

**Colistin non-susceptible *Pseudomonas aeruginosa* ST654 with
*bla*_{NDM-1} arrives in North America**

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Running title: *P. aeruginosa* with *bla*_{NDM-1} in North America

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Abstract

This study describes 3 different *bla*_{NDM-1} genetic platforms in 3 different species obtained from the same patient who was directly transferred to an institution in Calgary, Canada, following a prolonged hospital stay in India. The *bla*_{NDM-1} in the *Escherichia coli* was located on a 176kb IncA/C plasmid contained within an ISCR1 region. The *bla*_{NDM-1} in the *Providencia rettgeri* was located on a 117kb IncT plasmid contained within Tn3000, while the *bla*_{NDM-1} in *Pseudomonas aeruginosa* was located on the chromosome within an ISCR3 region. This report highlights the plasticity of the genetic regions and environments associated with *bla*_{NDM-1}. To the best of our knowledge, this is the first report of *P. aeruginosa* with *bla*_{NDM-1} identified in North America and the first report of *bla*_{OXA-181} in *P. rettgeri*. The *P. aeruginosa* belonged to the international high risk clone ST654 and was non-susceptible to colistin. This case emphasizes the need for appropriate infection prevention and control measures and vigilant screening for carbapenem resistant Gram negative bacteria in patients with a history of travel to endemic areas, such as the Indian subcontinent.

Introduction

The metallo- β -lactamase, NDM 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 with NDM-1 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) and certain countries within the Middle East and the Balkans (3).

NDM has most commonly been reported in *K. pneumoniae* and *E. coli*, but has also been found in a variety of other members of the Enterobacteriaceae, *Acinetobacter* spp., *Pseudomonas* spp. and *Vibrio cholerae* (4, 5). The treatment of infections caused by NDM-producing Enterobacteriaceae is causing serious therapeutic challenges for the medical community because isolates are often also resistant to non- β -lactam antibiotics, including the aminoglycosides and fluoroquinolones (6). Bacteria with NDMs often remain only susceptible to agents such as colistin, fosfomicin and tigecycline (7).

Infections with NDM-producing bacteria in non-endemic areas such as Europe and North America have most often been associated with visiting and being hospitalized in endemic regions, such as the Indian subcontinent (8). During April 2015, *Pseudomonas aeruginosa*, *Providencia rettgeri* and *E. coli* with bla_{NDM} were isolated from an elderly Canadian patient who was directly transferred to an institution in Calgary following a prolonged hospital stay in India. A study was designed to characterize the isolates and their respective plasmids using traditional and next generation sequencing techniques.

Materials and methods

Patient and isolates. During March 2015, an elderly male in his late seventies from Calgary, Alberta, Canada, travelled to India. Soon after his arrival, he developed a sudden onset of slurred speech, left sided weakness, and decreased level of consciousness associated with respiratory distress. He was admitted to a local hospital in New Delhi. A CT scan of the head showed a basilar artery thrombosis with an acute right cerebellar and pontine infarct. He was then transferred to a speciality hospital Intensive Care Unit (ICU), and received comprehensive stroke management with antiplatelet medications, statins, anti-hypertensive medications, insulin and intensive rehabilitation. During his complex hospital course, he required a tracheostomy, and subsequently developed nosocomial pneumonia caused by *P. aeruginosa* that was resistant to carbapenems. He was treated with intravenous colistin and meropenem, weaned from the ventilator, and directly transferred to Calgary for ongoing care and management approximately 6 weeks after his initial hospital admission in India.

He was admitted to a single room in a Calgary hospital and immediately placed on contact precautions. Routine admission screening was done for antimicrobial resistant organisms, including methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococcus and MDR Gram-negative organisms. Urine and rectal swab cultures revealed 3 different MDR Gram-negative bacteria including; 1) carbapenem-resistant *P. aeruginosa* [Pa15-1092] (urine), 2) carbapenem-resistant *P. rettgeri* [Pr15-1091] (urine), and 3) carbapenem-resistant *E. coli* [Ec15-1078] (rectal swab). The isolates were identified using MALDI-TOF MS (Vitek AMS; bioMerieux Vitek Systems Inc., Hazelwood, MO).

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 (9). The following drugs were tested: : piperacillin-tazobactam (TZP), 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) MICs were determined using E-tests (bioMérieux Inc., Hazelwood, MO, United States) according to the manufacturer's instructions. The European Committee for Antimicrobial Susceptibility Testing (EUCAST) breakpoint was used for COL and the FDA breakpoint was used for TGC.

β -lactamase 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 (10) (Mast Group Ltd., Merseyside, United Kingdom). All isolates were initially screened by an in-house PCR for *bla*_{KPCs}, *bla*_{VIMs}, *bla*_{IMP}s, *bla*_{NDM}s, and *bla*_{OXA-48-like} (10) and then referred to the National Microbiology Laboratory (NML)[Winnipeg, Manitoba, Canada] for further characterization. At the NML, isolates were screened for additional β -lactamase genes including *bla*_{SHV}, *bla*_{TEM}, *bla*_{CTX-M}, *bla*_{OXA-1}, *bla*_{CMY-2} and sequenced using conditions and primers as previously described (11).

Plasmid analysis. Plasmid sizes were determined as previously described (12) and assigned to plasmid incompatibility groups by PCR-based replicon typing (13, 14) <http://pubmlst.org/plasmid/primers/incF.shtml>. Conjugation experiments were performed by mating-out assays with nutrient agar containing MER 1 μ g/ml and using *E. coli* DH10B as recipient.

Multilocus sequencing typing (MLST). Detailed protocols of the MLST procedures used in this study for typing the *P. aeruginosa* and *E. coli*, including the allelic types and

sequence types (ST) assignment methods, are available at MLST Databases at <http://pubmlst.org/paeruginosa> for *P. aeruginosa* and <http://mlst.ucc.ie/mlst/dbs/Ecoli> for *E. coli*.

Next Generation Sequencing. Total cellular DNA was prepared using Epicentre MaterPure™ Complete kits (Madison, WI) and submitted for MiSeq™ (Illumina, San Diego, CA) and PacBio RS II (Pacific Biosciences, Menlo Park, CA) sequencing. De novo assembly of Illumina reads were done using Spades v3.5. De novo assembly of Pacific Biosystems reads were done on the Pacific Biosystems single molecule real-time (SMRT) portal using the hierarchal genome assembly process (HGAP) 3 workflow, including consensus polishing with Quiver. The final circularized assembly for each strain was polished using RS sequencing. Genomes and plasmids were initially annotated by Prokka v1.1 and edited in Sequin v13.7. Closed plasmids were obtained for Ec15-1078 (pNDM15-1078 accession number CP012902), Pr15-1091 (pNDM15-1091 accession number CP012903 and pOXA181-15-1091 accession number CP012904). A closed genome was obtained for *P. aeruginosa* 15-1092 (accession number CP012901). Sequence data was run through the Centre for Genomic Epidemiology (<http://www.genomicepidemiology.org/>) using Plasmid Finder, ResFinder, Virulence Finder and to confirm the multi-locus sequence type (MLST).

Results and Discussion

Overseas travel, as a risk factor for the acquisition of infections due to antimicrobial resistant organisms, has recently been described for infections due to CTX-M-producing *E. coli*, as well as various carbapenemase-producing Gram negative Enterobacteriaceae, including the NDMs (8). In Canada, out-of-country hospitalization due to medical tourism or of an unexpected nature during travel, has been the most important risk factor for colonization or infection with an NDM-1-producing organism (15, 16). In order to prevent the introduction and spread of

multidrug resistant (MDR) and extensively drug resistant (XDR) bacteria by returning travellers into the health care systems of their respective home countries, it is essential to rapidly identify patients colonized or infected by these bacteria and place them on appropriate infection control precautions (8, 17, 18). Contact precautions should be rigorously maintained for patients confirmed to be colonized or infected with carbapenemase-producing bacteria.

This study describes the characteristics of XDR *P. aeruginosa*, *P. rettgeri* and MDR *E. coli* obtained from an elderly Canadian patient who was directly transferred to our institution following a prolonged hospital stay in India. This patient was immediately placed on contact precautions in a single room upon admission in Calgary. No secondary spread has been documented during his prolonged stay in our institution. Our health region recommends careful attention to routine practices (i.e. hand hygiene, point-of-care risk assessment, gowns and gloves for contact with any blood, body fluids, secretions or drainage, cleaning of shared equipment, effective environmental cleaning) and additional precautions (usually contact precautions) as appropriate to the presenting illness. Routine screening is also done on admission and includes cultures from multiple body sites for carbapenem-resistant organisms from all patients who have received hemodialysis or have been hospitalized for > 24 hrs outside of Canada within the past 6 months, with a specific focus on known endemic areas with a high prevalence of carbapenemase-producing Gram-negative bacteria.

P. aeruginosa 15-1092 (Pa15-1092) and *P. rettgeri* 15-1091 (Pr15-1091) tested non-susceptible (NS) [i.e. intermediate or resistant] to all the antibiotics (including COL [MIC 3µg/ml for both) except for ATM [MIC ≤ 4µg/ml for both]; *E. coli* 15-1078 (Ec15-1078) was NS to TZP, FOX, CRO, CAZ, FEP, MER, ERT, AMK, GEN, TOB, SXT and tested susceptible to ATM, AMK, CIP, TIM and COL (MIC 0.19µg/ml) [Table 1]. All three isolates tested positive

Table 1. Characteristics of extensively drug resistant *Pseudomonas aeruginosa*, *Providentia rettgeri* and multidrug resistant *Escherichia coli* from Calgary, Canada

	<i>P. aeruginosa</i> (15-1092)	<i>P. rettgeri</i> (15-1091)	<i>E. coli</i> (15-1078)
Specimen source	Urine	Urine	Rectal swab
Susceptibilities			
TZP	>64/4	>64/4	>64/4
FOX	>16	>16	>16
CRO	>32	>32	>32
CAZ	>16	>16	>16
FEP	>16	>16	>16
ATM	≤ 4	≤ 4	≤ 4
MEM	>8	>8	>8
ERT	-	>4	>4
AMK	>32	>32	8
GEN	>8	>8	>8
TOB	>8	>8	>8
CIP	>2	>2	≤0.5
SXT	>2/38	>2/38	>2/38
TGC	>8	4	≤0.5
COL	3	3	0.19
MHT	pos	pos	pos
MASTDISCS™	MBL pos	MBL pos	MBL pos
β-lactamases	NDM-1, OXA-50, PDC-3	NDM-1, OXA-181, TEM-1B	NDM-1, OXA-10
Additional antimicrobial resistance genes	<i>aph(3')-IIb, strA, strB, catB7, fosA, sul1</i>	<i>mph(E), msr(E), armaA, sul1, aadA2, dfrA12, qnrB1, catA1, arr-3, cmlA1, tet(A), strA, strB</i>	<i>mph(E), msr(E), armaA, sul1, aadA1, aadA2, dfrA12, qnrA1, cmlA1, arr-3</i>
MLST	ST654	-	ST522
Plasmids (sizes [kb])	none	12, 117	176
Transconjugant (plasmid size[kb])	none	pNDM15-1091 (120)	pNDM15-1078 (180)
Replicon typing	-	IncT	IncA/C

piperacillin-tazobactam (TZP), 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), modified Hodge test (MHT), Multilocus sequencing typing (MLST)

with the modified Hodge test, and MASTDISCS™ ID inhibitor combination disks indicated that the isolates produced MBLs. PCR and sequencing results showed that Pa15-1092 and Ec15-1078 were positive for *bla*_{NDM-1}, while Pr15-1091 was positive for *bla*_{NDM-1} and *bla*_{OXA-181} (Table 1).

MLST showed Pa15-1092 belonged to ST654, while Ec15-1078 belonged to ST522 (Table 1). MDR *P. aeruginosa* ST654 is an international high risk clone present in Poland [among MDR isolates] (19), Sweden [with VIM-2] (20) and United Kingdom [with VIM, IMP] (21). No reports of *E. coli* ST522 have been published.

P. aeruginosa with *bla*_{NDM} is relatively uncommon and had previously been described in Serbia (22), France (23), Italy (24), India (25, 26), Egypt (27), Denmark (28) and United Kingdom (29). Moreover, very little is known regarding the molecular epidemiology of *P. aeruginosa* that contains NDM genes. The *P. aeruginosa* from Serbia (30), France (31) and India (32) underwent additional molecular characterization. The surrounding regions of the *bla*_{NDM-1} in the French and Serbian isolates were similar and had chromosomal locations contained within a class 1 integron bearing IS common region 1 (*ISCR1*) located in a Tn402-like structure. The *bla*_{NDM-1} in some of the Indian isolates was transferred to *E. coli* (32). In the current study a 7Mb single circular chromosome was sequenced and closed for Pa15-1092 (GenBank accession number CP012901). The following antimicrobial resistant determinants were identified: *bla*_{OXA-50}, *bla*_{NDM-1}, *bla*_{PDC-3}, *aph(3')-IIB*, *strA*, *strB*, *catB7*, *fosA*, and *sulI* (Table 1). The *bla*_{NDM-1} gene was found to be integrated into the *P. aeruginosa* chromosome within a 12kb variable region. Upstream to this region was the chromosomal gene *aniA* and downstream a hypothetical protein. The *bla*_{NDM-1} gene was flanked by *ISCR3* and *ISCR5-like* transposase genes. Downstream of *ISCR5* was *TniA*, *TniB*, *TniQ* (a family of proteins believed to be involved in integron dissemination) and *sulI* (Figure 1). This arrangement was highly similar to the surrounding

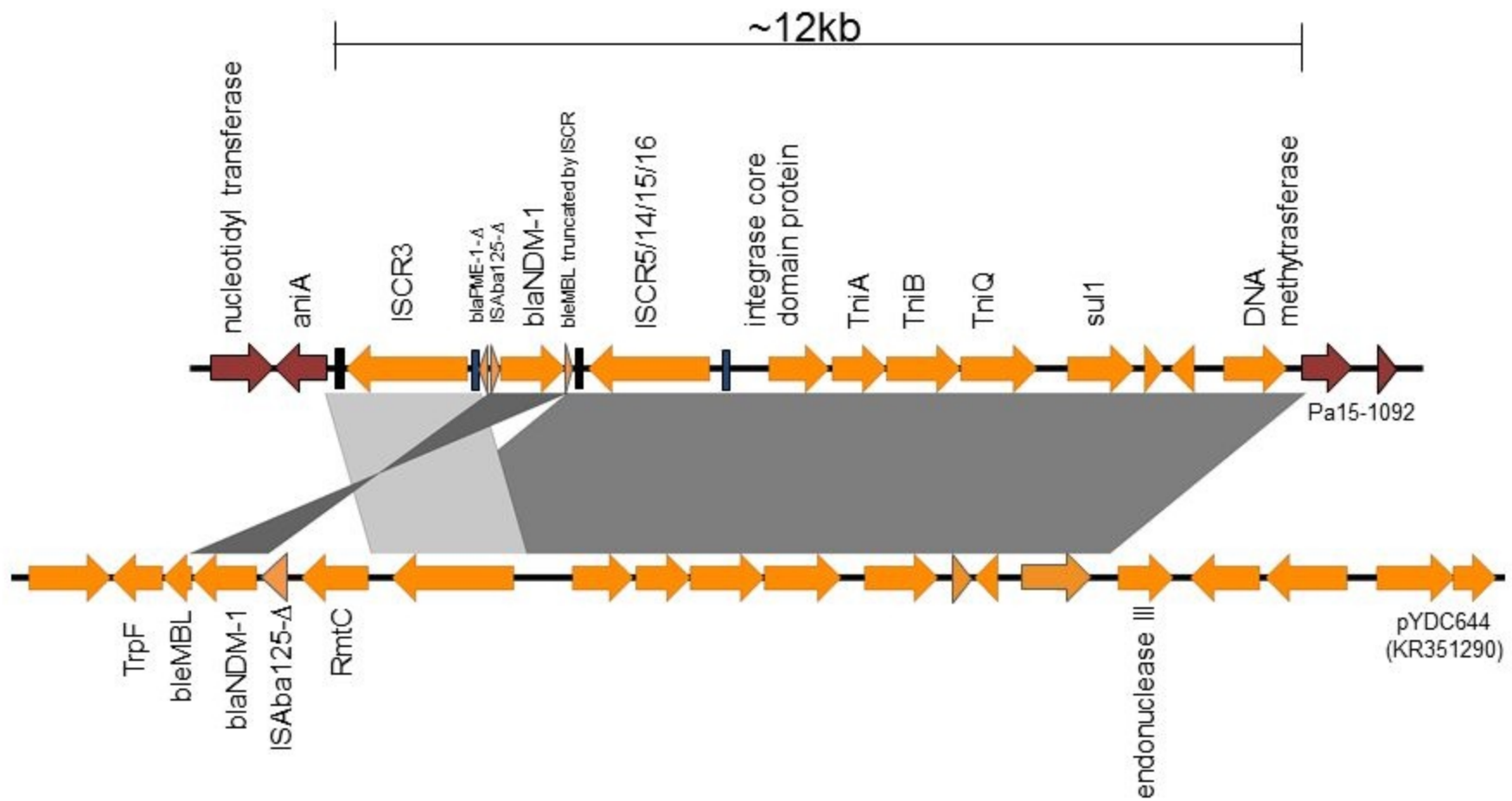


Figure 1. Schematic representation of the blaNDM-1 region from *P. aeruginosa* 15-1092 compared to a *K. pneumoniae* IncFII plasmid region. The genes and their corresponding transcriptional orientations are indicated by horizontal arrows; orange are plasmid associated genes and red are chromosomal. Black rectangles represent oriIS of ISCR-elements and blue rectangles represent terIS (gccg-cggc) of ISCR-elements. Delta (Δ) indicates an interrupted gene. Shaded regions are DNA sequences shared by both isolates, dark grey are identical sequences. Accession number for Pa15-1092 was CP012901.

regions of *bla*_{NDM-1}, present on an IncFII plasmid from *K. pneumoniae* isolated in Iran (GenBank accession number KR351290) (Figure 1). It is possible that a transposition event occurred previously that was responsible for mobilizing the *bla*_{NDM-1} from a chromosomal location within *P. aeruginosa* onto an IncFII plasmid, that was transferred to a *K. pneumoniae* isolate (or *vice versa*).

Molecular epidemiological studies indicated that the IncA/C-type broad-host range plasmids associated with mosaic genetic structures (e.g. *ISAbal1*) are mainly responsible for spreading *bla*_{NDM-1}, among Enterobacteriaceae (4, 33). Moreover, IncFII, IncN, IncH and IncL/M types of plasmids have also been associated with *bla*_{NDM} (34-36). In addition, IncA/C plasmids with the *bla*_{NDM} often contain various clinically-relevant antibiotic resistance genes, such as those encoding RmtA or RmtC (i.e. 16S rRNA methylases encoding high level resistance to aminoglycosides), QnrA (quinolone resistance), and other β -lactamases (i.e. CMY, CTX-M responsible for broad-spectrum cephalosporin resistance). In this study we describe a 176kb *bla*_{NDM-1} plasmid belonging to IncA/C which was sequenced and closed from Ec15-1078 (pNDM15-1078, GenBank accession number CP012902). All resistance determinants in this isolate were associated with the NDM plasmid: *bla*_{OXA-10}, *bla*_{NDM-1}, *mph(E)*, *msr(E)*, *armA*, *sul1*, *aadA1*, *aadA2*, *dfrA12*, *qnrA1*, *cmlA1*, and *arr-3*. Upstream of the *bla*_{NDM-1} gene was *ISCR1* followed by a class 1 integron and *IS26* (Figure 2a). Downstream on the *bla*_{NDM-1} gene was a second copy of *ISCR1*. Similar *bla*_{NDM-1} arrangements have been described in *Citrobacter freundii* which belonged to IncHI1 (35), and an *E. coli* belonging to IncFII (34), which shows the diverse host and plasmid range for this element. In addition, the virulence associated genes *prfB* and *gad* were identified on the *E.coli* chromosome.

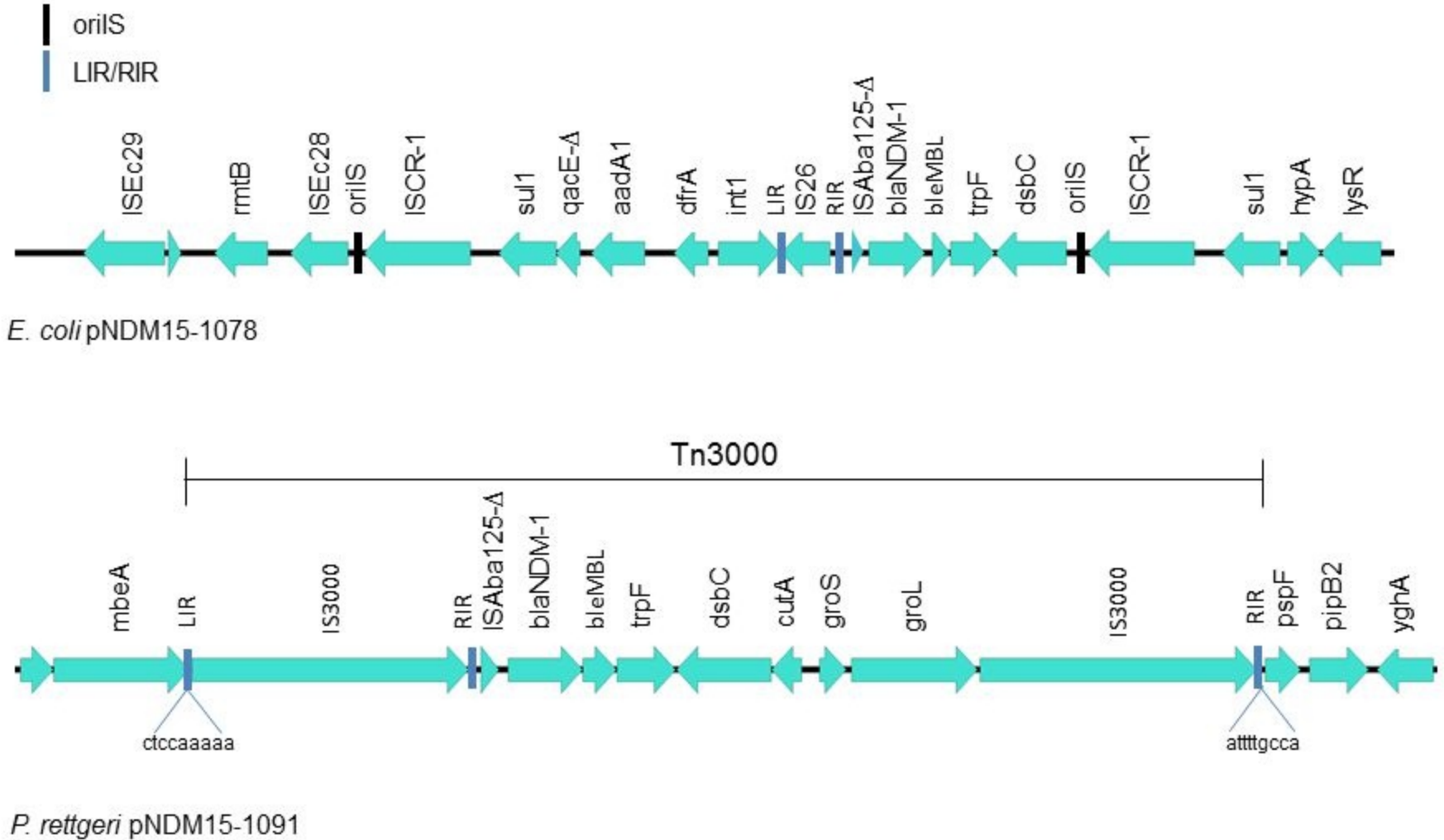


Figure 2. Schematic representation of the blaNDM-1 region from a) *E. coli* pNDM-1078 and b) *P. rettgeri* pNDM15-1091. The genes and their corresponding transcriptional orientations are indicated by horizontal arrows. Black rectangles represent oriIS of ISCR-elements and blue rectangles represent left (LIR) or right (RIR) inverted repeats of insertion sequences. Delta (Δ) indicates an interrupted gene. The accession numbers were: pNDM15-1078 (genbank CP012902) and pNDM15-1091 (genbank CP012903).

Conversely, the structure of the NDM region from the *P. rettgeri* (Pr15-1091) isolate was quite different. The NDM-1 plasmid (pNDM15-1091, CP012903) was 117kb and belonged to the incompatibility group IncT, a narrow host range plasmid, which has been rarely reported. IncT has only previously been reported from an outbreak of *P. mirabilis* CTX-M-2 in Japan (37) and a *C. freundii* harbouring *bla*_{OXA-181} from France (38). To the best of our knowledge this is the first time IncT has been reported in a *Providencia* spp. and associated with *bla*_{NDM-1}. This plasmid contained the following resistance determinants: *bla*_{NDM-1}, *mph(E)*, *msr(E)*, *arma*, *sull*, *aadA2*, *dfrA12*, *qnrB1*, and *catA1*. The *bla*_{NDM-1} and surrounding genes were on Tn3000 bracketed by inverted repeats (Figure 2b). Tn3000 was recently described and has been implicated in the dissemination of *bla*_{NDM-1} among Enterobacteriaceae (39).

OXA-181, a derivative of OXA-48, had mostly been identified in *K. pneumoniae* and is endemic in the Indian subcontinent (40). This enzyme is also present among other members of the Enterobacteriaceae (including *E. coli*, *C. freundii*, *E. cloacae*, and *M. morgani*) from countries in Europe, North Africa, Asia and Oceania (41). In addition to the NDM-1 containing plasmid, Pr15-1091 also harboured an OXA-181 containing plasmid (Figure 3). The OXA-181 plasmid (pOXA-181-15-1091, GenBank accession number CP012904) was 12kb and did not belong to any known incompatibility group. A *repB* gene was identified with >97% identity to known replication initiation proteins found in various Gammaproteobacteria (GenBank accession numbers WP_044111722, KGH49506, WP_008916987, EKT52968). The *bla*_{OXA-181} was found on Tn2013 flanked by a 5-bp duplication of the target site (TATCA). As previously reported the Tn2013 was bracketed by inverted repeats IRL and IRR2 of *ISEcp1* (42). In addition the plasmid contained ISKpn21, the transcriptional regulator *copG*, and a plasmid stabilization protein, *relE*. There were no additional resistance determinants, other than the *bla*_{OXA-181} gene. The structure is

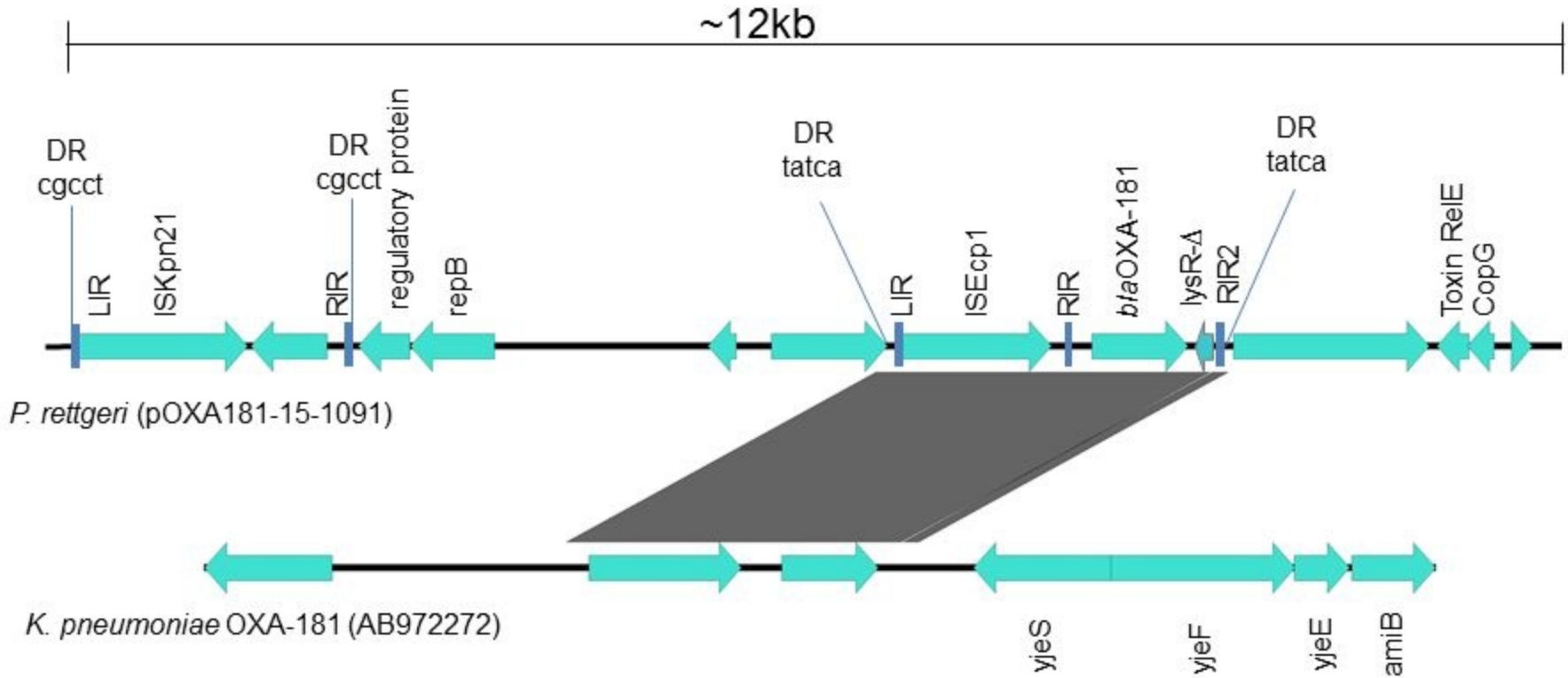


Figure 3. Schematic representation of the *bla*OXA-181 region from *P. rettgeri* pOXA-181-15-1091 compared to a *K. pneumoniae* OXA181 isolate. The genes and their corresponding transcriptional orientations are indicated by horizontal arrows. Blue rectangles represent left (LIR) or right (RIR) inverted repeats of insertion sequences. Delta (Δ) indicates an interrupted gene. Shaded regions are identical DNA sequences shared by both isolates. Accession number for pOXA-181-15-1091 was CP012904.

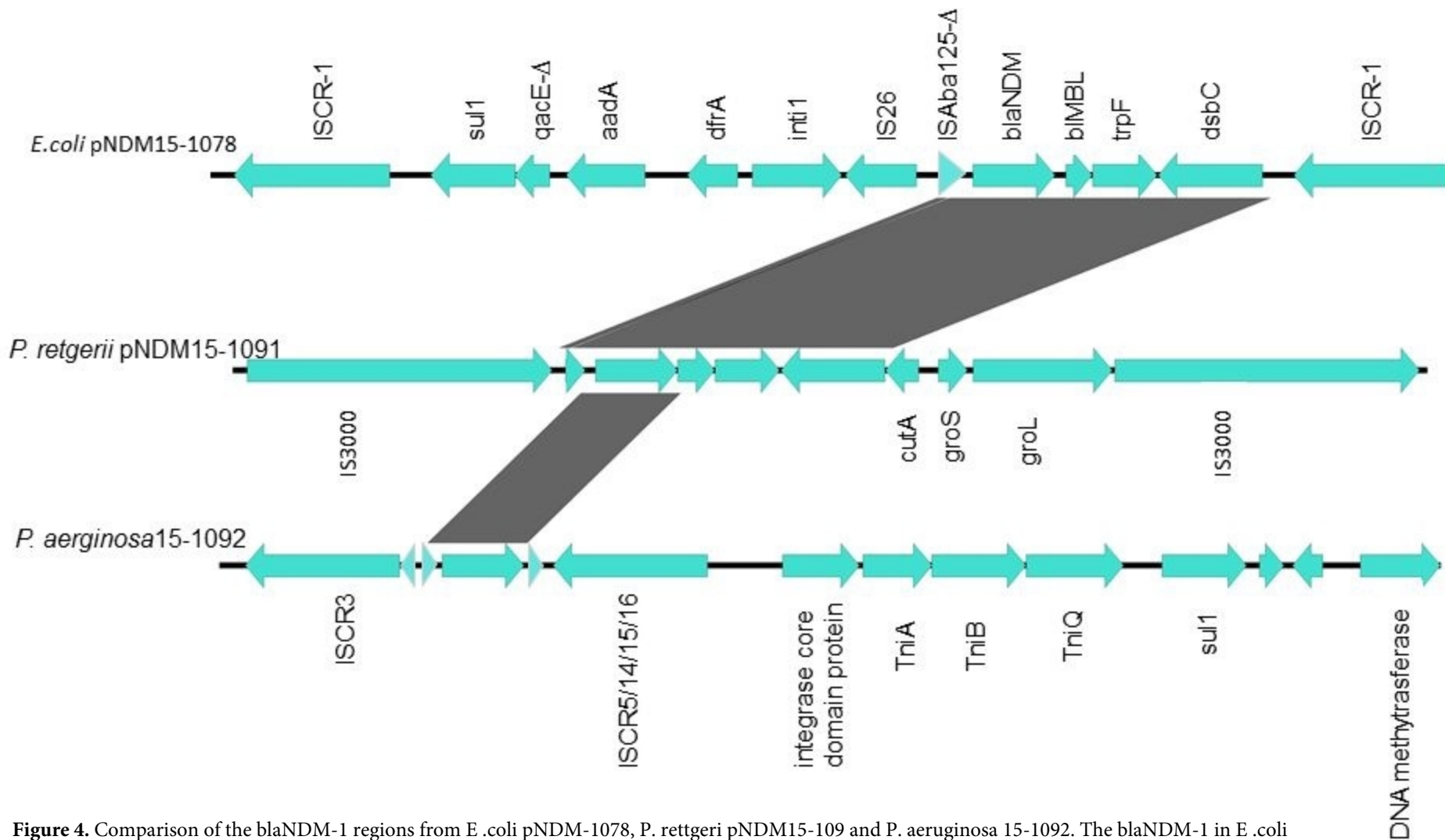


Figure 4. Comparison of the blaNDM-1 regions from *E. coli* pNDM-1078, *P. rettgeri* pNDM15-109 and *P. aeruginosa* 15-1092. The blaNDM-1 in *E. coli* pNDM-1078 was located on a 176kb IncA/C plasmid contained within an ISCR1 region. The blaNDM-1 in *P. rettgeri* pNDM15-109 was located on a 117kb IncT plasmid contained within Tn3000. The blaNDM-1 in *P. aeruginosa* 15-1092 was located on the chromosome within an ISCR3 region. The common 15 regions between all 3 species include ISAbal25, bleMBL, trpF and dsbC.

very similar to that of a recent report of *K. pneumoniae* with *bla*_{OXA-181} obtained from a Japanese patient that was transferred from Mumbai to an intensive care unit in Hiroshima, Japan (43) (Figure 3).

In summary, we describe 3 different *bla*_{NDM-1} genetic platforms in 3 different species present in the same patient that highlights the plasticity of the genetic regions and environments surrounding this enzyme (Figure 4). The common regions between all 3 species include IS*Aba*₁₂₅, *ble*_{MBL}, *trpF* and *dsbC*. We believe this represents the first report of *P. aeruginosa* with *bla*_{NDM-1} from North America, the first report of *bla*_{OXA-181} in *P. rettgeri* and the first IncT plasmid associated with *bla*_{NDM-1}. It is of special concern that the *P. aeruginosa* belonged to the high risk clone ST654 and tested non-susceptible to colistin. This case emphasizes the need for appropriate infection prevention and control measures and vigilant screening for carbapenem resistant Gram negative bacteria in patients who have recently travelled to endemic areas, such as the Indian subcontinent.

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Transparency declaration

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