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Molecular Characterization by Using Next-Generation Sequencing of Plasmids Containing bla_{NDM-7} in *Enterobacteriaceae* from Calgary, Canada

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***Enterobacteriaceae* with bla_{NDM-7} are relatively uncommon and had previously been described in Europe, India, the United States, and Japan. This study describes the characteristics of *Enterobacteriaceae* (*Klebsiella pneumoniae* [$n = 2$], *Escherichia coli* [$n = 2$], *Serratia marcescens* [$n = 1$], and *Enterobacter hormaechei* [$n = 1$] isolates) with bla_{NDM-7} obtained from 4 patients from Calgary, Canada, from 2013 to 2014. The 46,161-bp IncX3 plasmids with bla_{NDM-7} are highly similar to other bla_{NDM}-harboring IncX3 plasmids and, interestingly, showed identical structures within the different isolates. This finding may indicate horizontal transmission within our health region, or it may indicate contact with individuals from areas of endemicity within the hospital setting. Patients infected or colonized with bacteria containing bla_{NDM-7} IncX3 plasmids generate infection control challenges. Epidemiological and molecular studies are required to better understand the dynamics of transmission, the risk factors, and the reservoirs for bacteria harboring bla_{NDM-7}. To the best of our knowledge, this is the first report of *S. marcescens* and *E. hormaechei* with bla_{NDM-7}.**

The metallo-β-lactamase (MBL) NDM-1 was first described in *Klebsiella pneumoniae* and *Escherichia coli* recovered from a Swedish patient who was previously hospitalized in New Delhi, India (1). Subsequently, bacteria carrying NDM have been recognized in over 50 countries on every continent, except Antarctica (2). Gram-negative bacteria carrying bla_{NDM} are endemic in South Asia (especially the Indian subcontinent), South-East Asia (3, 4), and certain countries within the Middle East and the Balkans (5). Infections with NDM-producing bacteria in areas where these organisms are nonendemic, such as Europe and North America, have most often been associated with patients who required hospitalization while visiting an area where NDM-producing bacteria are endemic (6).

NDM carbapenemases are commonly reported in *K. pneumoniae* and *E. coli* but have also been found in a variety of other members of the *Enterobacteriaceae* family, including *Acinetobacter* spp., *Pseudomonas* spp., and *Vibrio cholerae* (7, 8). The treatment of infections caused by multidrug-resistant NDM-producing *Enterobacteriaceae* is causing serious therapeutic challenges for the medical community because isolates are often also resistant to non-β-lactam antibiotics (9). Bacteria with NDMs often remain susceptible only to agents such as colistin (CST), fosfomycin (FOF), and tigecycline (TGC) (10).

During 2013 and 2014, six *Enterobacteriaceae* with bla_{NDM-7} were isolated from four different Calgary patients over a period of 18 months. One patient had recently been hospitalized in India, while the remaining three did not have a history of recent travel outside Alberta, Canada. NDM-7 is an infrequent bla_{NDM} allele; therefore, a study was designed to characterize these isolates and their respective plasmids using traditional and next-generation sequencing techniques.

MATERIALS AND METHODS

Patients and isolates. For a summary of the clinical features of patients 1 to 4 and a timeline of events, please see Table 1 and Fig. 1.

The isolates were identified using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Vitek AMS; bioMérieux Vitek Systems Inc., Hazelwood, MO). Further identification of *Enterobacter cloacae* was performed by partial sequencing of the *leuS* gene (11).

Antimicrobial susceptibilities. MICs were determined using the MicroScan NEG 38 panel (Siemens, Burlington, Ontario, Canada) and were interpreted by using CLSI guidelines for broth dilution (12). The following drugs were tested: piperacillin-tazobactam (TZP), cefoxitin (FOX), ceftriaxone (CRO), ceftazidime (CAZ), cefepime (FEP), aztreonam (ATM), meropenem (MEM), ertapenem (ERT), amikacin (AMK), gentamicin (GEN), tobramycin (TOB), ciprofloxacin (CIP), tigecycline (TGC), and trimethoprim-sulfamethoxazole (SXT). Colistin (CST), fosfomycin (FOF), imipenem (IPM), MEM, and ERT MICs were determined using Etests (bioMérieux Inc., Hazelwood, MO, USA) according to the manufacturer's instructions. The IPM, MEM, ERT, and FOF Etests were also performed on the *E. coli* J53 transconjugants (see below). The Euro-

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TABLE 1 Characteristics of patients infected with *Enterobacteriaceae* harboring bla_{NDM-7} in Calgary, Canada

Characteristic	Patient 1	Patient 2	Patient 3	Patient 4
Sex	Male	Male	Male	Female
Hospital	A	B	A	A
Clinical diagnosis	Asymptomatic bacteriuria	Lower UTI[a]	Septicemia	Lower UTI
Date of clinical presentation	June 2013	November 2013	October 2014	December 2014
Treatment	In-out catheterization	Ciprofloxacin	Gentamicin and colistin	Ciprofloxacin
Travel history	None	India	None	None

[a] UTI, urinary tract infection.

pean Committee for Antimicrobial Susceptibility Testing (EUCAST) breakpoint was used for CST, and the FDA breakpoint was used for TGC.

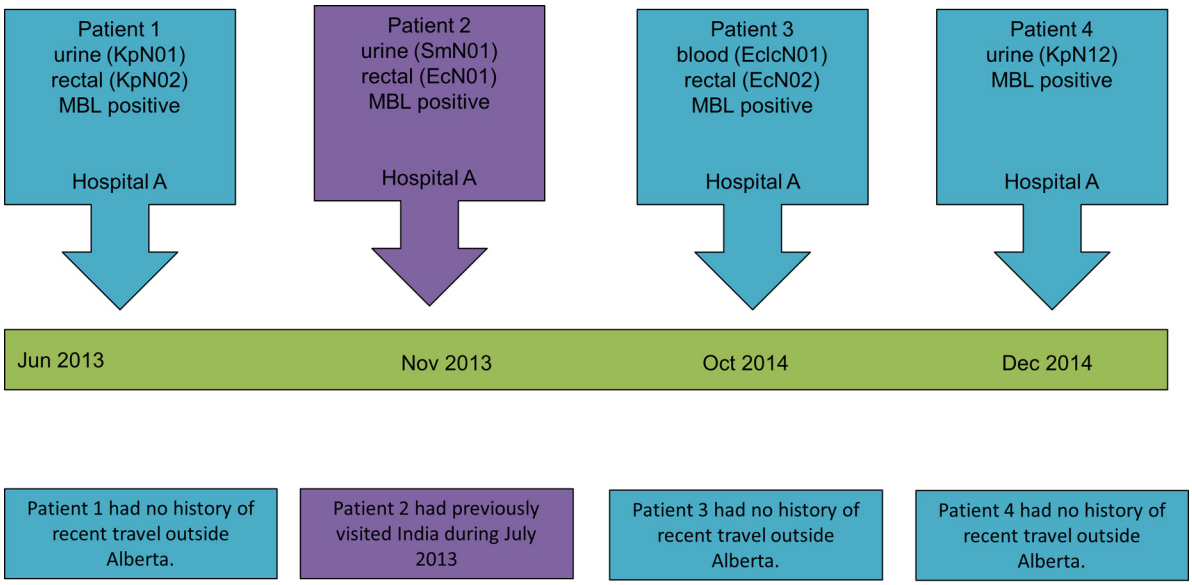
Carbapenemase gene identification. The presence of carbapenemases was detected using the CLSI guidelines for the modified Hodge test (MHT) and Mastdiscs ID inhibitor combination disks (13) (Mast Group Ltd., Merseyside, United Kingdom). PCR amplification and sequencing for bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{NDM}, and $bla_{OXA-48-like}$ genes were undertaken using primers and conditions as previously described (13).

Plasmid analysis. Plasmid sizes were determined as previously described (14) and were assigned to plasmid incompatibility (Inc) groups by PCR-based replicon typing (15–17). Conjugation experiments were performed by mating-out assays with nutrient agar containing 1 μg/ml MEM and by using *E. coli* J53 (azide, 100 μg/ml) as the recipient. Plasmids from the transconjugants were sequenced using the Pacific Biosciences RS II platform (Menlo Park, CA, USA) and the Illumina MiSeq system (San Diego, CA, USA) (see details below).

MLST. Multilocus sequencing typing (MLST) of the *K. pneumoniae* (18), *E. coli* (19), and *E. cloacae* (20) isolates was performed as previously described.

Complete sequencing of bla_{NDM-7}-harboring plasmids. The genome of isolate KpN01 from patient 1 (chromosome and plasmids) was sequenced using the Pacific Biosciences RS II platform (Menlo Park, CA, USA). Assembly of the data was performed using the hierarchical genome assembly process (HGAP) compiled specifically for quality trimming, *de novo* assembly, and polishing of PacBio data. The bla_{NDM-7}-harboring plasmids from isolates SmN01 (*Serratia marcescens* isolate from patient 2), EcN01 (*E. coli* isolate from patient 2), EclcN01 (*Enterobacter cloacae/hormaechei* isolate from patient 3), EcN02 (from patient 3), and KpN12 (*K. pneumoniae* isolate from patient 4) were sequenced using a previously described method (21). In brief, plasmid DNA from *E. coli* J53 transconjugants with a single bla_{NDM-7}-harboring plasmid was extracted using a Qiagen plasmid maxikit (Qiagen, Valencia, CA) and was sequenced using the Illumina MiSeq system (San Diego, CA, USA). Sequencing reads were *de novo* assembled into consensus contigs using Velvet algorithms (22), and sequence gaps were closed by PCR and standard Sanger sequencing. The resultant plasmids were annotated using the Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) available at NCBI (http://www.ncbi.nlm.nih.gov/). The plasmid replicon type (InC) was examined by PlasmidFinder (23), and antibiotic resistance genes were identified with the Comprehensive Antibiotic Resistance Database (CARD) (24). The plasmids were compared to each other and were then compared to publicly available plasmid references using BLAST at GenBank (www.ncbi.nlm.nih.gov/GenBank/). The plasmid comparison and visualization were generated by Easyfig (25) according to the online protocol (http://easyfig.sourceforge.net/).

Nucleotide sequence accession number. The complete nucleotide sequence of pKpN01-NDM-7 was deposited under GenBank accession number CP012990.



MBL; metallo-β-lactamase, Kp; *Klebsiella pneumoniae*, Sm; *Serratia marcescens*, Ec; *Escherichia coli*, Eclc; *Enterobacter cloacae/hormaechei*. KpN02 was not available for this study

FIG 1 The timeline of events of patients infected with *Enterobacteriaceae* with bla_{NDM-7}, Calgary, Canada.

TABLE 2 Characteristics of *Enterobacteriaceae* with NDM-7 from Calgary, Canada, from 2013 to 2014

Characteristic	KpN01	SmN01	EcN01	EclcN01	EcN02	KpN12
Patient	1	2	2	3	3	4
Hospital	A	B	B	A	A	A
Specimen	Urine	Urine	Rectal swab	Blood	Rectal swab	Urine
Date of clinical presentation	June 2013	November 2013	November 2013	October 2014	October 2014	December 2014
Susceptibilities (MIC, μg/ml)						
TZP	>64/4	>64/4	>64/4	>64/4	>64/4	>64/4
FOX	>16	>16	>16	>16	>16	>16
CRO	>32	>32	>32	>32	>32	>32
CAZ	>16	>16	>16	>16	>16	>16
FEP	>16	>16	>16	>16	>16	>16
ATM	>16	≤4	>16	>16	>16	>16
MEM	>8	>8	>8	>8	>8	>8
ERT	>4	>4	>4	>4	>4	>4
AMK	≤4	≤4	16	≤4	≤4	≤4
GEN	≤1	≤1	≤1	≤1	≤1	≤1
TOB	≤1	≤1	>8	≤1	≤1	≤1
CIP	1	≤0.5	>2	>2	≤0.5	≤0.5
SXT	>2/38	≤2/38	>2/38	>2/38	≤2/38	≤2/38
TGC	≤1	2	≤1	1	1	≤1
CST	0.19	>256	0.125	0.09	0.125	0.125
FOF	16	>256	1	32	1	16
MHT	Positive	Positive	Positive	Positive	Positive	Positive
Mastdiscs	MBL positive	MBL positive	MBL positive	MBL positive	MBL positive	MBL positive
Carbapenemase	NDM-7	NDM-7	NDM-7	NDM-7	NDM-7	NDM-7
MLST	ST654		ST44	ST113	ST91	ST138
Plasmid size(s) (kb)	190, 130, 50	50	175, 105, 80, 55, 50	170, 80, 50	135, 50	200, 50
Transconjugant (plasmid size, kb)	KpN01T (50)	SmN01T (50)	EcN01T (50)	EclcNT01T (50)	EcN02T (50)	KpN12T (50)
ERT MIC, μg/ml	16	10	16	8	16	16
MER MIC, μg/ml	8	6	4	4	8	4
IPM MIC, μg/ml	>32	>32	>32	>32	>32	>32
FOS MIC, μg/ml	0.5	0.5	0.5	0.5	0.5	0.5
Replicon typing	IncX	IncX	IncX	IncX	IncX	IncX

RESULTS AND DISCUSSION

Susceptibilities and carbapenemase genes. Table 2 shows the susceptibilities of the clinical isolates. EclcN01 was identified as *Enterobacter hormaechei*. All of the isolates tested positive with the modified Hodge test, and the Mastdiscs ID inhibitor combination disks indicated that they were all MBL producers. All of the isolates were positive for $bla_{\text{NDM-7}}$ according to PCR and sequencing results (Table 2).

Enterobacteriaceae with $bla_{\text{NDM-7}}$ are relatively uncommon, having only been previously described in *E. coli* from France (26), Germany (27), India (28), the United States (29), and Japan (30) and in *K. pneumoniae* from the United States (29), Spain (31), and Denmark (32). The single biggest risk factor in all patients was a history of recent travel to India with two exceptions; the patient in the French case had previously traveled to Burma, while no connection with the Indian subcontinent was established among the Spanish patients (26, 31). One of the Calgary patients (patient 2) recently visited India (but he had no contact with the health care system in that country to the best of our knowledge), and the remaining patients (patients 1, 3, and 4) had no recent history of travel outside Alberta. The cases were not linked by time or place, and we were unable to establish any epidemiological linkage between the four patients. The patients from hospital A were admitted to different wards over different time periods and stayed in different parts of Calgary.

Plasmids and MLST. MLST showed that KpN01 belonged to sequence type 278 (ST278), EcN01 belonged to ST44, EclcN01 belonged to ST113, EcN02 belonged to ST91, and KpN12 belonged to ST138 (Table 1). The isolates contained several plasmids of different sizes, but interestingly, they all harbored a plasmid of approximately 50 kb (Table 1). The transconjugants obtained with the different isolates (i.e., KpN01T, SmN01T, etc.) contained the 50-kb plasmid, which was positive for bla_{NDM} and was typed with the IncX3 replicon (Table 1). The international case reports of *E. coli* with $bla_{\text{NDM-7}}$ belonged to various sequence types, including ST167 from France (26), ST599 from Germany (27), ST617 from the United States (29), and ST648 from Japan (30). The *K. pneumoniae* isolates from the United States and Denmark were typed as ST147 (29, 32), while the Spanish isolates belonged to ST437 (31). The conjugative plasmid with $bla_{\text{NDM-7}}$ from the French isolate was untypeable (26), and $bla_{\text{NDM-7}}$ from the German *E. coli* isolate was flanked upstream by IS*5* and IS*Aba125* and downstream by ble_{MBL} and was located on a 60-kb IncX3 plasmid (27).

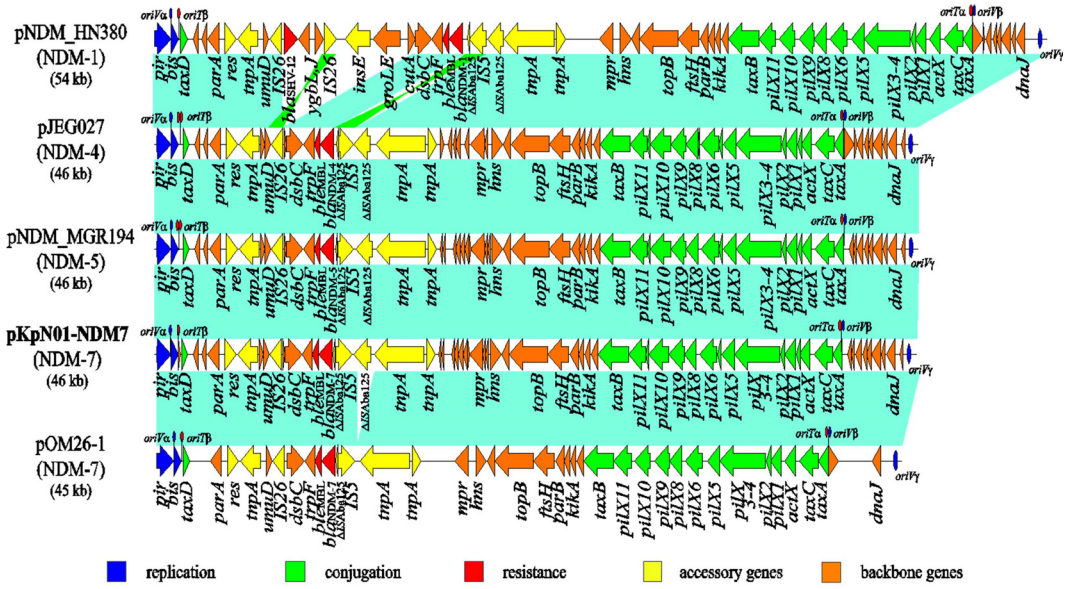


FIG 2 The comparative structures of IncX3 plasmids harboring bla_{NDM}: pKpN01-NDM7 (bla_{NDM-7}; GenBank accession number CP012990), pNDM-HN380 (bla_{NDM-1}; GenBank accession number JX104760), pNDM_MGR194 (bla_{NDM-5}; GenBank accession number KF220657), pJEG027 (bla_{NDM-4}; GenBank accession number KM400601), and pOM26-1 (bla_{NDM-7}; GenBank accession number KP776609). Light blue shading denotes shared regions of homology, while green shading indicates inversely displayed regions of homology. Open reading frames (ORFs) are portrayed by arrows and colored based on predicted gene function, while the putative *oriT* and *oriV* are shown by red and blue ellipses, respectively. The bla_{NDM-7}-harboring plasmid, pKpN01-NDM7, is in bold.

Plasmid sequencing and comparative plasmid analysis. The bla_{NDM-7}-harboring plasmid in KpN01 (namely pKpN01-NDM7) is 46,161 bp in size with an average GC content of 46.6% and contains 54 predicted open reading frames (ORFs) (Fig. 2). Complete sequencing of bla_{NDM-7}-harboring plasmids from the other five transconjugants (i.e., SmN01T, EcN01T, EclcN01T, EcN02T, and KpN12T) interestingly showed 100% identities to that of pKpN01-NDM7. This suggests that the same bla_{NDM-7}-containing plasmid was horizontally transferred to several different *Enterobacteriaceae* genera that colonized/infected the patients in the Calgary region. Alternatively, there may have been common connections with patients from settings where these organisms are endemic who harbored the same or a closely related plasmid. However, we were unable to establish such a link.

pKpN01-NDM7 is an IncX3 plasmid; IncX3 plasmids are usually self-transferable and have been associated with the spread of several antimicrobial-resistant genes, including carbapenemase genes, such as bla_{NDM} (33–37), bla_{KPC} (38), and $bla_{OXA-181}$ (39, 40). A BLAST search against the GenBank sequence database revealed that pKpN01-NDM7 is highly similar to other bla_{NDM}-harboring IncX3 plasmids, including pNDM-HN380 (bla_{NDM-1}) (36), p112298-NDM (bla_{NDM-1}; GenBank accession number KP987216), pEc1929 (bla_{NDM-5}; GenBank accession number KT824791), pNDM_MGR194 (bla_{NDM-5}) (34), pJEG027 (bla_{NDM-4}) (35), and pOM26-1 (bla_{NDM-7}; GenBank accession number KP776609) (Fig. 2). pKpN01-NDM7 has a typical backbone of IncX plasmids, including the genes encoding replication (*pir* and *bis*), partitioning (*par*), maintenance (*topB* and *hns*), and conjugal transfer (*pil* and *tax*) (17, 41). Similar to other IncX3 plasmids, pKpN01-NDM7 carries three putative origins of replication (*oriV*-α, *oriV*-β, or *oriV*-γ), and two origins of conjugal transfer (*oriT*-α and *oriT*-β) (41–43) (Fig. 1). The backbone regions among these bla_{NDM}-harboring IncX3 plasmids showed very high identities (>99.7% compared with each other), suggesting they likely evolved from the same ancestor plasmid.

Common to other IncX3 plasmids, the bla_{NDM-7}-containing accessory region in pKpN01-NDM7 was located downstream of the serine resolvase gene *res*. bla_{NDM-7} was carried by an IS*26*-*dsbC*-*trpF*-ble_{MBL}-bla_{NDM-7}-ΔIS*Aba125*-IS*5*-ΔIS*Aba125* genetic element; this is the same structure as that of the bla_{NDM-4}- and bla_{NDM-5}-containing elements reported previously (34, 35) and is similar to the bla_{NDM-7} plasmid described from Germany (27). The overall plasmid genome synteny in pKpN01-NDM7 is also identical to that of bla_{NDM-5}-harboring pNDM_MGR194 (from a ST11 *K. pneumoniae* isolate in India) (34) and bla_{NDM-4}-harboring pJEG027 (from a *K. pneumoniae* isolate in Australia) (35). Importantly, IS*5* was inserted into IS*Aba125* located upstream of bla_{NDM}, leading to the interruption of IS*Aba125* into two segments (1,018 bp and 73 bp) (Fig. 1). Downstream from bla_{NDM} are the genes for ble_{MBL} (encoding bleomycin-resistant protein), *trpF*, *dsbC*, and IS*26*.

There are two main differences between pKpN01-NDM7, pNDM_MGR194, and pJEG027. First, they carry different bla_{NDM} alleles; pKpN01-NDM7 carried bla_{NDM-7}, while pJEG027 and pNDM_MGR194 harbored bla_{NDM-4} and bla_{NDM-5}, respectively. Of note, bla_{NDM-7} and bla_{NDM-5} each differ from bla_{NDM-4} by a single nucleotide (G388A and G262T, respectively). Second, pKpN01-NDM7 (46,161 bp) is 92 bp shorter than pNDM_MGR194 and pJEG027 (both are 46,253 bp). This is due to an extra 92-bp palindromic sequence in pNDM_MGR194 and pJEG027 that is upstream of *taxD* and that forms an additional *oriT*-β site. It is not clear whether this extra *oriT*-β site will enhance plasmid transfer efficiency, because a previous study indicated that the IncX plasmid can simultaneously cleave multiple *nic* sites, thereby initiating conjugal transfer (42). In addition, pKpN01-NDM7 also showed high identity to a bla_{NDM-7}-bearing

plasmid pOM26-1 (GenBank accession number KP776609), which was isolated from an *E. coli* isolate in Oman. pOM26-1 differs from pKpN01-NDM7 by a 1,039-bp deletion, encompassing the aforementioned 1,018 bp ΔIS*Aba125* flanked by two 4-bp repeats (CTAA) (Fig. 2). This suggests that pOM26-1 evolved from the pKpN01-NDM7-like plasmid as a result of an ~1-kb ΔIS*Aba125*-bearing element deletion.

The first bla_{NDM}-harboring IncX3 plasmid (pNDM-HN380, bla_{NDM-1}) was reported in 2012 from a *K. pneumoniae* isolate discovered in China (36). Since then, IncX3 plasmids containing different bla_{NDM} alleles, including bla_{NDM-1}, bla_{NDM-4} (e.g., pJEG027), bla_{NDM-5} (e.g., pNDM_MGR194), and bla_{NDM-7} (e.g., pKpN01-NDM7 [current study] and pOM26-1) have been reported from different geographical regions (e.g., China, India, Australia, Germany, Canada, and Oman) and in different species (34–36, 44), dramatizing the significant role played by plasmids in the rapid worldwide dissemination of NDM-type carbapenemases. Our study showed that NDM-7 is present in several *Enterobacteriaceae* genera isolated in the Calgary region due to a single identical bla_{NDM-7}-harboring IncX3 plasmid, pKpN01-NDM7. The close sequence identities among these bla_{NDM}-harboring IncX3 plasmids also show their genetic evolution. Espedido et al. (35) hypothesized that pJEG027 (with bla_{NDM-4}) may have arisen from a pNDM-HN380-like plasmid (with bla_{NDM-1}) ancestor as a result of a different IS*5* insertion, an IS*26*-mediated flanking deletion of *cutA1-groL*, and acquisition of the A460C mutation in bla_{NDM-1} (Fig. 2). pNDM_MGR194 (bla_{NDM-5}) may have also evolved from pJEG027 by accumulation of an additional mutation (G262T). Our study suggested that pKpN01-NDM7 had arisen from a pJEG027-like plasmid (carrying bla_{NDM-4}) through acquisition of a different mutation (G388A) and that pOM26-1 is descended from pKpN01-NDM7 through an ~1-kb deletion.

In summary, this study describes the characteristics of *Enterobacteriaceae* with bla_{NDM-7} isolated from 4 Canadian patients from Calgary, Alberta without apparent epidemiological linkages. The 46,161-bp IncX3 plasmid with bla_{NDM-7} is highly similar to other bla_{NDM}-harboring IncX3 plasmids. The NDM-7-containing plasmids from this study, interestingly, showed identical structures within the different *Enterobacteriaceae* isolates (i.e., *K. pneumoniae*, *E. coli*, *Serratia marcescens*, and *E. hormaechei*). This suggests that very effective horizontal transfer events had occurred previously between these patients or that there were connections with patients from settings where these organisms are endemic that we were unable to establish. To the best of our knowledge, this is the first report of *S. marcescens* and *E. hormaechei* with bla_{NDM-7}.

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