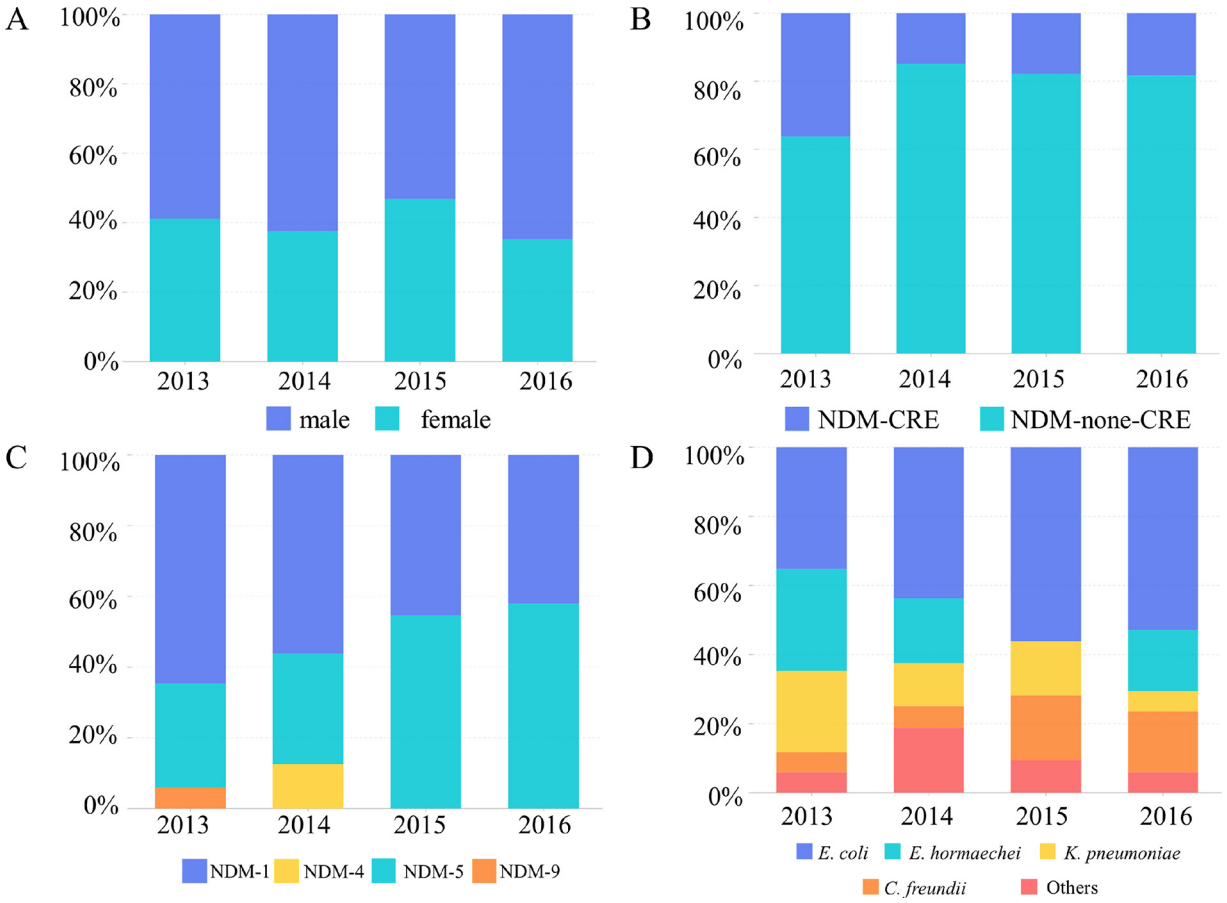


**FIG 1** Epidemiological description and impact factors of the 81 *bla*$_{NDM}$-positive strains used in this study. (A) The proportion carrying the NDM by gender in different years. (B) Isolation rates of NDM among CRE in different years. (C) Proportion of NDM subtypes isolated in different years. (D) Proportion of different species isolated in different years.

majority of *K. pneumoniae* carried *bla*$_{NDM-1}$ (9/12, 75%). However, carbapenemase gene *bla*$_{IMP-4}$ was only detected in two NDM-producing strains (KA-14-61 and KO-14-71).

We analyzed the clinical features of these 81 *bla*$_{NDM}$ carriers (Table 1). We found that most *bla*$_{NDM}$-positive strains were isolated from medical Intensive Care Units (ICUs). ICU patients usually have longer hospital stays, which increases the risk of infections and evolution of CRE pathogens. Comparatively, higher NDM-positive rates were also obtained among the Urinary Surgery and Pediatrics wards. The number of male patients was slightly higher compared with female patients (Fig. 1). We observed a wide age gap among these patients, ranging from 6 days to 89 years old; however, maximum cases (49.38%) were concentrated in the 50–79 age group. The mortality among the NDM-positive patients was 18.52%, which was lower compared to our previous report (7).

**Resistance phenotype, determinants, and bacterial genotyping.** Antimicrobial susceptibility testing revealed that all the 81 *bla*$_{NDM}$-positive strains were MDR strains, and they were resistant to multiple categories of antimicrobials ($n \geq 3$) (Table S2 in the supplemental material). Therefore, each isolate carried at least three categories of resistance genes associated with the resistance phenotype (Fig. 2 and Fig. S1). The MIC values of meropenem or imipenem were distributed between 16 and 64 $\mu$g/mL. Given that most NDM-producing isolates (92.59%) were resistant to aztreonam, we detected $\beta$-Lactamase encoding genes other than carbapenemase. Therefore, various AmpC (CMY, ACT, DHA) and ESBL (CTX-M, TEM, SHV, VEB, SFO, OXA) genes were identified in different species (Fig. 2 and Fig. S1). Moreover, four strains (EC-15-3, CF-15-2-29, ECL-16-5, and ECL-16-79) also contained plasmid-borne colistin resistance genes (*mcr-1* or

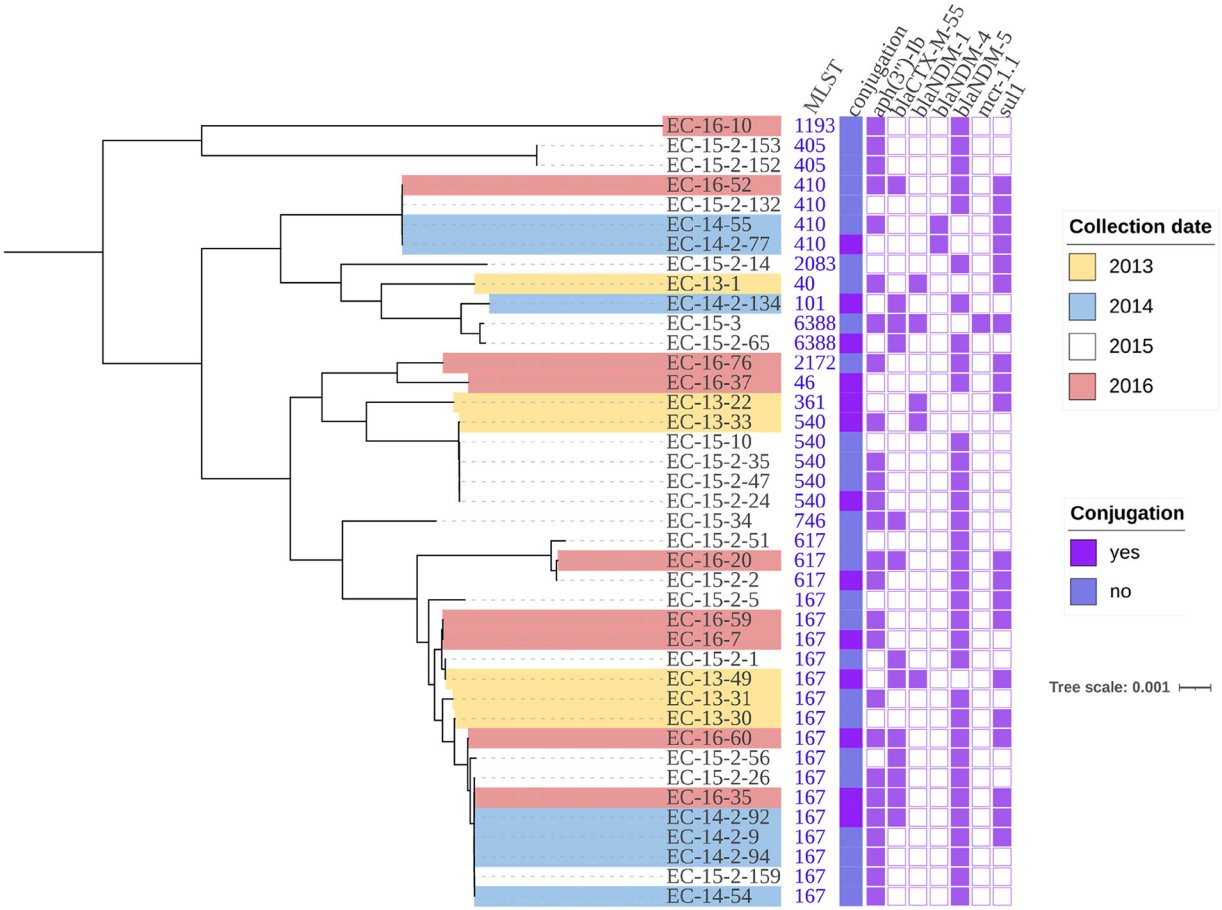


**FIG 2** Phylogenetic tree of all 40 *bla*$_{NDM}$-positive *E. coli* isolates from 2013–2016. Resistance genes are indicated by squares: solid square indicates has; hollow square indicates does not have.

*mcr-9*). The abundance of antibiotic resistance genes in strains increases the risk of *bla*$_{NDM}$ cotransmission. To evaluate the transferability of *bla*$_{NDM}$ genes, conjugation assays were performed for the 81 *bla*$_{NDM}$-positive strains with *E. coli* (EC600 or J53). The *bla*$_{NDM}$ genes carried by 46 strains were successfully transferred to the recipient, suggesting that the *bla*$_{NDM}$ genes carried by these 46 strains were located in conjugative plasmids or other mobilizable genetic elements. The conjugation frequencies ranged from $2.5 \times 10^{-3}$ to $1.8 \times 10^{-7}$ (Table 1).

As the most abundant species carrying *bla*$_{NDM}$, evolutionary relationships between the 40 *E. coli* isolates were investigated and a phylogenetic tree based on SNPs of the core genome data was constructed (Fig. 2). These isolates were assigned to 14 distinct sequence types (STs), and ST167 (16/40, 40%) was the most prevalent ST (Table 1). This finding was in agreement with the previous results (11), which suggest that ST167 appears to be the predominant type of *bla*$_{NDM}$-positive *E. coli* in China. To further investigate the evolutionary relationship between these ST167 *E. coli* and other ST167 *E. coli* collected from the NCBI database (Table S3), a phylogenetic tree based on SNPs of the core genomes was constructed. ST167 *E. coli* carrying *bla*$_{NDM}$ were mainly found in humans. However, they are also found in pets and environmental samples (Fig. S2). *bla*$_{NDM-5}$ was dominant in this subtype. Observation of diverse STs in *E. coli* indicated plasmids or other horizontal mobile elements to be considered as the main vehicles for *bla*$_{NDM}$ transmission. Similarly, four STs were identified among the eight *C. freundii*. Moreover, *K. pneumoniae* ($n$ = 12) and *E. hormaechei* ($n$ = 11) contained 12 and 8 different STs, respectively. The wide distribution of NDM-producing strains illustrates that in

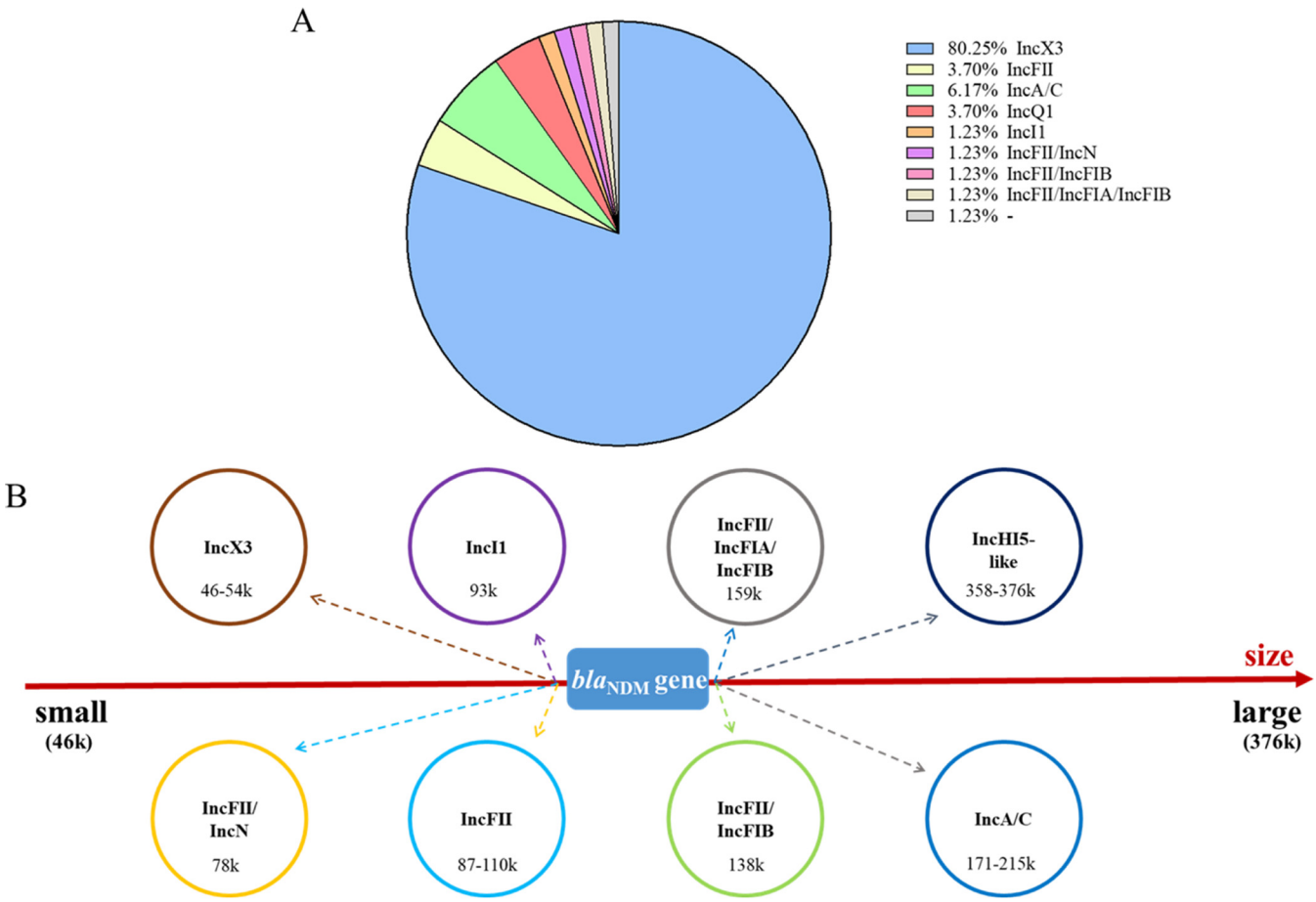


**FIG 3** The distribution of different Inc group plasmids in all *bla*$_{NDM}$-positive strains. (A) The percentage of Inc groups found in all *bla*$_{NDM}$-positive strains. (B) Diversity of *bla*$_{NDM}$-bearing plasmids in terms of replicon types and sizes. Eight different plasmids with various replicon combinations were identified, and each of them was labeled in different circle colors with plasmid types and sizes highlighted.

inter- and intraspecies, horizontal gene transfer plays the most important role in the transmission of *bla*$_{NDM}$ genes.

**Systematic analysis of the predominant IncX3 *bla*$_{NDM}$-bearing plasmids.** S1-PFGE and Southern blot analysis showed 77 *bla*$_{NDM}$-positive strains located on plasmids. The Pr-15-2-50 was an exception, encoding a chromosomal *bla*$_{NDM}$ gene, and four strains (KA-14-61, EC-14-2-92, EC-15-34, and EC-15-2-153) failed to produce a visible band; however, they were confirmed on plasmids during the transfer experiments and whole-genome sequencing (WGS) analysis. Notably, two different *bla*$_{NDM}$-bearing plasmids, pECL-14-60-NDM-1-IncAC (IncC, 171,038 bp) and pECL-14-60-NDM-1 (IncX3, 53,023 bp), were identified in the strain ECL-14-60. These 81 *bla*$_{NDM}$-harboring plasmids were categorized into nine different replicon types (Fig. 3A) with sizes ranging from ~46 to ~370 kb (Fig. 3B). The isolated Inc types of plasmids carrying *bla*$_{NDM}$ genes were different each year; however, IncX3 *bla*$_{NDM}$-positive plasmids were dominant through the period (Fig. 4 and Table 1). The bacteria carrying *bla*$_{NDM}$-positive IncX3-type plasmids were diverse. Sixty-five NDM-producing IncX3 type plasmids with different sizes 54 kb and 46 kb (lacking the *bla*$_{SHV-12}$-bearing segment) were found in 10 different bacterial species.

In total, the environment around the *bla*$_{NDM}$ gene located on the IncX3 plasmid can be classified into five major groups. These regions carrying the *bla*$_{NDM}$ genes were all inserted into the *umuD* gene, and a 3-bp (TGT) direct repeat sequence formed at the insertion site. Group A ($n$ = 1) is the simplest among several groups (Fig. 5). Compared with group A, group B ($n$ = 29) had one more IS*Aba125* insertion downstream from the *bla*$_{NDM}$. Group C ($n$ = 20) had more 7,874 bp regions carrying the *bla*$_{SHV}$ gene downstream from the IS*26* compared with group B. Group D had the reverse IS*5* arrangement

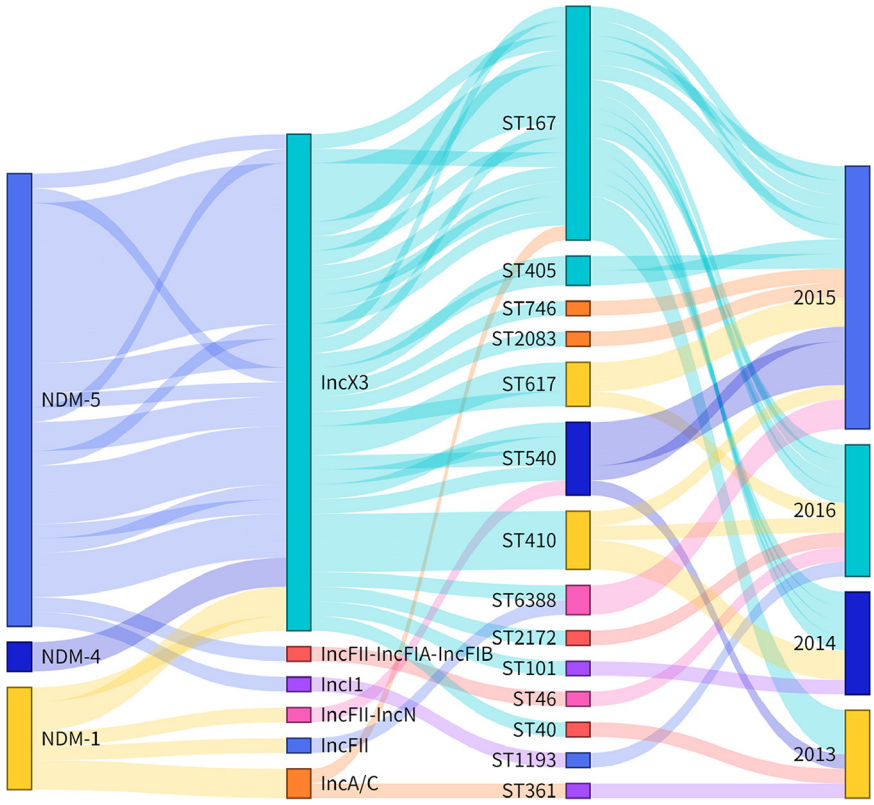


**FIG 4** Sankey diagram combining different NDM subtypes, plasmid Inc types, ST types, and collection date. The diameter of the line is proportional to the number of isolates, which is also labeled at the consolidation points.

compared with group C. Compared with group D, the region in group E lost the IS*Aba125* gene downstream from the *bla*$_{NDM}$.

By connecting the *bla*$_{NDM}$ subtypes of *E. coli* to plasmid types, ST types as well as the year of isolation (Fig. 4), we illustrated a complex combination of multiple genetic vehicles and diverse hosts in the spreading of the *bla*$_{NDM}$ gene. Most of the *bla*$_{NDM-5}$ genes were distributed on the IncX3 plasmids. Moreover, *bla*$_{NDM-1}$ and *bla*$_{NDM-4}$ were also found on the IncX3 plasmids. According to Fig. 4, IncX3 plasmids are the main *bla*$_{NDM}$-positive plasmids isolated each year, and these plasmids are distributed in many different STs of *E. coli*. However, compared with other Inc-type NDM positive plasmids (Fig. S3), IncX3 type plasmids carried only a few antibiotic resistance genes, which may incur a low fitness cost to the host.

**Characterization of novel Inc-type and hybrid *bla*$_{NDM}$-bearing plasmids.** In addition to IncX3 plasmids, other Inc-types of NDM-bearing plasmids were also detected in these strains (Fig. 6). To the best of our knowledge, the IncI1 plasmid pEC-16-10-NDM-5 characterized in this study is a novel *bla*$_{NDM}$-bearing plasmid (Fig. S4 and Fig. 6). Plasmid pEC-16-10-NDM-5 was 92,260 bp in size and had an average G+C content of 50.6%. The BLAST comparison against the GenBank database showed that pEC-16-10-NDM-5 exhibited similarities to IncI1 plasmid pEC224_2 (CP018946). The main difference is that plasmid pEC-16-10-NDM-5 has an additional 8,698 bp complex transposon structure composed of two IS*5* and a *bla*$_{NDM-5}$-bearing region (IS*5*-*hp*-*hp*-Δ*umuD*-IS*26*-*dsbC*-*trpF*-*ble*$_{MBL}$-*bla*$_{NDM-5}$-ΔIS*Aba125*-IS*5*). This additional transposon structure is similar to the IncX3 plasmid pNDM-HK3473 (MH234506) carrying the *bla*$_{NDM-5}$ gene. It is flanked by 15 bp inverted repeats (TAGGGAAGGTGCGAA) on either side. This phenomenon indicates that the *bla*$_{NDM-5}$ could be transferred through this complex transposon and integrated into the IncI1 plasmid (Fig. S5).

Five IncFII *bla*$_{NDM}$-bearing plasmids were also identified among the 22 plasmids

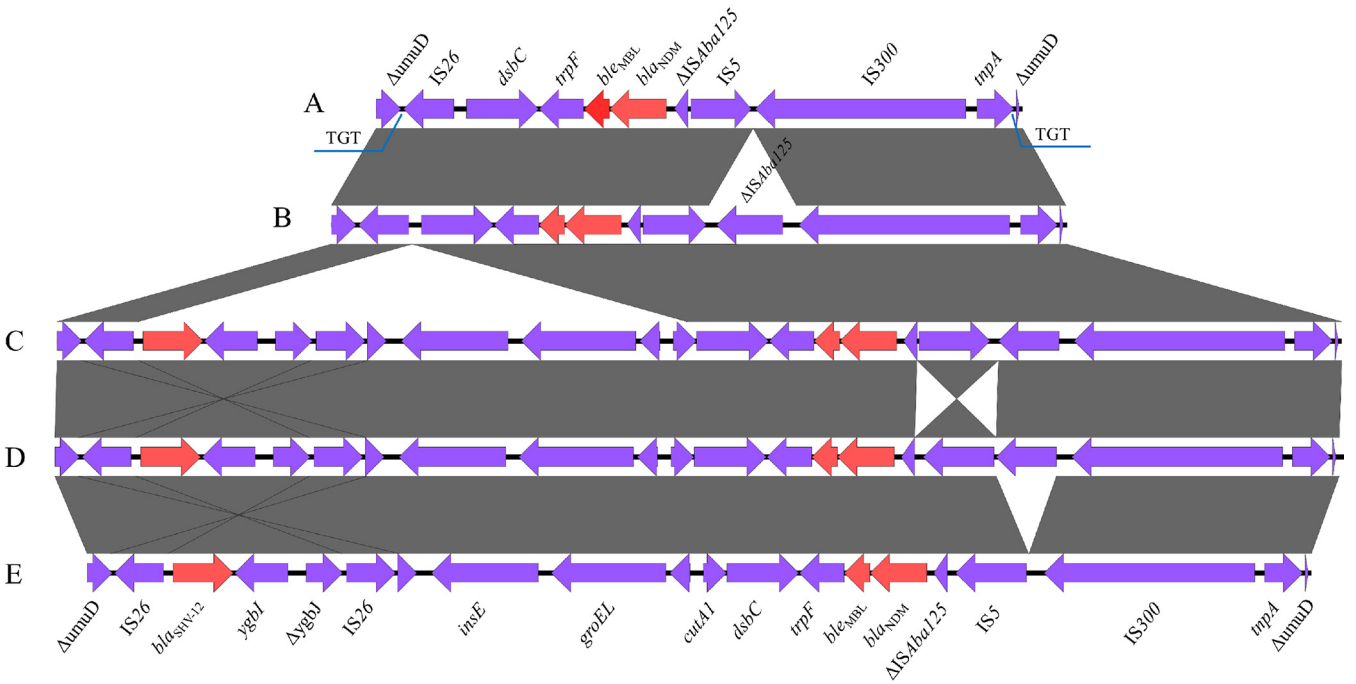


**FIG 5** Different *bla*$_{NDM}$ gene core genetic environments of the IncX3-type plasmids. A total of five (A–E) major types of *bla*$_{NDM}$-bearing genetic contexts among the 42 *bla*$_{NDM}$-bearing plasmids. Red arrows represent resistance genes.

with complete and circular sequences using Nanopore sequencing (Table 2). The pMLST of the pEC-15-3-NDM-1 plasmid was F2:A-:B-, and the size of the plasmid is 109,944 bp. BLASTn analysis of the pEC-15-3-NDM-1 plasmid showed that it had 99% nucleotide identity at more than 95% coverage to pMC-NDM (HG003695). The main difference between them was the copy number of *bla*$_{NDM-1}$. Three 10,461 bp region repeats were found on the pEC-15-3-NDM-1 plasmid, which carried a variety of resistance genes, including *bla*$_{NDM-1}$, *dfrA12*, *aadA2*, *sul1*, and *ble*$_{MBL}$. According to the result

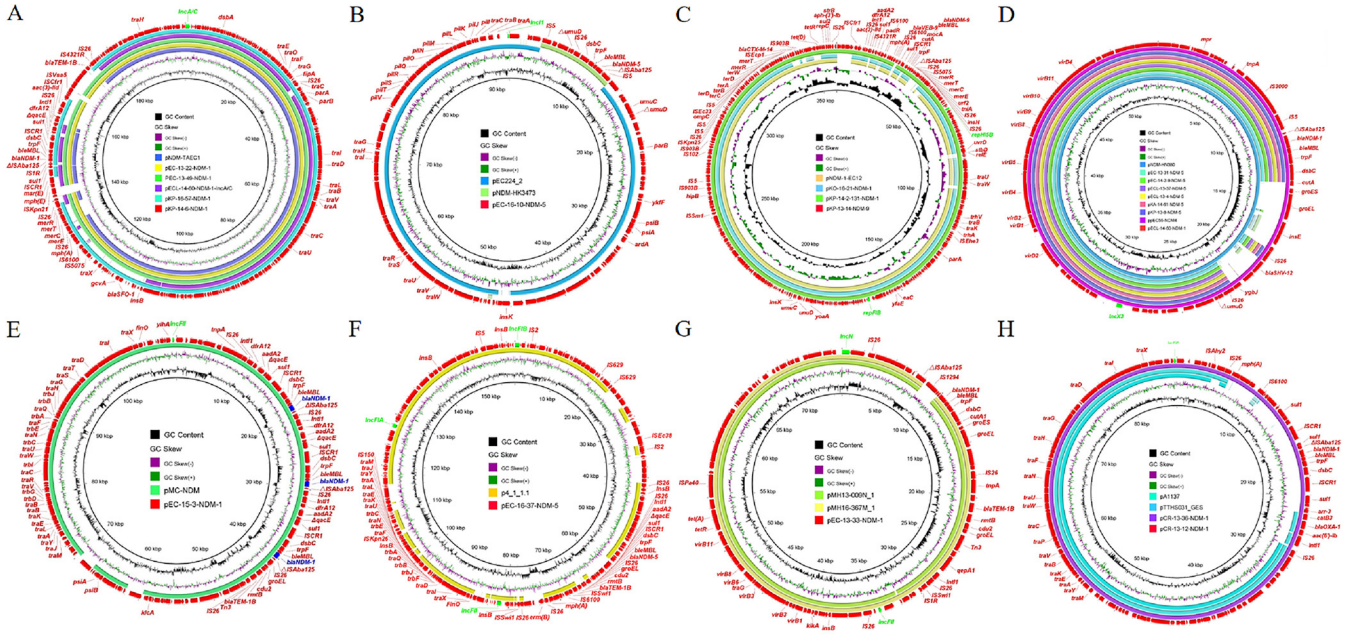


**FIG 6** Circular comparison of different *bla*$_{NDM}$-bearing plasmids with similar online plasmids. A–H represent different *bla*$_{NDM}$-bearing plasmids with various replicons IncC, IncI1, IncHI5-like, IncX3, IncFII, IncFIA-IncFIB-IncFII, IncFII-IncN, and IncFII(p14).

**TABLE 2** Basic information of 22 *bla*$_{NDM}$-bearing plasmids resolved by Illumina and Nanopore long-read sequencing

| Plasmid | Strain | Status | Size (bp) | Inc-type | Assembly method | Sequencing technology | Accession no. | Resistance genes |
|---|---|---|---|---|---|---|---|---|
| pCR-13-12-NDM-1 | CR-13-12 | complete | 86 619 | IncFII | Unicycler | Oxford Nanopore MinION, Illumina | NZ_MN175388 | *aac(6′)-Ib*, *arr-3*, *bla*$_{NDM-1}$, *bla*$_{OXA-1}$, *ble*$_{MBL}$, *catB3*, *mph*(A), *sul1* |
| pCR-13-36-NDM-1 | CR-13-36 | complete | 86 619 | IncFII | Unicycler | Oxford Nanopore MinION, Illumina | MZ857202 | *aac(6′)-Ib*, *arr-3*, *bla*$_{NDM-1}$, *bla*$_{OXA-1}$, *ble*$_{MBL}$, *catB3*, *mph*(A), *sul1* |
| pEC-13-22-NDM-1 | EC-13-22 | complete | 212 551 | IncC | Unicycler | Oxford Nanopore MinION, Illumina | MZ836796 | *aac(3)-IId*, *aph(3′)-VI*, *bla*$_{NDM-1}$, *bla*$_{SFO-1}$, *bla*$_{TEM-1}$, *ble*$_{MBL}$, *dfrA12*, *mph*(A), *mph*(E), *msr*(E), *sul1* |
| pEC-13-31-NDM-5 | EC-13-31 | complete | 49 021 | IncX3 | Unicycler | Oxford Nanopore MinION, Illumina | MZ836797 | *bla*$_{NDM-5}$, *ble*$_{MBL}$ |
| pEC-13-33-NDM-1 | EC-13-33 | complete | 74 978 | IncFII-IncN | Unicycler | Oxford Nanopore MinION, Illumina | MZ836798 | *bla*$_{NDM-1}$, *bla*$_{TEM-1}$, *ble*$_{MBL}$, *qepA1*, *rmtB1*, *tet*(A) |
| pEC-13-49-NDM-1 | EC-13-49 | complete | 214 323 | IncC | Unicycler | Oxford Nanopore MinION, Illumina | MZ836799 | *aac(3)-IId*, *aph(3′)-VI*, *bla*$_{NDM-1}$, *bla*$_{SFO-1}$, *bla*$_{TEM-1}$, *ble*$_{MBL}$, *dfrA12*, *mph*(A), *mph*(E), *msr*(E), *sul1* |
| pEC-14-2-9-NDM-5 | EC-14-2-9 | complete | 46 161 | IncX3 | Unicycler | Oxford Nanopore MinION, Illumina | MZ836800 | *bla*$_{NDM-5}$, *ble*$_{MBL}$ |
| pEC-15-3-NDM-1 | EC-15-3 | complete | 109 944 | IncFII | Unicycler | Oxford Nanopore MinION, Illumina | NZ_MN061455 | *aadA2*, *bla*$_{NDM-1}$, *bla*$_{TEM-1}$, *ble*$_{MBL}$, *dfrA12*, *rmtB1*, *sul1* |
| pEC-16-10-NDM-5 | EC-16-10 | complete | 92 260 | IncI1 | Unicycler | Oxford Nanopore MinION, Illumina | MZ836801 | *bla*$_{NDM-5}$, *ble*$_{MBL}$ |
| pEC-16-37-NDM-5 | EC-16-37 | complete | 157 578 | IncFII-IncFIA-IncFIB | Unicycler | Oxford Nanopore MinION, Illumina | MZ836802 | *aadA2*, *bla*$_{NDM-5}$, *bla*$_{TEM-1}$, *ble*$_{MBL}$, *dfrA12*, *erm*(B), *mph*(A), *rmtB1*, *sul1* |
| pEC55-NDM4 | EC-14-55 | complete | 54 035 | IncX3 | Unicycler | Oxford Nanopore MinION, Illumina | NZ_KX470734 | *bla*$_{NDM-4}$, *bla*$_{SHV-12}$, *ble*$_{MBL}$ |
| pECL-13-37-NDM-5 | ECL-13-37 | complete | 46 161 | IncX3 | Unicycler | Oxford Nanopore MinION, Illumina | MZ836804 | *bla*$_{NDM-5}$, *ble*$_{MBL}$ |
| pECL-13-4-NDM-5 | ECL-13-4 | complete | 46 161 | IncX3 | Unicycler | Oxford Nanopore MinION, Illumina | MZ836803 | *bla*$_{NDM-5}$, *ble*$_{MBL}$ |
| pECL-14-60-NDM-1-IncAC | ECL-14-60 | complete | 171 038 | IncC | Unicycler | Oxford Nanopore MinION, Illumina | MZ836805 | *aac(6′)-Ib*, *aph(3′)-Ia*, *armA*, *arr-3*, *bla*$_{NDM-1}$, *bla*$_{OXA-1}$, *ble*$_{MBL}$, *catB3*, *mph*(E), *msr*(E), *qnrA7*, *sul1*, *sul2* |
| pECL-14-60-NDM-1 | ECL-14-60 | complete | 53 023 | IncX3 | Unicycler | Oxford Nanopore MinION, Illumina | NZ_MN061454 | *bla*$_{NDM-1}$, *bla*$_{SHV-12}$, *ble*$_{MBL}$ |
| pKA-14-61-NDM-5 | KA-14-61 | complete | 46 161 | IncX3 | Unicycler | Oxford Nanopore MinION, Illumina | MZ836806 | *bla*$_{NDM-5}$, *ble*$_{MBL}$ |
| pKO-16-21-NDM-1 | KO-16-21 | complete | 376 570 | IncHI5-like | Unicycler | Oxford Nanopore MinION, Illumina | MZ836807 | *aac(3)-IId*, *aadA16*, *aph(3′′)-Ib*, *aph(6)-Id*, *arr-3*, *bla*$_{NDM-1}$, *bla*$_{TEM-1}$, *ble*$_{MBL}$, *dfrA27*, *mph*(A), *qnrB6*, *sul1*, *sul2* |
| pKP-13-14-NDM-9 | KP-13-14 | complete | 358 655 | IncHI5-like | Unicycler | Oxford Nanopore MinION, Illumina | NZ_MN175386 | *aac(3)-IId*, *aadA2*, *aph(3′′)-Ib*, *aph(6)-Id*, *bla*$_{CTX-M-14}$, *bla*$_{NDM-9}$, *bla*$_{TEM-1}$, *ble*$_{MBL}$, *dfrA12*, *mph*(A), *tet*(D), *sul1*, *sul2* |
| pKP-13-8-NDM-5 | KP-13-8 | complete | 46 161 | IncX3 | Unicycler | Oxford Nanopore MinION, Illumina | NZ_MN175389 | *bla*$_{NDM-5}$, *ble*$_{MBL}$ |
| pKP-14-2-131-NDM-1 | KP-14-2-131 | complete | 358 158 | IncHI5-like | Unicycler | Oxford Nanopore MinION, Illumina | MZ836808 | *aac(3)-IId*, *aadA2*, *aph(3′′)-Ib*, *aph(6)-Id*, *bla*$_{CTX-M-14}$, *bla*$_{NDM-1}$, *bla*$_{TEM-235}$, *ble*$_{MBL}$, *dfrA12*, *mph*(A), *tet*(D), *sul1*, *sul2* |
| pKP-14-6-NDM-1 | KP-14-6 | complete | 199 120 | IncC | Unicycler | Oxford Nanopore MinION, Illumina | NZ_MN175387 | *aac(3)-IId*, *bla*$_{NDM-1}$, *bla*$_{SFO-1}$, *bla*$_{TEM-1}$, *ble*$_{MBL}$, *dfrA12*, *mph*(A), *mph*(E), *msr*(E), *sul1* |
| pKP-16-57-NDM-1 | KP-16-57 | complete | 180 309 | IncC | Unicycler | Oxford Nanopore MinION, Illumina | MZ836809 | *aac(3)-IId*, *aadA2*, *bla*$_{NDM-1}$, *bla*$_{SFO-1}$, *ble*$_{MBL}$, *dfrA12*, *mph*(A), *sul1* |

of BLASTn, two Y2:A-:B- (pCR-13-12-NDM-1 and pCR-13-36-NDM-1) plasmids of IncFII were similar to the pA1137 (NZ_MF190369) and pTTHS031_GES (NZ_LC589514) plasmids in the NCBI database. In contrast to the plasmids in this study, they all lacked regions carrying the *bla*$_{NDM}$ gene, implying that the regions carrying the *bla*$_{NDM}$ may insert progenitors before forming these plasmids. Moreover, two-hybrid plasmids pEC-16-37-NDM-5 (IncFII-IncFIA-IncFIB) and pEC-13-33-NDM-1 (IncFII-IncN) were also found. The pEC-16-37-NDM-5 plasmid was similar to the online IncFII-IncFIA-IncFIB plasmid p4_1_1.1 (NZ_CP023845) in *E. coli*. The plasmid pEC-13-33-NDM-1 was similar to both of the online IncFII-IncN plasmids pMH13-009N_1 (AP018566) and pMH16-367M_1 (AP018565) found in *Proteus mirabilis* and *Morganella morganii*, respectively. The core genetic environment of *bla*$_{NDM-5}$ in pEC-16-37-NDM-5 is IS*CR1-dsbC-trpF-ble*$_{MBL}$-*bla*$_{NDM-5}$-ΔIS*Aba125*-IS*26*. Although there are only four IncFII-type plasmids carrying *bla*$_{NDM-1}$, the gene environment around *bla*$_{NDM-1}$ could be divided into three categories: Tn*As3-groEL-cutA-dsbC-trpF-ble*$_{MBL}$-*bla*$_{NDM-1}$-ΔIS*Aba125*-IS*1294* (pEC-13-33-NDM-1), IS*CR1-dsbC-trpF-ble*$_{MBL}$-*bla*$_{NDM-1}$-ΔIS*Aba125*-IS*26* (pEC-15-3-NDM-1 and pCR-13-12-NDM-1), and IS*CR1-dsbC-trpF-ble*$_{MBL}$-*bla*$_{NDM-1}$-ΔIS*Aba125*-IS*CR1* (pCR-13-36-NDM-1).

The characteristics of the five NDM-positive plasmids (171 kb–215 kb) of the IncC type were also analyzed (Fig. 6). The *bla*$_{NDM}$ subtypes carried by these plasmids were all *bla*$_{NDM-1}$, and most of them shared similar backbones. Despite their similar backbones, there are three types of genetic environments around *bla*$_{NDM-1}$: IS*CR1-dsbC-trpF-ble*$_{MBL}$-*bla*$_{NDM-1}$-ΔIS*Aba125*-IS*1R* (pECL-14-60-NDM-1 and pKP-14-6-NDM-1), IS*CR1-dsbC-trpF-ble*$_{MBL}$-*bla*$_{NDM-1}$-IS*Aba125* (pEC-13-22-NDM-1 and pEC-13-49-NDM-1), and IS*CR1-dsbC-trpF-ble*$_{MBL}$-*bla*$_{NDM-1}$-ΔIS*Aba125*-IS*26* (pKP-16-57-NDM-1). Among these five IncC plasmids, pEC-13-22-NDM-1 and pEC-13-49-NDM-1 were isolated from *E. coli*, pKP-16-57-NDM-1 and pKP-14-6-NDM-1 from *K. pneumoniae*, and pECL-14-60-NDM-1 from *E. hormaechei*. BLASTn comparison with the NCBI database showed similarities to pNDM-TAEC1 (NZ_MH001166) found in *E. coli*.

It is worth noting that three plasmids belonged to the recently discovered IncHI5-like plasmids (Fig. 6). The three *bla*$_{NDM}$-harboring IncHI5-like plasmids ranged from 358 to 376 kb and possessed the same plasmid backbone structure. The BLAST comparison against the GenBank database showed that plasmid pKO-16-21-NDM-1 from *K. oxytoca* exhibited similarities to the same Inc-type plasmids pKP19-3023-374k (CP063748) and pKP19-3088-375k (CP063149), which were collected from *K. pneumoniae*. The core genetic environment of *bla*$_{NDM}$ (IS*CR1-sul1-*Δ*qacE-bla*$_{NDM-1}$-*ble*$_{MBL}$-*trpF-dsbC*-IS*CR1*) carried on the plasmid pKO-16-21-NDM-1 was similar to the pKP19-3023-374k plasmid. This is the first time that a *bla*$_{NDM}$-positive IncHI5-like plasmid has been found in *K. oxytoca*. The pKP-13-14-NDM-9 plasmid that was isolated from *K. pneumoniae* was 358,655 bp in size. Although IncHI5-like plasmids were reported to carry *bla*$_{NDM-1}$ in previous studies (12, 13), pKP-13-14-NDM-9 was the first IncHI5-like plasmid positive for *bla*$_{NDM-9}$. The core genetic environment of *bla*$_{NDM-9}$ is IS*26*-ΔIS*Aba125-bla*$_{NDM-9}$-*ble*$_{MBL}$-*trpF-mocA-cutA*-IS*CR1*, and a similar genetic environment (IS*26*-ΔIS*Aba125-bla*$_{NDM-1}$-*ble*$_{MBL}$-*trpF-mocA-cutA*-IS*CR1*) was found in pKP-14-2-131-NDM-1.

Four of the 81 strains were found to carry both *bla*$_{NDM}$ and *mcr* genes (*mcr-1*, $n = 1$, *mcr-9*, $n = 3$). The *mcr-1* gene was located on a 60,961 bp plasmid designated as pEC-15-3-mcr-1 in the incompatibility group IncI2 (Fig. S6). The plasmids similar to pEC-15-3-mcr-1 in the public database were the *E. coli* plasmid pAH62-1 (NZ_CP055260) and *Salmonella* plasmid pS304_2 (NZ_CP061128), which showed 100% coverage and identity. Moreover, three strains were found (CF-15-2-29, ECL-16-5, and ECL-16-79) carrying the *mcr-9*. Online BLAST (Fig. S7) showed that *mcr-9*-positive plasmids all belonged to IncHI2A-IncHI2 and showed similarities to the pBSI034-MCR9 (NZ_MN937241) plasmid. Strains carrying *mcr-9* were usually resistant to polymyxin; however, ECL-16-79 was susceptible to polymyxin. It has been reported that the deletion of the two-component system *qseCB* may silence the *mcr-9* gene (14). However, the ECL-16-79 strain contains the two-component system *qseCB*, and other genes or molecules may regulate the expression of *mcr-9*. Further investigations are needed to decipher the underlying molecular mechanisms.

**Two tandem copies of *bla*$_{NDM-1}$ in the chromosome.** In addition to the plasmid-mediated *bla*$_{NDM}$ genes, we also found the *bla*$_{NDM-1}$ on the chromosome of the *P. rettgeri* strain Pr-15-2-50. The size of the genome was 4,648,900 bp, with 40.3% GC content. Two copies of *bla*$_{NDM-1}$ were found on the chromosome of the Pr-15-2-50. On comparing the Pr-15-2-50 chromosome with FZB001 (CP060821) and AR0156 (CP021852), we found a 40,775 bp Tn*7*-like transposon structure carrying the *bla*$_{NDM}$ gene, inserted into the chromosomal region (Fig. S8). This Tn*7*-like transposon had an average GC content of 48.8% and similarly to the p2BJAB07104 (CP003907) plasmid, it was surrounded by 11 bp inverted repeats (ACAAAATAGAT), implying that the transposon could translocate between chromosomes and plasmids. However, this plasmid lacked the *bla*$_{NDM}$-bearing region. The 5,250 bp *bla*$_{NDM}$-carrying region (IS*CR1-dsbC-trpF-ble*$_{MBL}$-*bla*$_{NDM-1}$-ΔIS*Aba125*-Δ*sul1*) may be incorporated because of the IS*CR1*-mediated insertion, similar to previous reports(15). Moreover, a 4,390 bp integron (*intI2-lnu*(F)-*dfrA1-aadA1*-Δ*qacE-sul1*) was found downstream to the *bla*$_{NDM-1}$ gene. Despite these reports, IS*CR1*-mediated copies of *bla*$_{NDM}$ have been found on these chromosomes. However, the IS*CR1*-mediated transposable units on *P. aeruginosa* MMA83 (IS*CR1-aph(3′)-VIa*-IS*Aba125-bla*$_{NDM-1}$-*sul1*), *E. coli* Y5 (IS*CR1-traF-ble*$_{MBL}$-*bla*$_{NDM-1}$-ΔIS*Aba125-catB3-arr-3*-Δ*qacE-sul1*), and *P. mirabilis* XH1653 (*sul1-arr-3-cat-bla*$_{NDM-1}$-*bleo*-IS*CR1*) are different from the Pr-15-2-50 (IS*CR1-dsbC-trpF-ble*$_{MBL}$-*bla*$_{NDM-1}$-ΔIS*Aba125*-Δ*sul1*) strain in this study (15–17). The *ble* and *sul1* genes were also detected in these transposable units. This suggests that *bla*$_{NDM}$ may cotransfer with other resistance genes.

## DISCUSSION

*bla*$_{NDM-1}$ was discovered in 2009. Since then, CRE strains carrying *bla*$_{NDM-1}$ and its variants have spread in more than 55 countries worldwide. Asian countries such as India, Pakistan, and China are considered major reservoirs of *bla*$_{NDM}$ (6). The *bla*$_{NDM-1}$-positive strains were first isolated in clinical stool samples in China in 2010, followed by an increasing number of *bla*$_{NDM}$-positive strains. In 2013, 17 *bla*$_{NDM}$-positive strains (38.64%) were obtained from 44 CRE strains isolated from hospitals in Henan, which was an increase compared to 2011–2012 (7). However, the positive rate decreased to 18.89% (17/90) and 17.13% (31/181) in 2014 and 2015, respectively. This may be the result of the effective clinical infection control measures. However, there was an increase in 2016, with the isolation rates reaching up to 21.79% (17/78).

ST11 is the most common type of *bla*$_{NDM}$-positive *K. pneumoniae* that was reported (18, 19). Moreover, ST11 *K. pneumoniae* often had hypervirulent and/or multidrug resistant phenotypes (20). However, only one strain of ST11 *K. pneumoniae* was found in this study. The ST types of *K. pneumoniae* were more diverse, indicating that *K. pneumoniae* carrying *bla*$_{NDM}$ in Henan is not clonally transferred. Moreover, we found diverse *E. coli* STs, and ST167 was predominant among them. This phenomenon is similar to previous domestic reports (21, 22). ST167 NDM-producing *E. coli* strains are not only widely disseminated in China (11, 23); they also cause infections worldwide (6, 24, 25), which has gained much attention. Consistent with this study, the *bla*$_{NDM-5}$ gene is mainly carried by *E. coli* of ST167 (26, 27), suggesting that ST167 *E. coli* is an important repository of *bla*$_{NDM-5}$. More importantly, ST167 *bla*$_{NDM}$-positive *E. coli* strains have been found in companion animals (28, 29), which suggests that the ST167 *E. coli* carrying *bla*$_{NDM-5}$ gene could be transmitted between animals and humans.

Four NDM subtypes (NDM-1, NDM-4, NDM-5, and NDM-9) were found in 81 NDM-producing strains; however, from 2011 to 2012, all *bla*$_{NDM}$-positive strains isolated from Henan were *bla*$_{NDM-1}$ (7). Since the isolation of *bla*$_{NDM-5}$ in Henan in 2013, the detection rate has gradually increased. It has now become the main subtype of *bla*$_{NDM}$. The *bla*$_{NDM-1}$ detection rates have been decreasing each year; however, it remains the main epidemic subtype. Previous studies have shown that NDM-5 exhibits higher hydrolytic activity toward carbapenems and cephalosporins compared with NDM-1 (30). This may be caused by the increase in the usage of carbapenems in clinical treatment. It has been shown that IncX3 plasmids could promote the transmission of NDM-5, and the

plasmids carrying NDM-5 demonstrated high stability (31, 32). In this study, most of the *bla*$_{NDM-5}$ genes were carried by IncX3 plasmids, which led to a higher prevalence. The increasing prevalence of *bla*$_{NDM-5}$-positive strains should be of high concern.

Carbapenem and colistin are considered the last line of defense in the treatment of severe infections caused by extensively drug-resistant bacteria. Only a few articles have previously reported the coexistence of *bla*$_{NDM-1}$ and *mcr-9* genes (33, 34). Four strains with the coexistence of *bla*$_{NDM}$ and *mcr* genes were found in this study. This phenomenon greatly increases the risk of treatment failure. The *bla*$_{IMP-4}$-producing *Enterobacterales* have been reported sporadically in China (11, 35). Only two strains harboring the *bla*$_{IMP-4}$ gene were found among the 81 *bla*$_{NDM}$-positive strains. Moreover, multiple resistance genes are often present on plasmids carrying *bla*$_{NDM}$ genes, which greatly increases the risk of cotransmission of multiple resistance genes.

Except for the strain Pr-15-2-50, the *bla*$_{NDM}$ gene was located on the plasmids, which might be the main mode of *bla*$_{NDM}$ transmission. A variety of *bla*$_{NDM}$-positive plasmids with different Inc types and sizes were found in the 80 strains, mainly IncX3 type, which is similar to previous reports. In this study, the ST type of NDM-producing strains carrying the IncX3 plasmid was mainly ST167. This highly prevalent ST and plasmid type promotes the transmission of *bla*$_{NDM}$ further and seriously threatens public health. We also discovered a novel *bla*$_{NDM}$-bearing plasmid pEC-16-10-NDM-5 (IncI1). IncI1 plasmid belongs to the narrow-host range plasmid type (36) and was only found in *Enterobacterales*. Several articles have pointed out that IncI1 plasmids frequently carry genes encoding antibiotic resistance, especially the extended-spectrum beta-lactamase genes (37–39). These plasmids are widely distributed in animals and patients worldwide (40, 41). The IncHI plasmid has a wide host range and plays an important role in the transmission of resistance genes (42, 43). Previously, it was shown that a variety of carbapenemase genes were found on the IncHI5 plasmids (44), which poses a great threat to clinical treatment. The two IncHI5-like plasmids, carrying both carbapenem and tigecycline resistance genes, were found in our recent study (12), severely restricting the clinical treatment options. In this study, the new *bla*$_{NDM}$ core genetic environment was found in the IncHI5-like plasmids, suggesting that this plasmid has evolved as a novel MDR plasmid and needs to be continuously monitored.

**Conclusion.** To date, NDM is the predominant mechanism for CRE in humans. Carbapenem, polymyxin, and tigecycline are regarded as the last line of defense in the clinical treatment of MDR infections. In recent years, several studies have found that *bla*$_{NDM}$ coexists with mobile colistin (*mcr*) and tigecycline resistance genes (*tet*(X) and *tmexCD-toprJ*), making clinical treatment extremely difficult. Therefore, continuous long-term surveillance for pathogens that clinically harbor *bla*$_{NDM}$ is important. This study conducted an in-depth analysis of *bla*$_{NDM}$-positive clinical strains and confirmed that the vast majority of *bla*$_{NDM}$ genes were distributed on plasmids of different Inc types, and are transmitted by horizontal transfer of plasmids. The emergence of *Enterobacterales* carrying both *bla*$_{NDM}$ and other resistance genes, such as *mcr*, is worrying. These isolates can seriously limit clinical treatment options. Therefore, there is an urgent need for large-scale monitoring and the development of effective control measures.

## MATERIALS AND METHODS

**Bacterial isolates.** The samples in this study were obtained between 2013 and 2016 at an affiliated hospital of Zhengzhou University. This study did not exclude patients based on age, gender, or symptoms. Moreover, the samples collected were nonduplicate isolates from different patients. CRE was defined as *Enterobacterales* resistant to at least one carbapenem (meropenem or imipenem). A total of 391 CRE strains were collected from blood, urine, sputum, wound, tissue, pus, swab, drainage liquid, secreta, bile, ascites, sanies, joint fluid, and urine tube tips. Clinical data of each patient were collected from the clinical and medical record system. Extracted clinical information included the date of collection, patient age, sex, source of isolate, ward type, and outcome (alive or dead). The *bla*$_{NDM}$-positive strains were screened and confirmed using PCR and Sanger sequencing, respectively. All *bla*$_{NDM}$-positive isolates were sent to Zhengzhou University for subsequent experiments. This study was approved by the Ethics Committee of Zhengzhou University with a waiver of informed consent because of the retrospective nature of the study.

**PCR screening and antimicrobial susceptibility testing.** The presence of carbapenem resistance genes (*bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{KPC}$, *bla*$_{VIM}$, and *bla*$_{OXA-48}$) and other important resistance genes (*mcr-1*, *bla*$_{SHV}$,

and $bla_{TEM}$) was investigated using PCR with the primers (Table S1). The PCR amplified products were confirmed using gel electrophoresis and Sanger sequencing. All CRE species identification was carried out by the automated Vitek 2 system. Antimicrobial susceptibility testing of clinical strains was performed against 17 antimicrobials by determining the MICs using the broth microdilution method, and *E. coli* ATCC 25922 was used as the quality control. All antibiotic breakpoints were interpreted according to CLSI guidelines (45); however, tigecycline (>2 mg/L) was interpreted according to the EUCAST criteria.

**Conjugation, S1-PFGE, and Southern blot.** The conjugation experiment was performed with each of the $bla_{NDM}$-positive strains using a rifampicin-resistant *E. coli* EC600 or sodium azide-resistant *E. coli* J53 recipients. The donor and recipient were mixed in a ratio of 1:1 and incubated statically in an LB broth at 35°C for 24 h. Transconjugants on the LB agar plates containing double antibiotics (meropenem 2 mg/L and rifampicin 100 mg/L, or meropenem 2 mg/L and sodium azide 200 mg/L) were selected and confirmed using PCR and PFGE, respectively. Transfer frequencies were calculated as the number of transconjugants/total number of recipients.

S1-PFGE and Southern blot analyses were performed to determine the plasmid sizes and genomic positions of $bla_{NDM}$. To elucidate the genetic environments of $bla_{NDM}$ genes, 22 representative $bla_{NDM}$-carrying plasmids were selected based on the plasmid replicon types and sizes to perform Nanopore sequencing to obtain the complete plasmid sequences.

**WGS procedures and analyses.** We characterized the genetic features and resistomes of the $bla_{NDM}$-positive CRE. The genomes of all $bla_{NDM}$-positive strains were extracted with the FastPure bacterial DNA isolation minikit (catalog no. DC103; Vazyme) and evaluated using 1% (wt/vol) agarose gel electrophoresis. The concentration and purity were quantified using the Qubit 4 Fluorometer and Nanodrop. The genomic DNA samples were sequenced using the Illumina Hiseq 2500 platform generating 2 × 150 bp paired-end reads. Twenty-two representative strains were sequenced with the Nanopore long-read sequencing platform according to resistant phenotypes and genotypes (46). The Rapid Barcoding Kit RBK004 was used to construct the long-read sequencing libraries, which were subjected to Nanopore sequencing in MinION R9.4.1 flow cells.

The Illumina paired-end reads were *de novo* assembled using the SPAdes version 3.14.0, and contigs less than 200 bp in length were removed (47). Unicycler v. 0.4.8 was used for hybrid assembly of genomes with the combination of Illumina short reads and Nanopore long reads with default parameters (48). For intricate regions that could not be resolved using the hybrid assembly method, Nanopore sequencing data were assembled using the long-read assembler Flye v. 2.4.2 to acquire accurate structures of complex genomic regions (49). The genomes were annotated using the online tool RAST (http://rast.nmpdr.org/). ResFinder and PlasmidFinder (http://cge.cbs.dtu.dk/services/) were used to identify antimicrobial resistance genes and plasmid replicon types with default parameters. The virulence factors in the assembled genome sequences were identified using the Kleborate software (50) and the virulence factor database (last updated 14th October 2020) in abricate v.1.0.1 (https://github.com/tseemann/abricate) with default parameters. Multilocus sequence typing (MLST) of the 81 $bla_{NDM}$-positive isolates was conducted using mlst (https://github.com/tseemann/mlst). The plasmid comparison maps were constructed and displayed by using BRIG v. 0.95 and Easyfig v. 2.2.3 (51, 52), respectively. The core genes in the genomes of $bla_{NDM}$-positive CRE were identified using Roary (53). The phylogenetic trees of $bla_{NDM}$-positive strains were constructed using FastTree (54) based on the core single-nucleotide polymorphism (SNP) alignments with default parameter settings and visualized using iTOL (https://itol.embl.de).

**Data availability.** The sequence data generated in this study have been submitted to the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/) under accession numbers PRJNA752009, and individual accession numbers of 22 $bla_{NDM}$-bearing plasmids are listed in Table 2.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 1.3 MB.

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## REFERENCES

1. van der Bij AK, Pitout JDD. 2012. The role of international travel in the worldwide spread of multiresistant Enterobacteriaceae. J Antimicrob Chemother 67:2090–2100. https://doi.org/10.1093/jac/dks214.
2. Karaiskos I, Giamarellou H. 2014. Multidrug-resistant and extensively drug-resistant Gram-negative pathogens: current and emerging therapeutic approaches. Expert Opin Pharmacother 15:1351–1370. https://doi.org/10.1517/14656566.2014.914172.
3. Zhang Y, Wang Q, Yin Y, Chen H, Jin L, Gu B, Xie L, Yang C, Ma X, Li H, Li W, Zhang X, Liao K, Man S, Wang S, Wen H, Li B, Guo Z, Tian J, Pei F, Liu L, Zhang L, Zou C, Hu T, Cai J, Yang H, Huang J, Jia X, Huang W, Cao B, Wang H. 2018. Epidemiology of carbapenem-resistant *Enterobacteriaceae* infections: report from the China CRE network. Antimicrob Agents Chemother 62:e01882-17. https://doi.org/10.1128/AAC.01882-17.
4. Nordmann P, Poirel L, Walsh TR, Livermore DM. 2011. The emerging NDM carbapenemases. Trends Microbiol 19:588–595. https://doi.org/10.1016/j.tim.2011.09.005.
5. Poirel L, Bonnin RA, Boulanger A, Schrenzel J, Kaase M, Nordmann P. 2012. Tn125-related acquisition of $bla_{NDM}$-like genes in *Acinetobacter baumannii*. Antimicrob Agents Chemother 56:1087–1089. https://doi.org/10.1128/AAC.05620-11.

6. Wu W, Feng Y, Tang G, Qiao F, McNally A, Zong Z. 2019. NDM metallo-beta-Lactamases and their bacterial producers in health care settings. Clin Microbiol Rev 32:e00115-18. https://doi.org/10.1128/CMR.00115-18.
7. Qin S, Fu Y, Zhang Q, Qi H, Wen JG, Xu H, Xu L, Zeng L, Tian H, Rong L, Li Y, Shan L, Xu H, Yu Y, Feng X, Liu HM. 2014. High incidence and endemic spread of NDM-1-positive *Enterobacteriaceae* in Henan Province, China. Antimicrob Agents Chemother 58:4275–4282. https://doi.org/10.1128/AAC.02813-13.
8. Chen Y, Zhou Z, Jiang Y, Yu Y. 2011. Emergence of NDM-1-producing *Acinetobacter baumannii* in China. J Antimicrob Chemother 66:1255–1259. https://doi.org/10.1093/jac/dkr082.
9. Zhou G, Guo S, Luo Y, Ye L, Song Y, Sun G, Guo L, Chen Y, Han L, Yang J. 2014. NDM-1-producing strains, family *Enterobacteriaceae*, in hospital, Beijing, China. Emerg Infect Dis 20:340–342. https://doi.org/10.3201/eid2002.121263.
10. Wang Q, Wang X, Wang J, Ouyang P, Jin C, Wang R, Zhang Y, Jin L, Chen H, Wang Z, Zhang F, Cao B, Xie L, Liao K, Gu B, Yang C, Liu Z, Ma X, Jin L, Zhang X, Man S, Li W, Pei F, Xu X, Jin Y, Ji P, Wang H. 2018. Phenotypic and genotypic characterization of carbapenem-resistant *Enterobacteriaceae*: data from a longitudinal large-scale CRE study in China (2012–2016). Clin Infect Dis 67:S196–S205. https://doi.org/10.1093/cid/ciy660.
11. Zhang R, Liu L, Zhou H, Chan EW, Li J, Fang Y, Li Y, Liao K, Chen S. 2017. Nationwide surveillance of clinical carbapenem-resistant Enterobacteriaceae (CRE) strains in China. EBioMedicine 19:98–106. https://doi.org/10.1016/j.ebiom.2017.04.032.
12. Qin S, Peng J, Deng R, Peng K, Yan T, Chen F, Li R. 2021. Identification of two plasmids coharboring carbapenemase genes and *tmexCD1-toprJ1* in clinical *Klebsiella pneumoniae* ST2667. Antimicrob Agents Chemother 65:e00115-18. https://doi.org/10.1128/AAC.00625-21.
13. Liu Z, Chen R, Xu P, Wang Z, Li R. 2021. Characterization of a *bla*$_{NDM-1}$-bearing IncHI5-like plasmid from *Klebsiella pneumoniae* of infant origin. Front Cell Infect Microbiol 11:738053. https://doi.org/10.3389/fcimb.2021.738053.
14. Kieffer N, Royer G, Decousser JW, Bourrel AS, Palmieri M, Ortiz De La Rosa JM, Jacquier H, Denamur E, Nordmann P, Poirel L. 2019. mcr-9, an inducible gene encoding an acquired phosphoethanolamine transferase in *Escherichia coli*, and its origin. Antimicrob Agents Chemother 63:e00965-19. https://doi.org/10.1128/AAC.00965-19.
15. Shen P, Yi M, Fu Y, Ruan Z, Du X, Yu Y, Xie X. 2017. Detection of an *Escherichia coli* sequence type 167 strain with two tandem copies of *bla*$_{NDM-1}$ in the chromosome. J Clin Microbiol 55:199–205. https://doi.org/10.1128/JCM.01581-16.
16. He J, Sun L, Zhang L, Leptihn S, Yu Y, Hua X. 2021. A novel SXT/R391 integrative and conjugative element carries two copies of the *bla*$_{NDM-1}$ gene in *Proteus mirabilis*. mSphere. https://doi.org/10.1128/mSphere.00588-21.
17. Jovcic B, Lepsanovic Z, Begovic J, Rakonjac B, Perovanovic J, Topisirovic L, Kojic M. 2013. The clinical isolate *Pseudomonas aeruginosa* MMA83 carries two copies of the *bla*$_{NDM-1}$ gene in a novel genetic context. Antimicrob Agents Chemother 57:3405–3407. https://doi.org/10.1128/AAC.02312-12.
18. Jain A, Hopkins KL, Turton J, Doumith M, Hill R, Loy R, Meunier D, Pike R, Livermore DM, Woodford N. 2014. NDM carbapenemases in the United Kingdom: an analysis of the first 250 cases. J Antimicrob Chemother 69:1777–1784. https://doi.org/10.1093/jac/dku084.
19. Yoon EJ, Yang JW, Kim JO, Lee H, Lee KJ, Jeong SH. 2018. Carbapenemase-producing *Enterobacteriaceae* in South Korea: a report from the National Laboratory Surveillance System. Future Microbiol 13:771–783. https://doi.org/10.2217/fmb-2018-0022.
20. Gu D, Dong N, Zheng Z, Lin D, Huang M, Wang L, Chan EW, Shu L, Yu J, Zhang R, Chen S. 2018. A fatal outbreak of ST11 carbapenem-resistant hypervirulent *Klebsiella pneumoniae* in a Chinese hospital: a molecular epidemiological study. Lancet Infect Dis 18:37–46. https://doi.org/10.1016/S1473-3099(17)30489-9.
21. Zong Z, Fenn S, Connor C, Feng Y, McNally A. 2018. Complete genomic characterization of two *Escherichia coli* lineages responsible for a cluster of carbapenem-resistant infections in a Chinese hospital. J Antimicrob Chemother 73:2340–2346. https://doi.org/10.1093/jac/dky210.
22. Xu L, Wang P, Cheng J, Qin S, Xie W. 2019. Characterization of a novel *bla*$_{NDM-5}$-harboring IncFII plasmid and an *mcr-1*-bearing IncI2 plasmid in a single *Escherichia coli* ST167 clinical isolate. Infect Drug Resist 12:511–519. https://doi.org/10.2147/IDR.S192998.
23. Bi R, Kong Z, Qian H, Jiang F, Kang H, Gu B, Ma P. 2018. High prevalence of *bla*$_{NDM}$ variants among carbapenem-resistant *Escherichia coli* in Northern Jiangsu Province, China. Front Microbiol 9:2704. https://doi.org/10.3389/fmicb.2018.02704.
24. Chakraborty T, Sadek M, Yao Y, Imirzalioglu C, Stephan R, Poirel L, Nordmann P. 2021. Cross-border emergence of *Escherichia coli* producing the carbapenemase NDM-5 in Switzerland and Germany. J Clin Microbiol 59:e02238-20. https://doi.org/10.1128/JCM.02238-20.
25. Mari-Almirall M, Cosgaya C, Pitart C, Vines J, Munoz L, Campo I, Cusco A, Rodriguez-Serna L, Santana G, Del Rio A, Francino O, Ciruela P, Pujol I, Ballester F, Marco F, Martinez JA, Soriano A, Vila J, Roca I, Group MES, MERCyCAT Study Group. 2021. Dissemination of NDM-producing *Klebsiella pneumoniae* and *Escherichia coli* high-risk clones in Catalan healthcare institutions. J Antimicrob Chemother 76:345–354. https://doi.org/10.1093/jac/dkaa459.
26. Garcia-Fernandez A, Villa L, Bibbolino G, Bressan A, Trancassini M, Pietropaolo V, Venditti M, Antonelli G, Carattoli A. 2020. Novel insights and features of the NDM-5-producing *Escherichia coli* sequence type 167 high-risk clone. mSphere 5:e00269-20. https://doi.org/10.1128/mSphere.00269-20.
27. Yang P, Xie Y, Feng P, Zong Z. 2014. *bla*$_{NDM-5}$ carried by an IncX3 plasmid in *Escherichia coli* sequence type 167. Antimicrob Agents Chemother 58:7548–7552. https://doi.org/10.1128/AAC.03911-14.
28. Cole SD, Peak L, Tyson GH, Reimschuessel R, Ceric O, Rankin SC. 2020. New Delhi metallo-beta-lactamase-5-producing *Escherichia coli* in companion animals, United States. Emerg Infect Dis 26:381–383. https://doi.org/10.3201/eid2602.191221.
29. Hong JS, Song W, Park HM, Oh JY, Chae JC, Han JI, Jeong SH. 2019. First detection of New Delhi metallo-beta-lactamase-5-producing *Escherichia coli* from companion animals in Korea. Microb Drug Resist 25:344–349. https://doi.org/10.1089/mdr.2018.0237.
30. Mei YF, Liu PP, Wan LG, Liu Y, Wang LH, Wei DD, Deng Q, Cao XW. 2017. Virulence and genomic feature of a virulent *Klebsiella pneumoniae* sequence type 14 strain of serotype K2 harboring *bla*$_{NDM-5}$ in China. Front Microbiol 8. https://doi.org/10.3389/fmicb.2017.00335.
31. Zhu WJ, Wang X, Qin JX, Liang W, Shen Z. 2020. Dissemination and stability of the *bla*$_{NDM-5}$-carrying IncX3-type plasmid among multiclonal *Klebsiella pneumoniae* isolates. Msphere 5:e00917-20. https://doi.org/10.1128/mSphere.00917-20.
32. Ma T, Fu J, Xie N, Ma S, Lei L, Zhai W, Shen Y, Sun C, Wang S, Shen Z, Wang Y, Walsh TR, Shen J. 2020. Fitness cost of *bla*$_{NDM-5}$-carrying p3R-IncX3 plasmids in wild-type NDM-free *Enterobacteriaceae*. Microorganisms 8:377. https://doi.org/10.3390/microorganisms8030377.
33. Sun L, Zhao X, Wang L, Guo X, Shi X, Hu L. 2021. Coexistence of *mcr-9* and *bla*$_{NDM-1}$ in a multidrug-resistant *Enterobacter hormaechei* strain recovered from a bloodstream infection in China. J Glob Antimicrob Resist 24:440–442. https://doi.org/10.1016/j.jgar.2021.02.011.
34. Ding M, Shi J, Ud Din A, Liu Y, Zhang F, Yan X, Li Q, Bai J, Chen W, Zhou Y. 2021. Co-infections of two carbapenemase-producing *Enterobacter hormaechei* clinical strains isolated from the same diabetes individual in China. J Med Microbiol 70. https://doi.org/10.1099/jmm.0.001316.
35. Zhang X, Chen D, Xu G, Huang W, Wang X. 2018. Molecular epidemiology and drug resistant mechanism in carbapenem-resistant *Klebsiella pneumoniae* isolated from pediatric patients in Shanghai, China. PLoS One 13:e0194000. https://doi.org/10.1371/journal.pone.0194000.
36. Rangnekar VM, Banker DD, Jhala HI. 1983. Antimicrobial resistance and incompatibility groups of R plasmids in *Salmonella typhimurium* isolated from human sources in Bombay from 1978 to 1980. Antimicrob Agents Chemother 23:54–58. https://doi.org/10.1128/AAC.23.1.54.
37. Bortolaia V, Guardabassi L, Trevisani M, Bisgaard M, Venturi L, Bojesen AM. 2010. High diversity of extended-spectrum beta-lactamases in *Escherichia coli* isolates from Italian broiler flocks. Antimicrob Agents Chemother 54:1623–1626. https://doi.org/10.1128/AAC.01361-09.
38. Leverstein-van Hall MA, Dierikx CM, Cohen Stuart J, Voets GM, van den Munckhof MP, van Essen-Zandbergen A, Platteel T, Fluit AC, van de Sande-Bruinsma N, Scharinga J, Bonten MJ, Mevius DJ, National ESBL surveillance group. 2011. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. Clin Microbiol Infect 17:873–880. https://doi.org/10.1111/j.1469-0691.2011.03497.x.
39. Diestra K, Juan C, Curiao T, Moya B, Miro E, Oteo J, Coque TM, Perez-Vazquez M, Campos J, Canton R, Oliver A, Navarro F, Red Espanola de Investigacion en Patologia Infecciosa. 2009. Characterization of plasmids encoding *bla*$_{ESBL}$ and surrounding genes in Spanish clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*. J Antimicrob Chemother 63:60–66. https://doi.org/10.1093/jac/dkn453.
40. Carattoli A, Villa L, Fortini D, Garcia-Fernandez A. 2021. Contemporary IncI1 plasmids involved in the transmission and spread of antimicrobial

resistance in Enterobacteriaceae. Plasmid 118:102392. https://doi.org/10.1016/j.plasmid.2018.12.001.
41. Chong Y, Shimoda S, Shimono N. 2018. Current epidemiology, genetic evolution and clinical impact of extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae*. Infect Genet Evol 61:185–188. https://doi.org/10.1016/j.meegid.2018.04.005.
42. Cain AK, Hall RM. 2012. Evolution of IncHI2 plasmids via acquisition of transposons carrying antibiotic resistance determinants. J Antimicrob Chemother 67:1121–1127. https://doi.org/10.1093/jac/dks004.
43. Rozwandowicz M, Brouwer MSM, Fischer J, Wagenaar JA, Gonzalez-Zorn B, Guerra B, Mevius DJ, Hordijk J. 2018. Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. J Antimicrob Chemother 73:1121–1137. https://doi.org/10.1093/jac/dkx488.
44. Zhu Y, Liu W, Schwarz S, Wang C, Yang Q, Luan T, Wang L, Liu S, Zhang W. 2020. Characterization of a $bla_{NDM-1}$-carrying IncHI5 plasmid from *Enterobacter cloacae* complex of food-producing animal origin. J Antimicrob Chemother 75:1140–1145. https://doi.org/10.1093/jac/dkaa010.
45. Clinical and Laboratory Standards Institute. 2018. M100: Performance standards for antimicrobial susceptibility testing, 27th ed. CLSI, Wayne, PA.
46. Li R, Lu X, Liu Z, Liu Y, Xiao X, Wang Z. 2020. Rapid detection and characterization of *tet*(X4)-positive *Escherichia coli* strains with nanopore sequencing. J Antimicrob Chemother 75:1068–1070. https://doi.org/10.1093/jac/dkz528.
47. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455–477. https://doi.org/10.1089/cmb.2012.0021.
48. Wick RR, Judd LM, Gorrie CL, Holt KE. 2017. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol 13:e1005595. https://doi.org/10.1371/journal.pcbi.1005595.
49. Kolmogorov M, Yuan J, Lin Y, Pevzner PA. 2019. Assembly of long, error-prone reads using repeat graphs. Nat Biotechnol 37:540–546. https://doi.org/10.1038/s41587-019-0072-8.
50. Lam MMC, Wyres KL, Judd LM, Wick RR, Jenney A, Brisse S, Holt KE. 2018. Tracking key virulence loci encoding aerobactin and salmochelin siderophore synthesis in *Klebsiella pneumoniae*. Genome Med 10:77. https://doi.org/10.1186/s13073-018-0587-5.
51. Alikhan NF, Petty NK, Ben Zakour NL, Beatson SA. 2011. BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. BMC Genomics 12:402. https://doi.org/10.1186/1471-2164-12-402.
52. Sullivan MJ, Petty NK, Beatson SA. 2011. Easyfig: a genome comparison visualizer. Bioinformatics 27:1009–1010. https://doi.org/10.1093/bioinformatics/btr039.
53. Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MT, Fookes M, Falush D, Keane JA, Parkhill J. 2015. Roary: rapid large-scale prokaryote pan genome analysis. Bioinformatics 31:3691–3693. https://doi.org/10.1093/bioinformatics/btv421.
54. Price MN, Dehal PS, Arkin AP. 2009. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. Mol Biol Evol 26:1641–1650. https://doi.org/10.1093/molbev/msp077.