Molecular characterization using next generation sequencing of plasmids containing *bla*$_{NDM-7}$ in Enterobacteriaceae from Calgary, Canada

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Running title: Characterization of plasmids with *bla*$_{NDM-7}$,

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Abstract

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

Introduction

The metallo-β-lactamase, NDM-1 was first described in *Klebsiella pneumoniae* and *Echerichia coli* recovered from a Swedish patient who was previously hospitalized in New Delhi, India (1). Subsequently, bacteria with NDM have been recognized in over 50 countries on every continent, except Antarctica (2). Gram negative bacteria with bla_{NDMs} 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 non-endemic areas such as Europe and North America, have most often been associated with patients who required hospitalization while visiting an endemic area (6).

NDM carbapenemases are commonly reported in *K. pneumoniae* and *E. coli,* but also have been found in a variety of other members of the Enterobacteriaceae, 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 only susceptible to agents, such colistin, fosfomycin and tigecycline (10).

During 2013 and 2014, six Enterobacteriaceae with $bla_{\text{NDM-7}}$ were isolated from four different Calgary patients over a period of 18 months. One patient was recently 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.

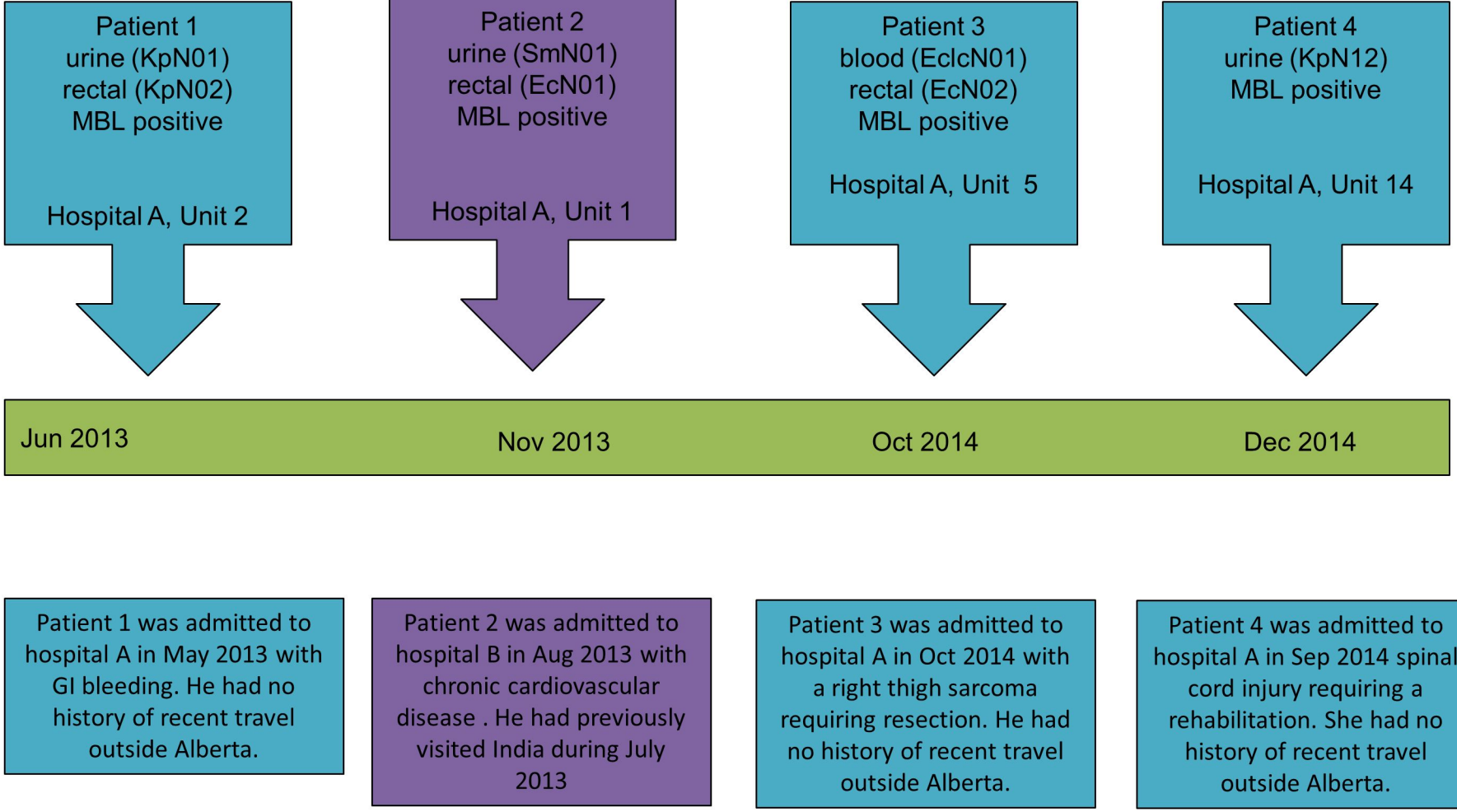
Materials and methods

Patient and isolates. For a summary of the clinical features of patients 1-4 and time line of events, please see Table 1 and Figure 1.

The isolates were identified using the MALDI-TOF MS (Vitek AMS; bioMerieux Vitek Systems Inc., Hazelwood, MO). Further identification of *E. cloacae* was performed by partial sequencing of the *leuS* gene (11).

Antimicrobial susceptibilities. Minimum inhibitory concentrations (MICs) were determined using the Microscan NEG 38 panel (Siemens, Burlington, Ontario, Canada) and 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 (COL), fosfomycin (FOS), imipenem (IPM), MEM, ERT MICs were determined using E-tests (bioMerieux Inc., Hazelwood, MO, United States) according to the manufacturer's instructions. The IPM, MEM, ERT, FOS E-tests were also performed on the *E. coli* J53 transcongugants (see below). The European Committee for Antimicrobial Susceptibility Testing (EUCAST) breakpoint was used for COL 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 the MASTDISCS™ ID inhibitor combination disks (13) (Mast Group Ltd., Merseyside, United Kingdom). PCR



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

Figure 1. The timeline of events of patients infected with Enterobacteriaceae harbouring $bla_{\text{NDM-7}}$, Calgary, Canada

Table 1. Characteristics of patients infected with Enterobacteriaceae harbouring *bla*$_{NDM-7}$, Calgary, Canada

Characteristic	Patient 1	Patient 2	Patient 3	Patient 4
Age	98	65	77	76
Sex	Male	Male	Male	Female
Hospital	A	B	A	A
Unit	2	1	5	14
Admission date	May 2013	Aug 2013	Oct 2014	Sep 2014
Clinical diagnosis	Asymptomatic bacteriuria	Lower UTI	Septicaemia	Lower UTI
Co-morbid conditions	Upper GI bleeding	Diabetes mellitus, PVD, hypertension	Sarcoma	Spinal cord injury
Date of clinical presentation	Jun 2013	Nov 2013	Oct 2014	Dec 2014
Treatment	in-out catheterization	ciprofloxacin	gentamicin and colistin	ciprofloxacin
Travel History	None	India	None	None

GI, Gastro-intestinal; UTI, urinary tract infection; PVD, peripheral vascular disease

amplification and sequencing for *bla*$_{KPCs}$, *bla*$_{VIMs}$, *bla*$_{IMPs}$, *bla*$_{NDMs}$, and *bla*$_{OXA-48-like}$ were undertaken using primers and conditions as previously described (13).

Plasmid analysis. Plasmid sizes were determined as previously described (14) and 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 MEM 1μg/ml and using *E. coli* J53 (azide 100 μg/ml) as recipient. Plasmids from the transconjugants were sequenced using Pacific Biosciences RSII platform (Menlo Park, CA, USA) and Illumina MiSeq system (San Diego, CA, USA) [see details below].

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

Complete sequencing of *bla*$_{NDM-7}$-harboring plasmids. The genome of isolate KpN01 from patient 1 (chromosome and plasmids) were sequenced using Pacific Biosciences RSII 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*$_{NDM-7}$-harbouring plasmids from isolate SmN01 (from patient 2), EcN01 (from patient 2), EclcN01 (from patient 3), EcN02 (from patient 3) and KpN12 (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 Maxi kit (Qiagen, Valencia, CA), and sequenced using 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 then compared to publicly available plasmid references using BLAST at GenBank. (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 as GenBank accession no. CP012990.

Results and Discussion

Susceptibilities, and carbapenemase genes. Table 2 shows the susceptibilities of the clinical isolates. EclcN01 was identified as *Enterobacter hormaechei.* All the isolates tested positive with the modified Hodge test, and the MASTDISCS™ ID inhibitor combination disks indicated that they all were 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), USA (29) and Japan (30), and *K. pneumoniae* from the USA (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 French case previously travelled to Burma, while no connection with the Indian subcontinent was established among the Spanish patients (26, 31). One of the Calgary patients (patient no 2) recently visited India (although he had no contact with the health-care system in that country to the best of our

Table 2. Characteristics of Enterobacteriaceae with NDM-7 from Calgary, Canada from 2013-14

	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	Jun 2013	Nov 2013	Nov 2013	Oct 2014	Oct 2014	Dec 2014
Susceptibilities (MIC)						
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
COL	0.19	>256	0.125	0.09	0.125	0.125
FOS	16	>256	1	32	1	16
MHT	pos	pos	pos	pos	pos	pos
MASTDISCS™	MBL pos	MBL pos	MBL pos	MBL pos	MBL pos	MBL pos
Carbapenemase	NDM-7	NDM-7	NDM-7	NDM-7	NDM-7	NDM-7

MLST	ST654	-	ST44	ST113	ST91	ST138
Plasmids (sizes [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	16	10	16	8	16	16
MER MIC	8	6	4	4	8	4
IPM MIC	>32	>32	>32	>32	>32	>32
FOS MIC	0.5	0.5	0.5	0.5	0.5	0.5
Replicon typing	IncX	IncX	IncX	IncX	IncX	IncX

Kp; *Klebsiella pneumonaie*, Sm; *Serratia marcescens*, Ec; *Escherichia coli,* Eclc; *Enterobacter cloacae/hormaechei*.
piperacillin-tazobactam (TZP), cefoxitin (FOX), ceftriaxone (CRO), ceftazidime (CAZ), cefepime (FEP), aztreonam (ATM), meropenem (MEM), ertapenem (ERT), Imipenem (IPM), amikacin (AMK), gentamicin (GEN), tobramycin (TOB), ciprofloxacin (CIP), tigecycline (TGC). Fosfomycin (FOS), trimethoprim-sulfamethoxazole (SXT), colistin (COL), modified Hodge test (MHT), Multilocus sequencing typing (MLST), Minimum inhibitory concentration (MIC), metallo-β-lactamase (MBL).

knowledge), and the remaining patients (patients 1, 3, 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 ST278, EcN01 to ST44, EclcN01 to ST113, EcN02 to ST91 and KpN12 belonged to ST138 (Table 1). The isolates contained several plasmids with different sizes, but interestingly, they all harboured a plasmid of approximately 50 kb (Table 1). The transconjugants obtained with the different isolates (i.e., KpN01T, SmN01T etc.) contained the 50kb plasmid, which was positive for bla_{NDM} and 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 USA (29) and ST648 from Japan (30).The *K. pneumoniae* from the USA and Denmark were typed as ST147 (29, 32) while the Spanish isolates belonged to ST437 (31). The conjugative plasmid with the $bla_{\text{NDM-7}}$ from the French isolate was untypeable (26) and $bla_{\text{NDM-7}}$ from the German *E. coli* was flanked upstream by IS*5*, IS*Aba125*, downstream by ble_{MBL} and located on a 60kb IncX3 plasmid (27).

Plasmid sequencing and comparative plasmid analysis. The $bla_{\text{NDM-7}}$ 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 ORFs (Figure 2). The complete sequencing of $bla_{\text{NDM-7}}$-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 $bla_{\text{NDM-7}}$-containing plasmid was horizontally transferred to several

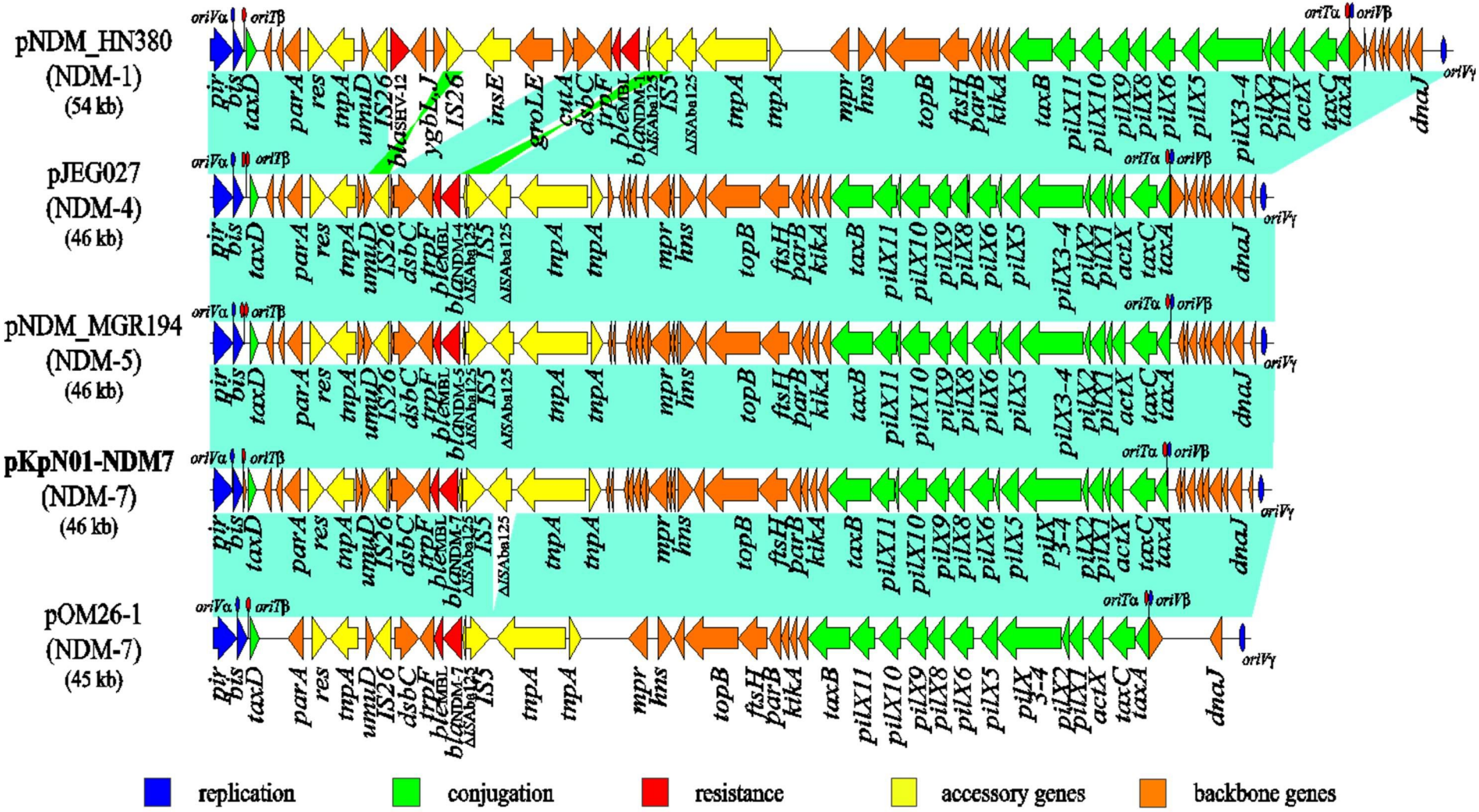


Figure 2. The comparative structures of IncX3 plasmids harboring bla_{NDM}: pKpN01-NDM7 (bla_{NDM-7}, CP012990), pNDM-HN380 (bla_{NDM-1}, JX104760), pNDM_MGR194 (bla_{NDM-5}, KF220657), pJEG027 (bla_{NDM-4}, KM400601) and pOM26-1 (bla_{NDM-7}, KP776609). Light blue shading denotes shared regions of homology, 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 showed by red and blue ellipses, respectively. The bla_{NDM-7} harboring plasmid, pKpN01-NDM7, is highlighted in bold case.

different Enterobacteriaceae genera that colonized/infected the patients in the Calgary region. Alternatively, there could have been common connections with patients from endemic settings who harbored the same or a closely related plasmid. However, we were unable to establish such a link.

pKpN01-NDM7 is a plasmid of IncX3 that 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*$_{NDM-1}$, KP987216), pEc1929 (*bla*$_{NDM-5}$, KT824791), pNDM_MGR194 (*bla*$_{NDM-5}$) (34), pJEG027 (*bla*$_{NDM-4}$) (35) and pOM26-1 (*bla*$_{NDM-7}$, KP776609) (Figure 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, - β or - γ), and two origins of conjugal transfer (*oriT*- α and - β) (41-43) [Figure 1]. The backbone regions amongst these *bla*$_{NDM}$-harboring IncX3 plasmids showed very high identities (> 99.7% when 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; the same structure as the *bla*$_{NDM-4}$ and *bla*$_{NDM-5}$ containing elements reported previously (34, 35), as well as being 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 an ST11 *K. penumoniae* in India) (34) and *bla*$_{NDM-4}$ –harboring pJEG027 (from a *K. pneumoniae* isolate in Australia) (35). Importantly, an 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) (Figure 1). Downstream from *bla*$_{NDM}$ are the genes for *ble*$_{MBL}$ (encoding bleomycin-resistant protein), *trpF*, *dsbC* gene and an IS*26* element.

There are two main differences between pKpN01-NDM7, pNDM_MGR194 and pJEG027. Firstly, they carry different *bla*$_{NDM}$ alleles; pKpN01-NDM7carried *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 differs from *bla*$_{NDM-4}$ by a single nucleotide (G388A and G262T, respectively). Secondly, 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*, which forms an additional *oriT-β* site. It is not clear whether this extra *oriT-β* 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 (KP776609), 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) (Figure 2). This suggests that pOM26-1 evolved from the pKpN01-NDM7-like plasmid as a result of ~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* isolated in China (36). Since then, IncX3 plasmids containing different *bla*$_{NDM}$ alleles, including bla$_{NDM-1}$, $_{-4}$ (e.g. pJEG027), $_{-5}$ (e.g. pNDM_MGR194) and $_{-7}$ (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, 45), dramatizing the significant role played by plasmids in the rapid worldwide dissemination of NDM-type carbapenemases. Our study showed that NDM-7 are 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 shows 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*$_{NDM-1}$ (Figure 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 (*bla*$_{NDM-4}$-carrying) through acquisition of a different mutation (G388A), and that pOM26-1 is descendant from pKpN01-NDM7 through a ~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*.

S. marcescens, *E. hormaechei*). This suggests that very effective horizontal transfer events had occurred previously between these patients or there were connections with patients from endemic settings that we were unable to establish. To the best of our knowledge, this is the first report of *S. marcescens,* and *E. hormaechei* with $bla_{\text{NDM-7.}}$

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Transparency declaration

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Figure headings

Figure 1. The timeline of events of patients infected with Enterobacteriaceae with $bla_{\text{NDM-7}}$, Calgary, Canada

Figure 2. The comparative structures of IncX3 plasmids harboring bla_{NDM}: pKpN01-NDM7 ($bla_{\text{NDM-7}}$, CP012990), pNDM-HN380 ($bla_{\text{NDM-1}}$, JX104760), pNDM_MGR194 ($bla_{\text{NDM-5}}$, KF220657), pJEG027 ($bla_{\text{NDM-4}}$, KM400601) and pOM26-1 ($bla_{\text{NDM-7}}$, 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 showed by red and blue ellipses, respectively. The $bla_{\text{NDM-7}}$ harboring plasmid, pKpN01-NDM7, is highlighted in bold case.

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