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High Incidence and Endemic Spread of NDM-1-Positive *Enterobacteriaceae* in Henan Province, China

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The emergence and spread of New Delhi metallo-β-lactamase 1 (NDM-1)-producing carbapenem-resistant *Enterobacteriaceae* (CRE) present an urgent threat to human health. In China, the bla_{NDM-1} gene has been reported mostly in *Acinetobacter* spp. but is rarely found in *Enterobacteriaceae*. Here, we report a high incidence and endemic spread of NDM-1-producing CRE in Henan Province in China. Sixteen (33.3%) of the 48 CRE isolates obtained from patients during June 2011 to July 2012 were positive for bla_{NDM-1}, and the gene was found to be carried on plasmids of various sizes (~55 to ~360 kb). These plasmids were readily transferrable to recipient *Escherichia coli* by conjugation, conferred resistance to multiple antibiotics, and belonged to multiple replicon types. The bla_{NDM-1}-positive CRE isolates were genetically diverse, and six new multilocus sequence typing (MLST) sequence types were linked to the carriage of NDM-1. Five of the isolates were classified as extensively drug-resistant (XDR) isolates, four of which also carried the *fosA3* gene conferring resistance to fosfomycin, an alternative drug for treating infections by CRE. In each bla_{NDM-1}-positive CRE isolate, the bla_{NDM-1} gene was downstream of an intact IS*Aba125* element and upstream of the ble_{MBL} gene. Furthermore, gene environment analysis suggested the possible transmission of bla_{NDM-1}-containing sequences from *Acinetobacter* spp. to *Klebsiella pneumoniae* and *Klebsiella oxytoca*. These findings reveal the emergence and active transmission of NDM-1-positive CRE in China and underscore the need for heightened measures to control their further spread.

Carbapenems are the last-resort antibiotics against drug-resistant Gram-negative bacterial pathogens (1). However, carbapenem-resistant *Enterobacteriaceae* (CRE) are increasingly reported in health care-associated infections (HAIs) and are considered an urgent threat to human health by the Centers for Disease Control in the United States (2). Multiple genes conferring carbapenem resistance have been reported, which encode various types of carbapenemases (1). New Delhi metallo-β-lactamase 1 (NDM-1), an Ambler class B metallo-β-lactamase (MBL), hydrolyzes all β-lactams (including carbapenems) but monobactams and was first identified in *Klebsiella pneumoniae* and *Escherichia coli* isolated from a Swedish patient who was hospitalized in India in 2008 (3). Subsequently, the wide distribution of NDM-1-producing *Enterobacteriaceae* in India, Pakistan, and the United Kingdom was reported (4), and the Indian subcontinent was considered the main reservoir of NDM-1 producers (5). To date, the NDM-1-producing pathogens have been reported in more than 40 countries and have become a significant threat for public health worldwide (6).

In China, the bla_{NDM-1} gene was first identified in four nonclonal *Acinetobacter baumannii* isolates (7). Subsequent studies found that *Acinetobacter* spp. were the main species carrying this gene, but the carriage of bla_{NDM-1} was at a low frequency (<1.5%) (7–9). Carbapenem resistance in *Enterobacteriaceae* in China has been mainly associated with *Klebsiella pneumoniae* carbapenemases (KPCs), such as KPC-2, and MBLs (such as IMP-4) other than NDM-1 (10, 11). NDM-1-mediated carbapenem resistance in *Enterobacteriaceae* in China has been rarely reported; so far there have been only two confirmed cases of NDM-1-producing *K. pneumoniae* and *E. coli* infections in China (12, 13). A recent study detected a high infection rate (14.8%) of NDM-1-producing bacteria in clinical fecal samples from multiple hospitals in China, but none of the NDM-1-carrying bacteria belonged to *Enterobacteriaceae* (14). These observations suggested that NDM-1 was not prevalent in CRE isolates from China. However, in this study, we demonstrated the high incidence and endemic spread of NDM-1-producing *Enterobacteriaceae* in Henan Province in China. Additionally, we conducted molecular characterization of the bla_{NDM-1}-positive CRE isolates, revealing new molecular epidemiological features of CRE in China.

MATERIALS AND METHODS

Bacterial isolates, identification, and antimicrobial susceptibility testing. A total of 48 CRE (imipenem [MIC, ≥4 μg/ml] or meropenem [MIC, ≥2 μg/ml]) isolates, including 6 *E. coli*, 33 *K. pneumoniae*, 1 *Klebsiella oxytoca*, 5 *Enterobacter cloacae*, and 3 *Citrobacter freundii* isolates, were collected from diagnostic laboratories in three hospitals located in the middle (Zhengzhou, $n = 40$), western (Sanmenxia, $n = 4$), and southern (Zhumadian, $n = 4$) regions of Henan Province (north-central China). These isolates were obtained from June 2011 to July 2012. All isolates were identified using the Vitek 2 system (bioMérieux, France) and 16S rRNA gene sequencing. Antimicrobial susceptibilities for the bla_{NDM-1}-positive

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TABLE 1 Characteristics of bla_{NDM-1}-positive CRE isolates

Isolate[a]	Clinical features				Additional resistance determinants[b]			MLST[c]	Plasmid type carrying bla_{NDM-1}/ plasmid size (kb)
	Patient age/sex	Specimen	Diagnosis/ward[d]	Outcome	β-Lactamases	16S rRNA methylase	Others		
EC-01	76 yr/female	Urine	Pyelonephritis complicated with diabetes/endocrinology	Discharge	−	−	−	ST1237	Untypeable/230
EC-03	58 yr/male	Blood	Severe acute pancreatitis/ICU	Death	TEM-1, CTX-M-55, CMY-30	RmtB	FosA3	ST361	A/C/180
EC-13	6 days/male	Blood	Neonatal sepsis/NICU	Discharge	TEM-1, CMY-30	−	−	ST40	FIB/310
EC-24	56 yr/female	Urine	Diabetes and urinary tract infections/endocrinology	Discharge	CTX-M-15	−	FosA3	ST205	A/C/230
EC-SMX3	42 yr/male	Blood	Multiple injuries/EICU	Discharge	TEM-1, CTX-M-15, CMY-30	−	−	ST410	I1/60
EC-SMX5	63 yr/female	Sputum	Lung cancer/oncology	Discharge	CTX-M-15, CMY-30	−	−	ST361	A/C/260
KP-07	72 yr/female	Blood	Intracranial hemorrhage associated with cerebral infections/neurosurgery	Discharge	−	−	−	ST11	Untypeable/55
KP-09	1 yr/male	Urine	Multiple contusions as a result of a car accident/PICU	Discharge	TEM-1	RmtB	−	ST889	A/C/245
KP-40A	10 days/male	Blood	Neonatal sepsis/NICU	Death	TEM-1, CTX-M-15	−	−	ST966	A/C/−[e]
KP-41	9 mo/female	Blood	Septicemia/PICU	Death	−	−	−	ST113	N/55
KO-ZMD8	75 yr/male	Urine	Nephrotic syndrome/nephrology	Discharge	−	−	−	ND	Untypeable/55
ECL- ZMD10	49 yr/male	Wound	Extensive burns/burn unit	Discharge	−	ArmA	FosA3	ND	Untypeable/360
ECL- ZMD12	21 yr/female	Blood	Severe aplastic anemia/hematology	Death	−	ArmA	−	ND	A/C/55
ECL-36	15 days/male	Sputum	Lung infections and asphyxia/NICU	Death	MIR-2	−	−	ND	A/C/160
CF-SMX4	67 yr/male	Urine	Nephropathy/nephrology	Discharge	CMY-73	−	−	ND	Untypeable/55
CF-25	62 yr/male	Urine	Cerebral hemorrhage and lung infection/neurosurgery	Discharge	CMY-73	ArmA	FosA3	ND	A/C/170

[a] EC, *E. coli* strains; KP, *K. pneumoniae* strains; KO, *K. oxytoca* strains; ECL, *E. cloacae* strains; CF, *C. freundii* strains.
[b] Resistance markers that are cotransferred with bla_{NDM-1} by conjugation are underlined. Minus signs indicate negative results. PCR screening included bla_{TEM}; bla_{SHV}; $bla_{OXA-1-like}$; bla_{CTX-M} groups 1, 2, 8, 9, and 26; $bla_{OXA-48-like}$; bla_{IMP}; bla_{VIM}; bla_{KPC}; bla_{NDM}; 6 groups of bla_{AmpC} β-lactamase genes; and fosfomycin resistance genes *fosA*, *fosB*, *fosC*, and *fosX* as well as the *armA*, *rmtA-rmtE*, and *npmA* 16S methylase genes.
[c] STs that are newly identified harboring bla_{NDM-1} in this study are underlined. MLST, multilocus sequence typing; ND, not determined.
[d] ICU, intensive care unit; NICU, newborn ICU; EICU, emergency ICU; PICU, pediatric ICU.
[e] S1-PFGE and Southern blotting failed to detect the bla_{NDM-1} plasmid in KP-40A and its transconjugant, although the bla_{NDM-1} gene was detected in them by PCR and sequencing.

isolates and transconjugants were initially tested using the Vitek 2 system (bioMérieux, France) and then were followed by measuring the MIC using the broth microdilution method (for ampicillin-sulbactam, piperacillin-tazobactam, cefazolin, cefotetan, ceftazidime, cefepime, imipenem, ertapenem, ciprofloxacin, levofloxacin, gentamicin, amikacin, and aztreonam), the Etest (AB bioMérieux, Sweden) (for chloramphenicol, tetracycline, tigecycline, and colistin), and the agar dilution method (for fosfomycin), respectively. The standard microbroth and agar dilution methods were performed according to the guideline M07-A9 of the Clinical and Laboratory Standards Institute (CLSI) (15). Etests were conducted according to packet insert instructions using Mueller-Hinton agar (MHA). Fresh bacterial colonies taken directly from MHA plates that were incubated at 37°C for 16 to 20 h were resuspended in sterile Mueller-Hinton broth to obtain a suspension with McFarland standard 0.5 turbidity. *E. coli* ATCC 25922 was used as the quality control. The MIC results were interpreted according to the CLSI guideline M100-S22 (15). The 2013 European Committee on Antimicrobial Susceptibility Testing breakpoints were used (available at http://www.eucast.org/clinical_breakpoints/) for colistin and tigecycline.

Detection of resistance determinants. The modified Hodge test and the imipenem-EDTA double-disk synergy test were performed according to the CLSI guidelines to detect carbapenemase activity (15). PCR and nucleotide sequencing were employed to screen for the presence of carbapenemase-encoding genes, including $bla_{OXA-48-like}$, bla_{IMP}, bla_{VIM}, bla_{KPC}, and bla_{NDM}. In addition, extended-spectrum β-lactamase (ESBL) genes, plasmid-mediated AmpC genes, 16S rRNA methyltransferase genes, and fosfomycin resistance determinants were also detected by use of the methods described previously (16–20).

Bacterial genotyping. Pulsed-field gel electrophoresis (PFGE) of XbaI-digested genomic DNA of bla_{NDM-1}-positive CRE and reference marker *Salmonella* H9812 was performed using a contour-clamped homogeneous electric field (CHEF)-Mapper XA PFGE system (Bio-Rad, USA) with a 5- to 35-s linear ramp for 22 h at 6 V/cm at 14°C. The running buffer was 0.5× Tris-boric acid-EDTA (TBE) without thiourea. The gel image was captured using a Bio-Rad Universal Hood II gel imaging system (Bio-Rad, USA). The dendrograms were constructed from the PFGE data by the unweighted-pair group method with arithmetic mean (UPGMA) with the Dice coefficient using InfoQuest FP software version 4.5 (Bio-Rad Laboratories, USA). Multilocus sequence typing (MLST) for clinical *K. pneumoniae* and *E. coli* isolates was performed following the methods described previously (21, 22). The allelic profiles and sequence types (STs) were assigned using online databases (see http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html for *K. pneumoniae* and http://mlst.warwick.ac.uk/mlst/dbs/Ecoli for *E. coli*).

Conjugation and plasmid analysis. Conjugative assays were performed according to the method described previously (23). The bla_{NDM-1}-positive CRE served as the donors, while *E. coli* J53 (sodium azide resistant) was used as the recipient strain. Transconjugants were selected on Mueller-Hinton (MH) agar supplemented with sodium azide (100 μg/ml) and imipenem (1 μg/ml). The presence of the bla_{NDM-1} gene and other resistance genes in transconjugants were identified using PCR and DNA sequencing, and antimicrobial susceptibility was also determined.

S1-PFGE and Southern blotting were conducted to estimate sizes of bla_{NDM-1} plasmids (24). Briefly, whole-cell DNA of clinical and transconjugant strains in agarose gel plugs was treated with S1 nuclease (TaKaRa, Dalian, China), then separated by PFGE under the following conditions: 0.5× Tris-borate-EDTA, 1% agarose solution for 18 h at 6 V/cm and 14°C, with a pulse angle of 120° and the pulse time linearly ramped from 2.16 s to 63.8 s. Linear plasmids generated by S1-PFGE were transferred to nylon membranes (Millipore, USA), hybridized with digoxigenin-labeled

TABLE 2 Antibiotic susceptibilities of bla_{NDM-1}-positive CRE and their transconjugants

Isolate[a]	Antibiotic[b] susceptibility (μg/ml) to:																		
	SAM	TZP	CFZ	CTT	CAZ	FEP	IPM	ETP	CIP	LVX	GEN	AMK	SXT	ATM	CHL	TET	FOF	TGC	CST
EC-01	>256	>256	>256	>256	>256	>256	8	16	2	2	64	16	40	8	256	256	8	0.75	0.5
EC-03	>256	>256	>256	>256	>256	256	>32	32	>32	>32	>256	>256	>320	256	256	256	512	0.5	0.5
EC-13	>256	>256	>256	>256	>256	>256	4	16	8	2	32	16	>320	64	256	256	8	0.38	1
EC-24	>256	>256	>256	>256	>256	>256	>32	32	>32	>32	32	8	>320	128	256	256	512	3	1
EC-SMX3	>256	>256	>256	>256	>256	>256	4	8	>32	>32	64	128	>320	256	4	2	32	0.5	2
EC-SMX5	>256	>256	>256	>256	>256	>256	32	16	>32	>32	32	<2	>320	128	256	256	8	0.5	2
KP-07	>256	>256	>256	>256	>256	>256	8	>32	>32	>32	<1	<2	>320	256	128	2	8	3	1
KP-09	>256	>256	>256	>256	>256	>256	>32	4	1	1	>256	>256	>320	32	256	256	32	3	1
KP-40A	>256	>256	>256	>256	>256	>256	>32	16	<0.25	<0.25	64	<2	>320	>256	256	256	32	6	1
KP-41	>256	>256	>256	>256	>256	>256	>32	>32	<0.25	<0.25	<1	<2	>320	<1	6	2	8	3	0.5
KO-ZMD8	>256	>256	>256	>256	>256	>256	8	16	<0.25	0.5	32	<2	>320	128	256	256	32	0.5	0.5
ECL-ZMD10	ND	64	ND	ND	>256	>256	8	32	>32	>32	>256	>256	>320	256	256	256	128	1	1
ECL-ZMD12	ND	>256	ND	ND	>256	>256	8	32	>32	>32	>256	>256	>320	>256	256	256	32	3	2
ECL-36	ND	>256	ND	ND	>256	>256	32	>32	<0.25	<0.25	32	<2	>320	256	8	4	8	2	1
CF-SMX4	ND	>256	ND	ND	>256	>256	>32	>32	8	4	16	<2	<20	256	6	128	8	1	1
CF-25	ND	>256	ND	ND	>256	>256	>32	>32	>32	>32	>256	>256	>320	64	48	256	512	0.75	0.5
E. coli transconjugant strains																			
EC-01-J53	>256	64	>256	>256	>256	32	8	16	<0.25	<0.25	32	<2	<20	4	256	256	8	1	0.5
EC-03-J53	>256	32	>256	>256	>256	8	>32	8	<0.25	<0.25	>256	>256	>320	128	256	2	512	0.19	1
EC-13-J53	>256	64	>256	32	>256	4	4	4	<0.25	<0.25	16	<2	>320	32	128	128	8	0.25	1
EC-24-J53	>256	>256	>256	>256	>256	>256	32	16	<0.25	<0.25	8	<2	<20	2	256	256	8	0.19	1
EC-SMX3-J53	>256	>256	>256	>256	>256	>256	4	8	8	16	32	32	>320	64	4	0.75	8	0.19	1
EC-SMX5-J53	>256	64	>256	>256	>256	16	32	16	<0.25	<0.25	32	<2	<20	<1	256	8	16	1	0.5
KP-07-J53	>256	>256	>256	>256	>256	>256	8	16	8	16	<1	<2	>320	128	24	2	8	0.5	1
KP-09-J53	>256	64	>256	4	>256	2	4	4	<0.25	<0.25	<1	<2	<20	<1	8	256	32	2	1
KP-40A-J53	>256	>256	>256	32	>256	8	8	4	<0.25	<0.25	32	<2	>320	128	256	256	8	1	1
KP-41-J53	>256	>256	>256	>256	>256	8	8	16	<0.25	<0.25	<1	<2	40	<1	4	2	8	0.75	1
KO-ZMD8-J53	>256	>256	>256	>256	>256	>256	8	16	<0.25	<0.25	8	<2	>320	128	256	256	16	0.75	1
ECL-ZMD10-J53	>256	64	>256	>256	>256	>256	4	16	32	16	>256	>256	>320	128	256	256	128	1.5	0.5
ECL-ZMD12-J53	>256	>256	>256	>256	>256	>256	8	32	32	32	>256	16	<20	>256	256	256	32	3	1
ECL-36-J53	>256	64	>256	>256	>256	>256	32	>32	<0.25	<0.25	32	<2	>320	<1	8	8	8	1.5	1
CF-SMX4-J53	>256	64	>256	32	>256	>256	8	16	<0.25	<0.25	2	<2	<20	2	4	128	8	1	1
CF-25-J53	>256	64	>256	16	>256	2	8	4	<0.25	<0.25	32	<2	<20	4	48	128	128	0.25	1
EC J53	<2	<4	<4	<4	<1	<1	<1	<0.5	<0.25	<0.25	<1	<2	<20	<1	8	2	2	0.25	0.5

[a] All of the bla_{NDM-1}-positive isolates were multidrug-resistant (MDR) strains, and the XDR isolates are highlighted in bold type. EC, *E. coli* strains; KP, *K. pneumoniae* strains; KO, *K. oxytoca* strains; ECL, *E. cloacae* strains; CF, *C. freundii* strains. For the transconjugants, all were *E. coli* J53 harboring plasmids from the respective clinical isolates.

[b] SAM, ampicillin-sulbactam (1/0.5–256/128) (the numbers in parentheses indicate the test range [μg/ml] for each agent); TZP, piperacillin-tazobactam (0.5/4–256/4); CFZ, cefazolin (0.5–256); CTT, cefotetan (0.03–256); CAZ, ceftazidime (0.03–256); FEP, cefepime (0.015–256); IPM, imipenem (0.06–32); ETP, ertapenem (0.004–32); CIP, ciprofloxacin (0.004–32); LVX, levofloxacin (0.008–32); GEN, gentamicin (0.25–256); AMK, amikacin (0.5–256); ATM, aztreonam (0.06–256); CHL, chloramphenicol (0.016–256); TET, tetracycline (0.016–256); FOF, fosfomycin (0.25–512); TGC, tigecycline (0.016–256); CST, colistin (0.016–256). The MICs of trimethoprim-sulfamethoxazole (SXT) were obtained by the Vitek 2 system. ND, not determined (*E. cloacae* and *C. freundii* are intrinsically resistant to SAM, CFZ, and CTT).

bla_{NDM-1}-specific probes, and detected using a nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) color detection kit (Roche Applied Sciences, Germany) according to the recommendations of the supplier. Plasmid replicons were determined using the PCR-based replicon typing method (25).

The genetic environment surrounding bla_{NDM-1} was investigated by PCR mapping and sequencing, and the *Acinetobacter lwoffii* plasmid of pNDM-BJ01 (accession no. JQ001791) and *E. coli* plasmid of pBJ01 (GenBank accession no. JX296013) were used as the references. PCR primers were designed from the reference sequences and are listed in Table S1 in the supplemental material. The locations of the primers are also shown (see Fig. 3).

Nucleotide sequence accession number. The sequence described in this paper has been submitted to GenBank under the accession no. KF985036 (*K. oxytoca* isolate ZMD8).

RESULTS AND DISCUSSION

Identification of bla_{NDM-1}-positive isolates from hospital patients. Among the 48 CRE, 16 (16/48 [33.3%]) were identified as bla_{NDM-1} positive, including 6 *E. coli*, 4 *K. pneumoniae*, 1 *K. oxytoca*, 3 *E. cloacae*, and 2 *C. freundii* isolates, which were obtained from specimens of blood ($n = 7$), urine ($n = 6$), and sputum ($n = 2$) and a burn site ($n = 1$) (Table 1). Additionally, 26 *K. pneumoniae* isolates harbored bla_{KPC-2}, 2 isolates (1 *K. pneumoniae* and 1 *E. cloacae*) carried bla_{IMP-4}, and the remaining isolates did not contain the carbapenemase genes (bla_{NDM}, bla_{KPC}, bla_{VIM}, bla_{IMP}, and $bla_{OXA-48-like}$) screened in this study. The 16 bla_{NDM-1}-positive CRE were from three hospitals in three different cities in Henan Province. The clinical data associated with the 16 isolates were summarized in Table 1. These patients were diagnosed with different clinical diseases, but none of the patients had a history of foreign travel. Notably, 5 patients, including 3 young children, died of infections. All of the patients who died, except an infant, had a positive blood culture for CRE and two of the patients had septicemia (Table 1). Thus, the death rate among the patients infected with a bla_{NDM-1}-positive isolate was 31%, which is higher than previously reported rates in confirmed cases associated with NDM-1-producing bacteria worldwide (26). These results

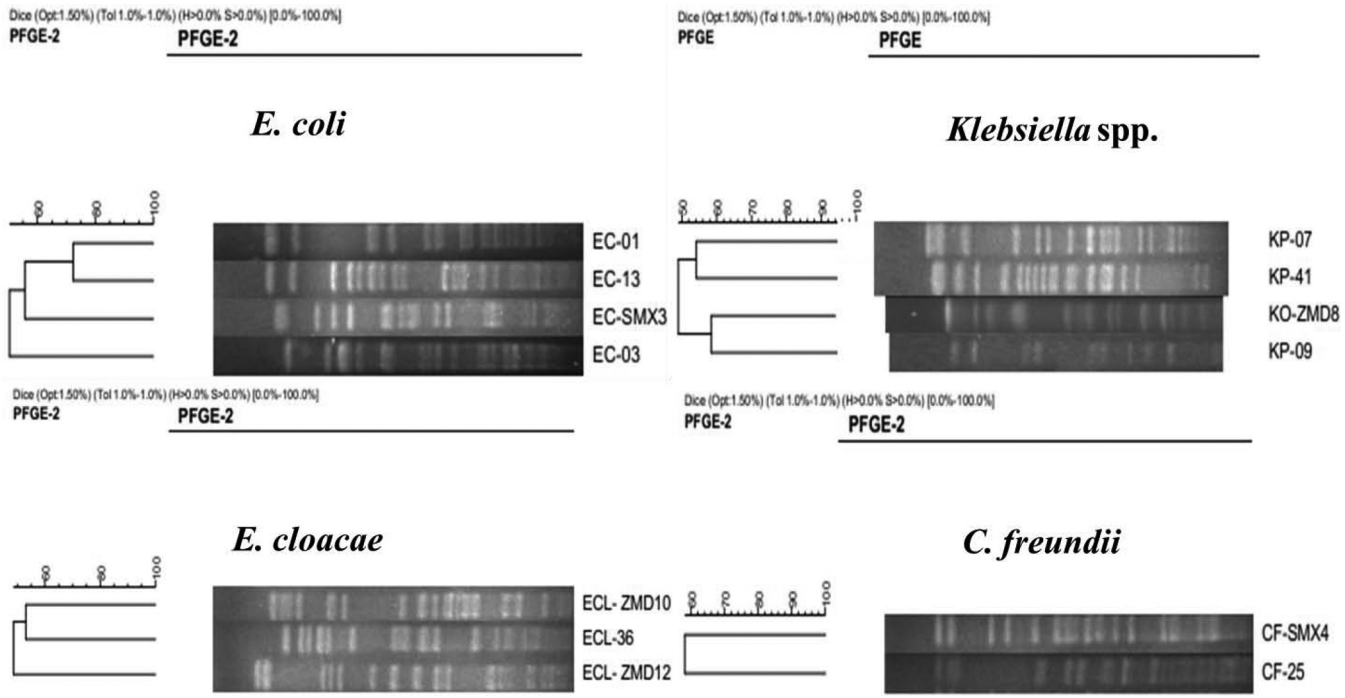

FIG 1 PFGE-based dendrograms showing the genetic relationships of 13 bla_{NDM-1}-positive CRE isolates. EC-24, EC-SMX5, and KP-40A failed to yield bands by XbaI-PFGE and were not included.

revealed the high prevalence of bla_{NDM-1}-positive CRE in the hospitals of Henan Province and their association with a high mortality rate.

Antimicrobial susceptibility patterns and resistance determinants. All of the NDM-1-positive CRE isolates were resistant to carbapenems and cephalosporins but susceptible to colistin (MICs of ≤2 mg/liter) (Table 2). Molecular characterization showed that most of the *E. coli* (5/6) and half of the *K. pneumoniae* isolates (2/4) harbored ESBL genes, AmpC genes, or both (Table 1). Other carbapenemase-encoding genes, including bla_{KPC}, bla_{VIM}, bla_{IMP}, and $bla_{OXA-48-like}$, were not detected in any of the NDM-1-positive CRE isolates. Six isolates (EC-24, KP-07, KP-09, KP-40A, KP-41, and ZMD12) demonstrated tigecycline resistance according to the EUCAST clinical breakpoint, with MICs of ≥2 μg/ml (Table 2). More significantly, five isolates, including EC-03, CF-25, ECL-ZMD10 (susceptible only to tigecycline and colistin), EC-24 (susceptible only to amikacin and colistin), and ECL-ZMD12 (susceptible only to fosfomycin and colistin), were identified as extensively drug-resistant (XDR) bacteria (nonsusceptible to ≥1 agent in all of the 17 but ≤2 antimicrobial categories, including glycylcyclines and polymyxins used for treatment of infections caused by *Enterobacteriaceae*) according to the definitions described by Magiorakos et al. (27). In addition, four isolates (EC-03, EC-24, ECL-ZMD10, and CF-25) harbored a plasmid-mediated fosfomycin resistance gene, *fosA3*. This gene was found only in CTX-M-producing *E. coli* isolated from Asian countries (28), and our finding represents the first report of NDM-1-producing CRE harboring the *fosA3* gene encoding fosfomycin resistance. Generally, the resistance rate of fosfomycin in *E. coli* was low (1% to 3%) worldwide, and fosfomycin is also used as an alternative choice for treating infections caused by ESBL-producing and even carbapenemase-producing *Enterobacteriaceae* (29). However, the occurrence of fosfomycin resistance in NDM-1-producing CRE observed in this study will further limit clinical therapeutic options.

Genotyping. PFGE was successfully performed with 4 *E. coli*, 3 *K. pneumoniae*, 1 *K. oxytoca*, 3 *E. cloacae*, and 2 *C. freundii* isolates, and their patterns were completely different (Fig. 1). Three isolates, including EC-24, EC-SMX5, and KP-40A, were nontypeable by PFGE, as their XbaI-digested genomic DNA failed to yield distinct bands despite multiple efforts to repeat the experiment. MLST was performed for all of the bla_{NDM-1}-positive *E. coli* and *K. pneumoniae* isolates, since the two species of *Enterobacteriaceae* were the major ones carrying the bla_{NDM-1} gene. Five MLST types were identified among the six *E. coli* isolates. Similarly, four MLST types were identified among the four *K. pneumoniae* isolates (Table 1). Two *E. coli* isolates (EC-03 and EC-SMX5) obtained from two different hospitals located in geographically separated areas (Zhengzhou and Sanmenxia) shared the same ST type (ST361), suggesting they were clonally related. Overall, our data showed that clonally diverse NDM-1-producing *Enterobacteriaceae* contributed to the dissemination of bla_{NDM-1}in Henan Province.

This study linked for the first time six new STs (ST40, ST205, and ST1237 [*E. coli*] and ST113, ST889, and ST966 [*K. pneumoniae*]) to the production of NDM-1. *E. coli* ST410 and *K. pneumoniae* ST11 were frequently detected in clinical isolates of NDM-1-producing *E. coli* and *K. pneumoniae* from different countries, suggesting that they play an important role in the dissemination of bla_{NDM-1} (30). In addition, the NDM-1-producing *E. coli* ST361 isolate was also found in New Zealand (31).

Plasmid analysis. S1-PFGE and Southern blot analysis showed that the presence of the bla_{NDM-1} gene in the 16 CRE isolates was located on diverse plasmids, with sizes ranging from ~55 to ~360

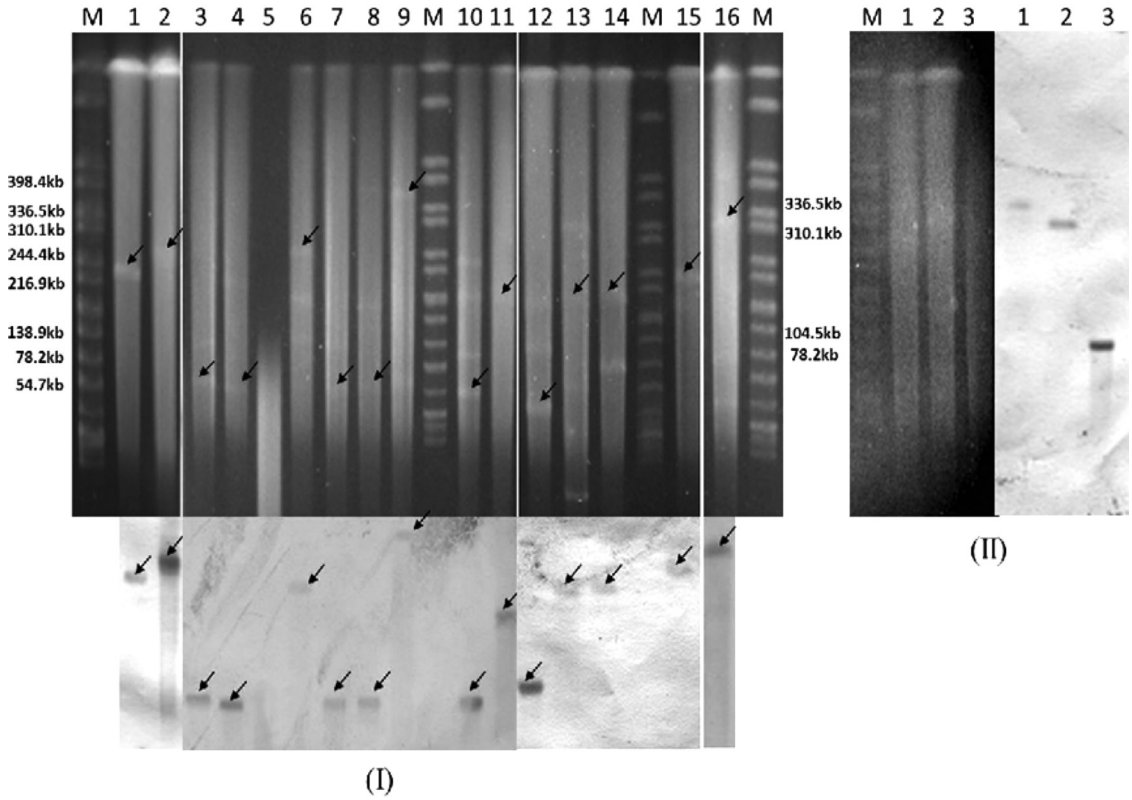

FIG 2 Detection of bla_{NDM-1}- (I) and *fosA3*- (II) carrying plasmids by PFGE and Southern hybridization. (I) S1-PFGE (top) and Southern blotting (bottom) with bla_{NDM-1} specific probe. Lanes M, marker (*Salmonella* H9812); lane 1, EC-01-J53; lane 2, EC-SMX5-J53; lane 3, EC-SMX3; lane 4, KP-41; lane 5, KP-40A (untypeable); lane 6, KP-09; lane 7, KP-07; lane 8, KO-ZMD8; lane 9, ECL-ZMD10; lane 10, ECL-ZMD12; lane 11, ECL-36; lane 12, CF-SMX4; lane 13,CF-25; lane 14, EC-03-J53; lane 15, EC-24-J53; lane 16, EC-13. The arrows indicate the locations of the plasmids hybridized to the NDM-1 probe. (II) S1-PFGE (left) and Southern blotting (right) with *fosA3*-specific probe. Lane M, marker (*Salmonella* H9812); lanes 1, ECL-ZMD10-J53; lanes 2, CF-25-J53; lanes 3, EC-03-J53.

kb. Furthermore, half of the bla_{NDM-1} plasmids belonged to plasmid replicon type IncA/C (Table 1 and Fig. 2). This broad-host-range plasmid was most frequently reported for carrying bla_{NDM-1} and other resistance genes, and it is widely disseminated among Gram-negative bacteria worldwide (6, 32).

Conjugative assays revealed that all of the bla_{NDM-1} plasmids were successfully transferred to *E. coli* J53 from the 16 donors by conjugation. The 16 transconjugants all showed resistance to carbapenems and cephalosporins (Table 2). Moreover, the bla_{NDM-1} plasmids in all the transconjugants remained stable after 10 passages in the absence of imipenem selection. In addition, resistance genes such as bla_{TEM-1}, $bla_{CTX-M-15/55}$, bla_{CMY-30}, *rmtB*, *armA*, and *fosA3* were cotransferred to *E. coli* J53 with the bla_{NDM-1} gene in several isolates (Table 2). Interestingly, we found that the bla_{NDM-1} and *fosA3* genes were carried by distinct IncA/C plasmids in EC-03 (~180 kb for bla_{NDM-1} and ~90 kb for *fosA3*) and CF-25(~170 kb for bla_{NDM-1} and ~310 kb for *fosA3*) (Fig. 2), but the two plasmids in each isolate were cotransferred to the *E. coli* J53 recipient, suggesting that both were mobile or one of them acted as a helper plasmid for the other to transfer. In contrast to the above two isolates, ECL-ZMD10 harbored a large plasmid (~360 kb) of an untypeable replicon type, which carried both bla_{NDM-1} and *fosA3* and was transferred to *E. coli* J53 by conjugation (Fig. 2 and Table 2). Several recent studies demonstrated that the *fosA3* gene was associated with bla_{CTX-M} genes and carried by plasmids belonging to different replicon types, including F, N, B/O, and I1 in *E. coli* and *K. pneumonia* (33–35). In our study, this gene was found to be carried by a conjugative bla_{NDM-1} plasmid in a species (*E. cloacae*) other than *E. coli* and *K. pneumonia*, implying the further spread of *fosA3* among *Enterobacteriaceae*. Moreover, the *E. coli* J53 recipient became an XDR strain after accepting the ECL-ZMD10 plasmid by conjugation, indicating multiple resistance determinants might be carried by this bla_{NDM-1} plasmid. Additional studies are ongoing to characterize this plasmid. *In vivo* interspecies dissemination of IncA/C plasmids carrying $bla_{NDM-1}$was described in previous studies (23, 36). However, all of the NDM-1 plasmids identified in the 16 bla_{NDM-1}-positive CRE isolates were different either in size or replicon type, suggesting that insertion elements or transposons may play important roles in the mobilization of bla_{NDM-1}.

Genetic environments of bla_{NDM-1}. The genetic environments surrounding the bla_{NDM-1} gene were detected by PCR mapping and DNA sequencing. The bleomycin resistance gene ble_{MBL}, followed by a truncated *trpF* gene, was located immediately downstream of the bla_{NDM-1} gene in all of the 16 CRE isolates (Fig. 3). The three-gene cluster (bla_{NDM-1}-ble_{MBL}-Δ*trpF*) was highly conserved and was also previously reported in *E. coli* plasmids (pNDM-HK, pBJ01, and pNDM_Dok01) and *A. lwoffii* plasmids (pNDM-BJ01 and pNDM-BJ02) (37) (Fig. 3). It was reported that bla_{NDM-1} and ble_{MBL} are under the control of the same promoter in front of bla_{NDM-1}, and this structure may facilitate the spread of NDM-1 when under the selective pressure of bleomycin-like mol-

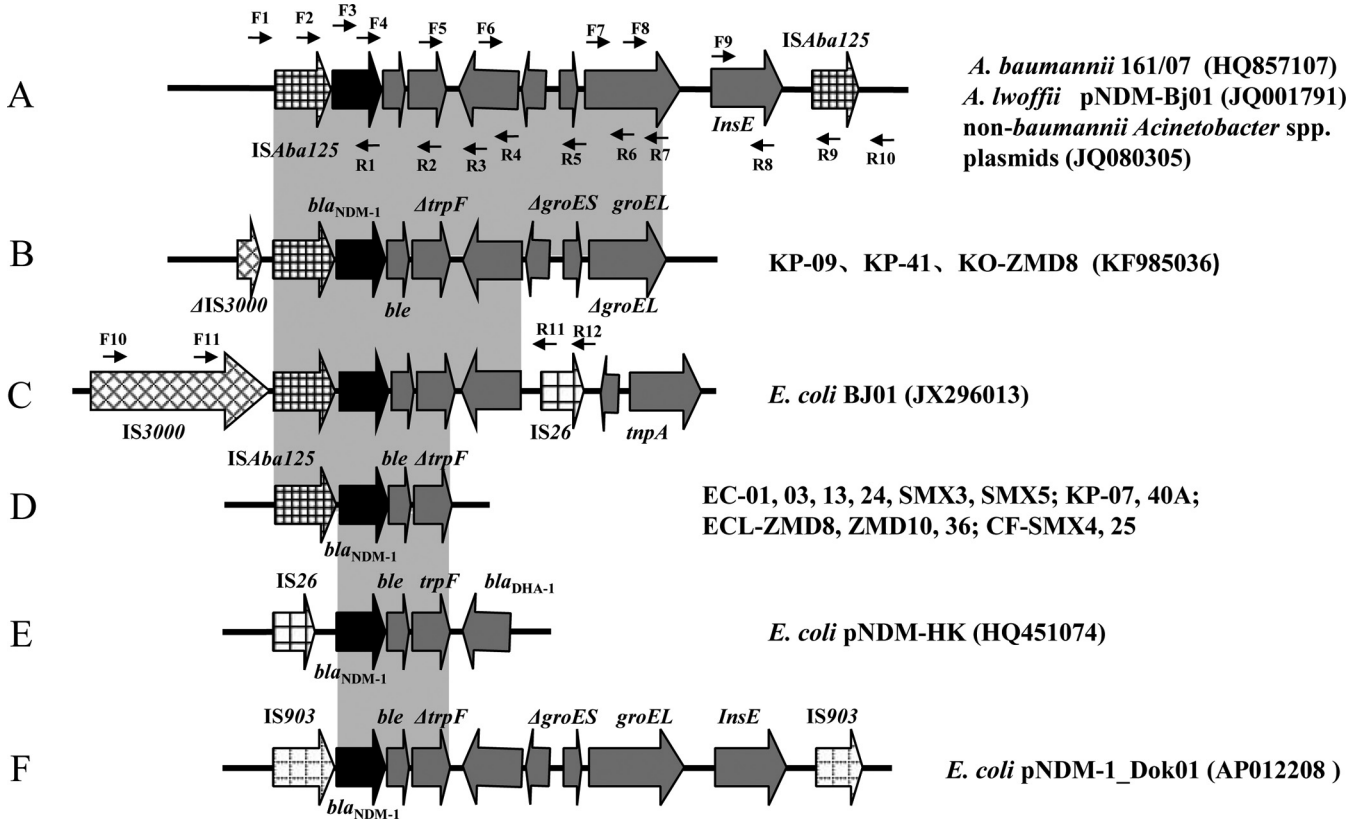

FIG 3 Comparison of the bla_{NDM-1} gene environments identified in this study with others published previously. (A) bla_{NDM-1}-surrounding sequences in *A. baumannii* 161/07 (GenBank accession no. HQ857107), *A. lwoffii* pNDM-Bj01 (JQ001791), and non -*baumannii Acinetobacter* spp. plasmids (JQ080305). (B and D) the bla_{NDM-1} gene environments identified in this study; the names of the isolates are listed on the right of each panel.(C, E and F) bla_{NDM-1}-surrounding sequences in *E. coli* BJ01 (JX296013), *E. coli* pNDM-HK (HQ451074), and *E. coli* pNDM-1_Dok01 (AP012208), respectively. The boxed arrows indicate the positions and directions of transcription of the genes. The gray-shaded areas represent regions sharing >99% DNA identity. Positions of the primers used for PCR mapping are indicated by arrows.

ecules in the environment (38). The entire IS*Aba125* element was detected immediately upstream of the bla_{NDM-1} gene in each of the 16 isolates. It has been documented that IS*Aba125* provides the −35 region of the promoter sequence for bla_{NDM-1} in all reported cases (37). For two *K. pneumonia* isolates (KP-09 and KP-41) and one *K. oxytoca* isolate (KO-ZMD8), a 5,859-bp DNA segment was sequenced and it contained eight genes, including IS*Aba125*, bla_{NDM-1}, ble_{MBL}, Δ*trp*F, *dsbC*, *cutA1*, *groES*, and *groEL* (Fig. 3). This DNA segment is highly homologous (>99%) to previously identified sequences in *Acinetobacter* spp. from China (9, 39) (Fig. 3), suggesting the possible transmission of bla_{NDM-1}-containing sequences between *Acinetobacter* spp. and *Enterobacteriaceae*. Considering that bla_{NDM-1} was mostly reported in *Acinetobacter* spp. in China, our finding suggests that *Acinetobacter* may have served as a reservoir for the dissemination of NDM-1 toward *Enterobacteriaceae*.

The sporadic emergence of NDM-1-producing *E. coli* and *K. pneumoniae* in mainland China has been reported in two recent studies (12, 13). Additionally, several cases of NDM-1-producing CRE reported from Hong Kong and Taiwan were also epidemiologically linked to mainland China (40–42). Here, we report our study of a cluster of bla_{NDM-1}-positive CRE from patients in Henan Province. Together, these findings strongly suggest that NDM-1-producing CRE is spreading in mainland China. Compared to previously reported studies, our work revealed several important findings on CRE in China. First, a high incidence (33.3%) of NDM-1-producing *Enterobacteriaceae* was observed among the examined CRE, which is much higher than previously realized and suggests that bla_{NDM-1}-positive CRE is endemic in Henan Province. Second, none of the patients carrying NDM-1-producing CRE had a history of foreign travel, suggesting that the NDM-1-carrying CRE were endogenous to the region and likely originated locally due to antibiotic selection and spread of the resistance gene. Third, most of the NDM-1-producing *E. coli* and *K. pneumoniae* isolates identified in this study belong to new MLST sequence types that have not been reported previously, suggesting the active spread of bla_{NDM-1} among *Enterobacteriaceae* in China. These findings provide new insights into the epidemiology of CRE in China.

In summary, our study demonstrated a high incidence of NDM-1-producing CRE from patients with different clinical diseases in Henan Province. These NDM-1-positive isolates were genetically diverse and carried plasmids of various sizes that were readily transferred by conjugation. Our results suggest that mainland China is becoming an endemic reservoir for NDM-1-producing *Enterobacteriaceae*. In addition, the emergence of XDR *Enterobacteriaceae* carrying conjugative bla_{NDM-1} plasmids coharboring other resistance determinants such as *fosA3* is alarming,

as spread of these isolates will seriously limit options for clinical treatment in future. Thus, enhanced efforts are urgently needed to control the further spread of NDM-1-producing *Enterobacteriaceae*.

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