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Characterization of Multiple NDM-1-Producing *Enterobacteriaceae* Isolates from the Same Patient

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A male patient was admitted to a community hospital in Ontario, Canada, with an infected sacral ulcer after returning from India, where he was hospitalized. Carbapenem-resistant *Escherichia coli* (isolated from blood cultures), *Enterobacter cloacae*, and *Providencia stuartii* (from urine samples), all positive for bla_{NDM-1}, were recovered. Comparative NDM-1 plasmid analysis suggests both lateral plasmid transfer and independent acquisition of the bla_{NDM-1} gene in these clinical isolates.

Carbapenem resistance in *Enterobacteriaceae* has emerged worldwide mainly by the production of carbapenemases (1). Carbapenemase genes are commonly carried on conjugative plasmids, representing a significant infection control challenge because of the potential horizontal transfer of resistance genes between bacterial isolates, species, and genera. The New Delhi metallo-β-lactamase (NDM), first described in 2008 (2), has been detected in different species and genera without a clear link to dominant plasmids or clones (3). In this study, we characterized three different NDM-producing enterobacterial species isolated from the same patient.

A 65-year-old man presented to the emergency department of a community hospital in Ontario, Canada, with fever and generalized weakness. He had been hospitalized in India with a urinary tract infection, and his stay was complicated by the development of a large sacral decubitus ulcer. The patient was admitted to the hospital after returning from India with a diagnosis of an infected sacral ulcer. His symptoms improved with the initial treatment (cefazolin and metronidazole), but he had a recurrence of fever. Blood cultures grew *Escherichia coli* GN568 resistant to all of the antibiotics tested in the hospital except tigecycline, with which the patient was treated for 14 days and recovered. However, 2 weeks after treatment, his fever recurred and blood and urine cultures were collected. His blood cultures grew an extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* strain. His urine grew *Enterobacter cloacae* GN574 sensitive only to tigecycline and *Providencia stuartii* GN576 sensitive to tigecycline, trimethoprim-sulfamethoxazole, and ciprofloxacin. The patient was asymptomatic for bacteriuria, and his urinary catheter was changed. He was diagnosed with an infected peripherally inserted central catheter, which was removed, and treated with ertapenem for 14 days against an ESBL-producing, carbapenem-susceptible *K. pneumoniae* strain detected in blood samples. He recovered from his infections and was discharged to a nursing home. These three carbapenem-resistant clinical isolates (*E. coli* GN568, *E. cloacae* GN574, and *P. stuartii* GN576) were submitted to the Public Health Ontario laboratories for further studies. They were positive for carbapenemase activity by the KPC/MBL Confirm kit (Rosco Diagnostica). They were resistant to all β-lactams and aminoglycosides, consistent with the detection of different β-lactamase-encoding genes (including $bla_{CTX-M-15}$ and bla_{CMY-6} in two of the isolates, in addition to bla_{NDM-1}) and the 16S rRNA methyltransferase *rmtC* gene (Table 1). *E. coli* GN568 (assigned to sequence type 1289 by multilocus sequence typing) (4) was also resistant to quinolones and co-trimoxazole; *E. cloacae* GN574 was also resistant to quinolones, co-trimoxazole, and tigecycline; and *P. stuartii* GN576 was also resistant to tetracycline and colistin. IncA/C plasmids were identified in *E. coli* GN568 (IncFIA and Frep were also detected in this isolate) and *P. stuartii* GN576 by PCR (5). *E. cloacae* GN574 was negative for all of the Inc groups tested. In conjugative assays with *E. coli* J53 as the acceptor (6), bla_{NDM-1} and bla_{CMY-6} were cotransferred on the IncA/C plasmid to strains J-568 (derived from *E. coli* GN568) and J-576 (from *P. stuartii* GN576) (Table 1). The only β-lactamase-encoding gene transferred to *E. coli* J-574 (transconjugant strain derived from *E. cloacae* GN574) was bla_{NDM-1}, consistent with its low aztreonam MIC.

NDM-1-carrying plasmids were extracted from the transconjugant *E. coli* strains with the Qiagen Large-Construct kit (Qiagen, Valencia, CA) and sequenced with the Illumina compact MiSeq system. Assembly of the contigs obtained was done with the CLC Genomics Workbench software (CLC bio, Qiagen). Gaps were filled by PCR amplification and Sanger sequencing. Open reading frames (ORFs) were predicted and annotated by the RAST server (available at rast.nmpdr.org) (7), followed by manual comparative curation and sequence similarity searches directed against the NCBI (www.ncbi.nlm.nih.gov/BLAST).

pNDM-EcoGN568 resulted in a closed circular sequence of 166,750 bp (average GC content of 51.8%). Its annotation revealed 205 predicted ORFs ranging from 117 to 5,487 bp, 98 of

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TABLE 1 Antibacterial drug susceptibility profiles, plasmid types, and resistance genes of clinical isolates and their *E. coli* transconjugant

	MIC (μg/ml)[b]											
Isolate[a]	Ampicillin	Cefoxitin	Ceftazidime	Cefotaxime	Cefepime	Aztreonam	Ertapenem	Meropenem	Imipenem	Amikacin	Gentamicin	Tobramycin
Eco GN568	≥256	≥256	≥256	≥256	≥256	256	16	32	6	≥256	≥1,024	≥256
Ecl GN574	≥256	≥256	≥256	≥256	128	128	≥32	≥32	≥32	≥256	≥1,024	≥256
Pst GN576	≥256	≥256	≥256	16	1.5	0.75	≥32	≥32	≥32	≥256	≥1,024	≥256
Eco J-568	≥256	≥256	≥256	256	16	6	≥32	4	8	≥256	≥1,024	≥256
Eco J-574	≥256	≥256	≥256	≥256	16	0.125	16	6	24	≥256	≥1,024	≥256
Eco J-576	≥256	≥256	≥256	192	16	6	≥32	4	12	≥256	≥1,024	≥256
Eco J53	6	8	0.19	0.094	0.064	0.125	0.008	0.023	0.38	1.5	1.5	1

TABLE 1 (Continued)

MIC (μg/ml)[b]						Rep type (plasmid size [kb])	PCR and sequencing results[d]					
Nalidixic acid	Ciprofloxacin	Tetracycline	Tigecycline	Colistin	Co-trimoxazole		bla_{NDM-1}	$bla_{CTX-M-15}$	bla_{OXA-1}	bla_{TEM-1}	bla_{CMY-6}	*rmtC*
≥256	≥32	1.5	0.64	0.047	≥32	A/C FIA Frep	+	+	−	+	+	+
64	8	12	3	0.064	≥32	Neg[c]	+	+	+	+	−	+
4	0.12	96	0.5	≥256	0.25	A/C	+	−	−	−	+	+
3	0.012	0.75	0.064	0.047	0.094	A/C (~160)	+	−	−	−	+	+
3	0.012	1	0.094	0.047	0.064	Neg[c] (~100)	+	−	−	−	−	+
3	0.016	0.75	0.094	0.047	0.25	A/C (~150)	+	−	−	−	+	+
3	0.012	1	0.047	0.047	0.064	NA[e]	NA	NA	NA	NA	NA	NA

[a] Eco, *E. coli*; Ecl, *E. cloacae*; Pst, *P. stuartii*; Eco J53, recipient *E. coli* J53. *E. coli* J53 transconjugant strains were derived from *E. coli* GN568 (J-568), *P. stuartii* GN576 (J-576), and *E. cloacae* GN574 (J-574).

[b] Susceptibility testing was performed by the Etest (bioMérieux) and agar dilution methods, and results were interpreted according to Clinical and Laboratory Standards Institute guidelines (13), except for colistin and tigecycline, for which the European Committee on Antimicrobial Susceptibility Testing breakpoints were used (http://www.eucast.org/clinical_breakpoints/).

[c] Nontypeable by the PCR-based replicon typing scheme described by Carattoli et al. (5). A new set of primers was designed for detection of this *repA* allele: FIB F2, 5′-CGTCTATC TCTCGCCAGAGC-3′; FIB R2, 5′-GTTCTCAGCACTCTCATCATGC-3′.

[d] Sequencing of complete genes was performed with samples positive by PCR. PCR included screening for bla_{TEM}; bla_{SHV}; $bla_{OXA-1-like}$; bla_{CTX-M} groups 1, 2, and 9; bla_{VEB}; bla_{PER}; bla_{GES}; $bla_{OXA-48-like}$; bla_{IMP}; bla_{KPC}; bla_{NDM}; and six groups of bla_{AmpC} β-lactamase genes, as well as the *armA*, *rmtA* to *-F*, and *npmA* 16S methylase genes (14, 15). A plus sign indicates a positive result, and a minus sign indicates a negative result.

[e] NA, not applicable.

which showed homology to proteins with known functions (Fig. 1; see Table S1 in the supplemental material). pNDM-EcoGN568 was almost identical to pNDM10-0505 (plasmid not yet published, GenBank accession no. JF503991), a 166,744-bp IncA/C plasmid detected in an *E. coli* isolate from a female patient hospitalized in Vancouver, British Columbia, Canada (8). Like the patient described here, that patient also received medical attention in India before being transferred to Canada. Moreover, pNDM-EcoGN568 also had ~89% identity with pNDM-KN (GenBank accession no. JN157804), another IncA/C plasmid of 162,746 bp detected in a *K. pneumoniae* isolate from Kenya (9, 10) (Fig. 1A). Differences between these two plasmids were detected mainly immediately upstream of the bla_{NDM-1} gene (Fig. 1B): the cassette content in a class I integron (four cassettes and *qacEΔ1* deleted from the 3′ conserved region in pNDM-KN versus only one cassette plus *qacEΔ1* in pNDM-EcoGN568) and a fragment of ~10 kb flanked by two copies of IS*Kpn14* between the *rmtC* and bla_{NDM-1} genes. Besides some transposase genes, this fragment also included two determinants of aminoglycoside resistance. One of these IS*Kpn14* copies in pNDM-EcoGN568 disrupts IS*Aba125*, part of which was absent from pNDM-KN (Fig. 1). Upstream of this region, a copy of IS*Ec23* inserted into the *sugE* gene (close to the bla_{CMY-6} gene) in pNDM-KN was not present in pNDM-EcoGN568.

pNDM-PstGN576 had a circular sequence of 147,886 bp with a GC content of ~52% and 190 predicted ORFs (from 117 to 5,487 bp in size) (Fig. 1; see Table S1 in the supplemental material). Compared to pNDM-EcoGN568, pNDM-PstGN576 showed a deletion of ~19 kb corresponding to a Tn*7*-like transposon (the missing fragment was flanked by the inverted repeats IR-L and -R, which define the ends of the transposon) (Fig. 1A). The rest of both plasmid sequences showed 99% identity. These similarities suggest possible *in vivo* horizontal transfer between *E. coli* and *P. stuartii* clinical isolates with their subsequent evolution in the colonized patient, although their independent acquisition by each isolate cannot be ruled out.

pNDM-EclGN574 was 110,625 bp in length, with an average GC content of 54.8% and harboring 146 predicted ORFs (114 to 3,045 bp in size) (Fig. 1; see Table S1 in the supplemental material). pNDM-EclGN574 was identical in sequence to pKOX_NDM1 isolated from a *Klebsiella oxytoca* strain recovered in Taiwan from a patient who underwent renal transplantation in China (11, 12). pNDM-EclGN574 showed high identity with pNDM-EcoGN568 and pNDM-PstGN576 only in the region containing the bla_{NDM-1} gene (Fig. 1B). As described for pKOX_NDM1, this region was flanked by 256-bp direct repeats (Fig. 1B, orange circles) with similarities to miniature inverted-repeat transposable elements (MITEs) suggested to be involved in the acquisition of this bla_{NDM-1} region (11). The low sequence identity of pNDM-EclGN574 with the other two NDM plasmids recovered from the

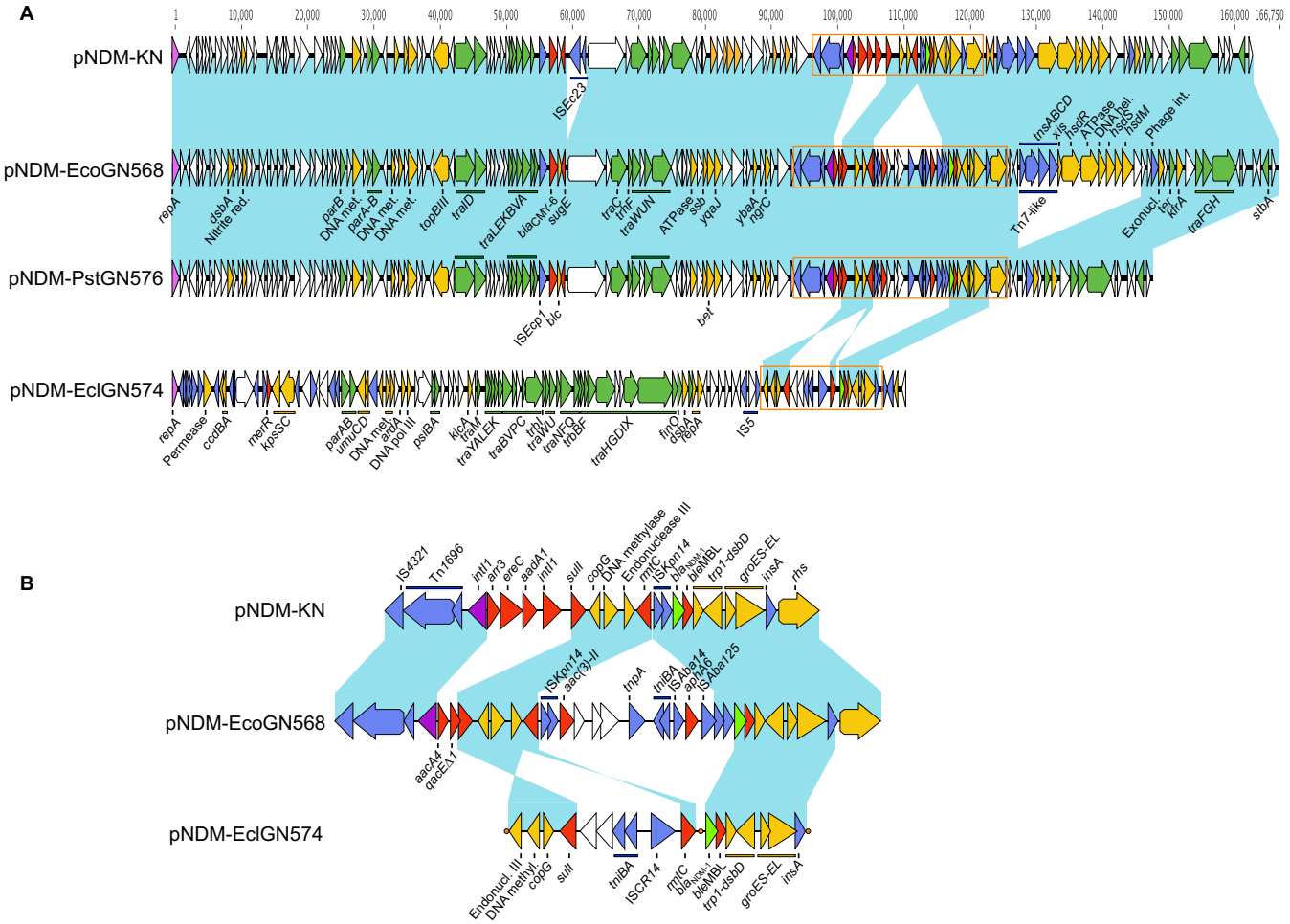

FIG 1 Comparison of the NDM plasmids characterized in this study with closely related IncA/C plasmid pNDM-KN (GenBank accession no. JN157804) (9). (A) Regions with high homology are shaded light blue. Arrows indicate the following predicted ORFs: conjugation, stability, and accessory genes (green, yellow), antimicrobial resistance genes (red, bla_{NDM-1} is light green), transposon-related genes (blue), and hypothetical proteins (white). The replicase gene is pink. The orange-boxed segments are detailed in panel B. (B) Detail of the bla_{NDM-1}-flanking regions (those of pNDM-PstGN576 are the same as those of pNDM-EcoGN568). The orange circles represent the 256-bp direct repeats with similarities to MITEs identified in bla_{NDM-1} and *insA*. A third MITE was found ~11 kb upstream of the bla_{NDM-1} gene, between a truncated copy of IS*5* and a putative endonuclease III gene.

same patient suggests its independent acquisition by *E. cloacae* GN574. We were unable to identify the incompatibility group of plasmids from *E. cloacae* GN574 and its *E. coli* transconjugant by the standard plasmid replicon typing method described by Carattoli et al. (5). However, the *repA* gene on pNDM-EclGN574 had the highest identity with RepFIB replication protein A. Analysis of the primers used for amplification of this replicon type indicated that one of them (FIB F) was not identical to the *repA* sequence in this plasmid, and the reverse primer had two mismatches.

In conclusion, plasmid sequencing and comparative analysis suggest both lateral plasmid transfer and independent acquisition of the bla_{NDM-1} gene in these three different enterobacterial genera recovered from the same patient. Plasmids with high sequence identity recovered from different bacterial species in different geographic areas support the idea of lateral dissemination as the main mechanism of the spread of this metallo-β-lactamase.

Nucleotide sequence accession numbers. The sequences of the plasmids reported here have been deposited in GenBank under accession numbers KJ802404, KJ812998, and KJ802405.

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