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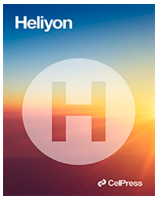

Research article

Characterization of *bla*$_{NDM-19}$-producing IncX3 plasmid isolated from carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae*

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ABSTRACT

The increase in the prevalence of carbapenem-producing Enterobacterales (CPE) is a major threat, with the New Delhi metallo-β-lactamase (NDM) enzyme-producing CPEs being one of the major causative agents of healthcare settings infections. In this study, we characterized an IncX3 plasmid harboring *bla*$_{NDM-19}$ in Lebanon, recovered from three *Escherichia coli* belonging to ST167 and one *Klebsiella pneumoniae* belonging to ST16 isolated from a clinical setting. Plasmid analysis using PBRT, Plasmid Finder, and PlasmidSPAdes showed that all four isolates carried a conjugative 47-kb plasmid having *bla*$_{NDM-19,}$ and was designated as pLAU-NDM19. We constructed a sequence-based maximum likelihood phylogenetic tree and compared pLAU-NDM19 to other representative IncX3 plasmids carrying NDM-variants and showed that it was closely linked to NDM-19 positive IncX3 plasmid from *K. pneumoniae* reported in China. Our findings also revealed the route mediating resistance transmission, the IncX3 dissemination among Enterobacterales, and the NDM-19 genetic environment. We showed that mobile elements contributed to the variability of IncX3 genomic environment and highlighted that clonal dissemination in healthcare settings facilitated the spread of resistance determinants. Antimicrobial stewardship programs implemented in hospitals should be coupled with genomic surveillance to better understand the mechanisms mediating the mobilization of resistance determinants among nosocomial pathogens and their subsequent clonal dissemination.

1. Introduction

Carbapenem-resistant Enterobacterales (CRE) are a major concern in the community and in healthcare settings [1]. Among the carbapenamases, NDM, with the exception of aztreonam, mediates resistance to all β-lactam antibiotics [2]. NDM enzymes have been reported worldwide, but being commonly detected in Asia and southeastern Europe [3]. The first NDM enzyme, *bla*$_{NDM-1}$, was identified in 2008 in *Klebsiella pneumoniae* from a Swedish patient previously hospitalized in India [4]. Since then, many variants in more than 60 species were reported [5]. In 2019, a new variant designated as NDM-19 was reported in China, recovered from an Egyptian patient and mediated less susceptibility to broad-spectrum cephalosporins and carbapenems. NDM-19 has three amino acid substitutions when compared to NDM-1 and one from NDM-7 [6,7]. The first NDM variant detected in Lebanon was NDM-1 in

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K. pneumoniae recovered in 2012 from a urine sample of an Iraqi patient hospitalized in Lebanon [8], followed by several others reporting NDM-1 and OXA-48 which became the most common carbapenemases in the country [8, 9, 10]. More recently, NDM-7 and NDM-5 were also detected in *K. pneumoniae* [11,12], and NDM-19 in *E. coli* [13]. The present study was initiated to determine the genomic environment of $bla_{\text{NDM-19}}$-carrying IncX3 plasmid recovered from *E. coli* and *K. pneumoniae* recovered from the same healthcare setting and showing carbapenem resistance and determine co-carriage of other plasmids and resistance determinants.

2. Materials and methods

2.1. Sample collection, identification, and susceptibility to antibiotics

Clinical samples were collected from the Al-Makassed Hospital in Lebanon and were stored as part of routine clinical care 16S rRNA gene sequencing and BLAST were used to identify the isolates [14]. The disk diffusion on Mueller-Hinton agar was used to determine the susceptibility profiles of the isolates against aztreonam, norfloxacin, tobramycin, sulfamethoxazole/trimethoprim, amikacin, gentamicin, tetracycline, and ciprofloxacin. Susceptibility to ertapenem, imipenem, and meropenem was determined by the E-test methodology (BioMérieux, France). Results were interpreted according to the CLSI guidelines (CLSI, 2023) [15]. Isolates undertaken in this study were three *E. coli* (designated as EC20, 21, and 22; Accession Numbers: NZ_JAAJRZ000000000.1 (EC20), NZ_JAAJSD000000000.1 (EC21), NZ_JAAJRY000000000.1 (EC22) and one *K. pneumoniae* (Accession Number: JBBJJS000000000.1).

2.2. DNA extraction and whole-genome sequencing

Nucleospin® tissue kit (Macherey-Nagel, Germany) was used to extract the DNA. Illumina Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA) was used for the preparation of libraries. The genomic DNA (gDNA) was end-repaired, followed by A-tailing and adaptor and sample-specific barcodes ligation. Libraries were quantified using Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA), multiplexed, clustered, and sequenced on Illumina MiSeq (Illumina, San Diego, CA, USA) using paired-end 500 cycles protocol and 2 x 250bp reads. Raw sequences were subjected to quality control using FastQC version 0.11.9 (Andrews, 2010), followed by *de novo* assembly with SPAdes v3.13 [16].

2.3. Genome analysis

Genome annotation was done using Rapid Annotation Subsystem Technology (RAST) database (rast.nmdpr.org) [17]. Resistance genes were identified using the ResFinder server v2.1 [18,19]. The Achtman Multilocus Sequence type available on the Center for Genomic Epidemiology (CGE) (www.genomicepidemiology.org), was used to determine the sequence MLST 2.0 [20]. *E. coli* PhyloTypeFinder on GoSeqIt (https://www.goseqit.com/goseqittools-faq/) was used to reveal the phylogroups while the serotype was determined through SerotypeFinder 2.0 available on CGE [21]. The *K. Pneumoniae* capsule type (K-type) was determined using Kaptive (https://github.com/katholt/Kaptive) [22].

2.4. Plasmid typing

Plasmids were extracted using QIAprep Spin Miniprep kit (Qiagen, USA). PCR-based replicon typing analysis (PBRT) was then used to determine the incompatibility (Inc) groups [23]. Plasmid incompatibility groups and plasmid MLST were further investigated *in silico* using the Plasmid Finder 1.3 (CGE) and pMLST [24], respectively. The presence of $bla_{\text{NDM-19}}$ was confirmed through a PCR assay using the following primer pairs: 5′-GTTGTCGATACCGCCTGGACCGAT-3′ and 5′-AGTCAGGCTGTGTTGCGCCGCAAC -3′ with the expected amplicon having a size of 200 bp. PlasmidSPAdes (v 3.12.0) [25] was used to reconstruct the plasmids, and Bandage was used to visualize the assembly graph [26]. BLAST was used to find and align to the closest matches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The IncX3 plasmids showing the highest similarity (identity >90%) were compared with pLAU-NDM19 plasmid of the present study (Genbank accession # CP074195) using the genome comparison tool BRIG (Blast Ring Generator) [27].

2.5. Plasmid conjugation assay

The conjugation assay was conducted as described previously [28], with minor modifications. $bla_{\text{NDM-19}}$ positive isolates were the donor strains, while azide-resistant *E. coli* J53 was the recipient. The mixture with the donor and recipient was incubated at 37 °C for 4 h or overnight. LB agar with sodium azide (100 μg/mL) and ampicillin (100 μg/mL) was used to screen for the transconjugants. Colonies showing resistance were picked and used for further $bla_{\text{NDM-19}}$ PCR validation using the protocol described above.

2.6. Phylogenetic analysis

Sequences of IncX3 plasmids carrying variants of the bla_{NDM} gene were retrieved from NCBI. Plasmid sequences, including the four isolates from this study, were annotated using Prokka v1.14.6. Roary v3.13.0 with its default parameters was used for genomic analysis. FastTree v2.1.10 with the general time-reversible model and a categorical model rate of heterogeneity (GTR-CAT) was used to build a maximum-likelihood phylogenetic tree. The tree was then visualized through iTOL (https://itol.embl.de).

3. Results

3.1. Identification and typing

Four CREs were recovered from hospitalized patients and identified using 16S rRNA gene sequencing; three were *E. coli* (EC20, 21, and 22) and one was *K. pneumoniae.* Genome characterization revealed a single clone of *E. coli* of sequence type ST167, belonging to phylogroup A, and serotype O89-like:H5-like. *K. pneumoniae* on the other hand, was typed as ST16 and K-type 51 (Table 1).

3.2. Resistance genes and susceptibility to antibiotics

Following the CLSI guidelines, all isolates exhibiting an intermediate/resistant phenotype against a tested antimicrobial agent were reported as non-susceptible, and which was the case with all the four against ertapenem, meropenem, and imipenem. EC20 and 21 were additionally non-susceptible to aztreonam, while all were susceptible to gentamicin. The resistance genes and profiles are summarized in Fig. 1. Other β-lactamases where also detected including $bla_{\text{CTX-M-15}}$, $bla_{\text{SHV-12}}$, $bla_{\text{SHV-26}}$, and $bla_{\text{TEM-1}}$.

3.3. Plasmid typing and comparative genomic analysis

Collectively the PBRT results and *in silico* plasmid typing, revealed the presence of a 47 kb IncX3 plasmid harboring $bla_{\text{NDM-19}}$ in all four isolates and was designated as pLAU-NDM19 (accession # CP074195). Following conjugation assays, *E. coli* J53 showed ampicillin resistance confirming the conjugative nature of the IncX3 plasmid. pLAU-NDM19 G + C content was 47.1%, and its sequence showed high similarity (identity 94%) to plasmid pSCM96-2 recovered from a $bla_{\text{NDM-19}}$ positive *K. pneumoniae* (GenBank accession #CP028718.1; China, 2018) and plasmid pHN4109c from a $bla_{\text{NDM-7}}$ positive *E. coli* (GenBank accession #MK088485.1; China, 2018).

Analysis of the genetic environment showed the following upstream of the $bla_{\text{NDM-19}}$ Tn*5403*-ΔIS*3000*-ΔIS*Aba125*-IS*5*, while downstream we identified ble_{MBL}-*trpF*-*dsbC*. The genetic features of this plasmid were very similar to the following previously sequenced IncX3 plasmids: pSCM96-2 ($bla_{\text{NDM-19;}}$ accession #CP028718), pHN4109c ($bla_{\text{NDM-7;}}$ accession #MK088485.1), pEC1929 ($bla_{\text{NDM-5;}}$ accession #KT824791), pNDM-20 ($bla_{\text{NDM-20;}}$ accession #MF458176), and pAD-19R ($bla_{\text{NDM-17}}$, accession #KX833071.1) (Fig. 2A). However, a 2497-kb fragment harboring *umuD*, a DNA polymerase V protein (UmuD) which regulates mutagenesis, and *tnpA* transposase, were missing in pLAU-NDM19, while a 3668-kb insertion harboring *tnpA* and *tnpR* was detected (Fig. 2B). We constructed a sequence-based maximum likelihood phylogenetic tree using Roary in which we compared pLAU-NDM19 to other representative IncX3 plasmids retrieved from NCBI, carrying NDM-variants, and recovered from different geographical regions. pLAU-NDM19 was closely linked to NDM-19 positive IncX3 plasmid from *K. pneumonia* reported in China [42] (Fig. 3). The tree additionally revealed the distribution of IncX3 plasmids over three main clusters with pLAU-NDM19 along with its closest neighbor (CP028718.1) being the only two representatives with the $bla_{\text{NDM-19}}$ gene while the other plasmids had different variants including bla_{NDM1}, bla_{NDM4}, bla_{NDM5}, and $bla_{\text{NDM-7}}$.

4. Discussion

The worldwide dissemination of bla_{NDM} is a substantial public health threat. The global spread of bla_{NDM} is linked to mobile genetic elements and specifically to IncX3 plasmids [5,29]. This work reported the detection of pLAU-NDM19, an IncX3 47-kb plasmid carrying $bla_{\text{NDM-19}}$ in four isolates recovered from the same healthcare setting, with all showing resistance to carbapenems and other multiple classes of antibiotics. High sequence similarity and similar $bla_{\text{NDM-19}}$ genetic environment were detected when compared to other IncX3 plasmids carrying bla_{NDM} variants ($bla_{\text{NDM-5}}$, $bla_{\text{NDM-7}}$, $bla_{\text{NDM-17}}$, and $bla_{\text{NDM-20}}$). IS*3000*, IS*Aba125* and IS*5* insertion sequences were detected upstream of the $bla_{\text{NDM-19}}$. IS*3000* was disrupted by a copy of Tn*5403* and IS*Aba*125 by IS*5*, and which previously were linked to bla_{NDM} mobilization [7,30]. Downstream of $bla_{\text{NDM-19,}}$ and in agreement with other studies we detected, ble_{MBL}, *trpF*, and *dsbC* [6,7].

The three *E. coli* in this study were of sequence type ST167, commonly linked to NDM-positive isolates [5], and which showed that NDM spread was linked to mobile elements and clonal dissemination. IncX3-linked NDM-5 was previously detected in *E. coli* ST167 with the isolates additionally having IncFII plasmid. Only EC21 in our study harbored both IncX3 and IncFII but was also positive for three other plasmids. $bla_{\text{NDM-19}}$ variant, was first detected in an *E. coli* from Switzerland, having also sequence type ST167 and IncX3 plasmid [6], while in *K. pneumoniae* NDM-19 was first linked to ST16; an emergent isolate increasingly associated with outbreaks and

Table 1
Clinical information and molecular characteristics of the NDM-19-producing isolates.

						Patient Information			
Isolate	Organism	ST	Phylogroup	Serotype	K-type	Isolation Date	Sex	Age	Specimen
EC20	*E. coli*	167	A	O89-like:H5-like		July 27, 2018	F	66	Urine
EC21	*E. coli*	167	A	O89-like:H5-like		September 01, 2018	F	76	Urine
EC22	*E. coli*	167	A	O89-like:H5-like		September 19, 2018	F	6	Urine
KP1	*K. pneumoniae*	16			51	May 07, 2019	M	66	Wound

Sex: M = male, F = female; ST = sequence type; K-type = capsule type in *Klebsiella*; NDM = New Delhi metallo-β-lactamase.

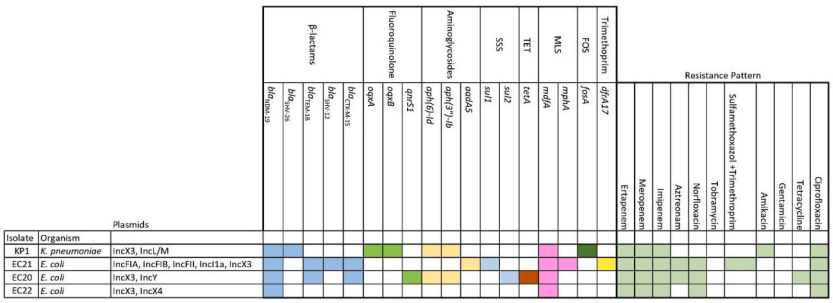


Fig. 1. Plasmid content and distribution of resistance determinants and antimicrobial susceptibility results. Resistance determinants were grouped as: β-lactam, fluoroquinolone, aminoglycoside, sulfonamide (SSS), tetracycline (TET), macrolide-associated (MLS), fosfomycin (FOS) and trimethoprim; gray: gene present. Resistance patterns: green: non-susceptible, white: susceptible. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

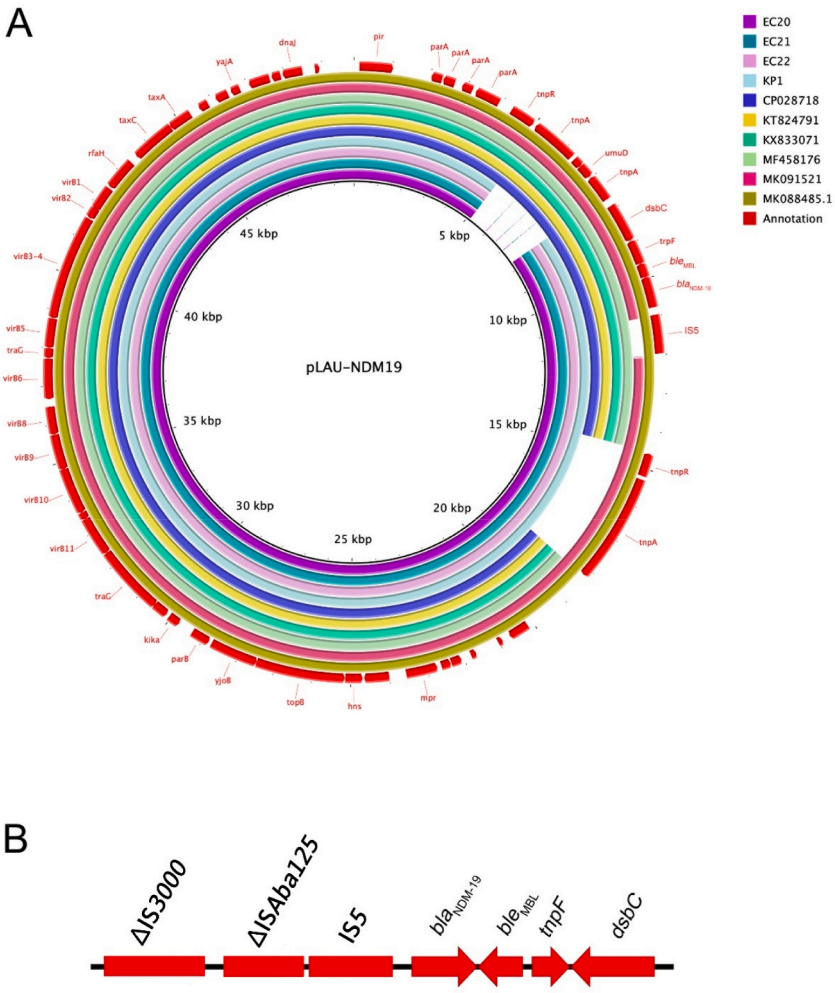
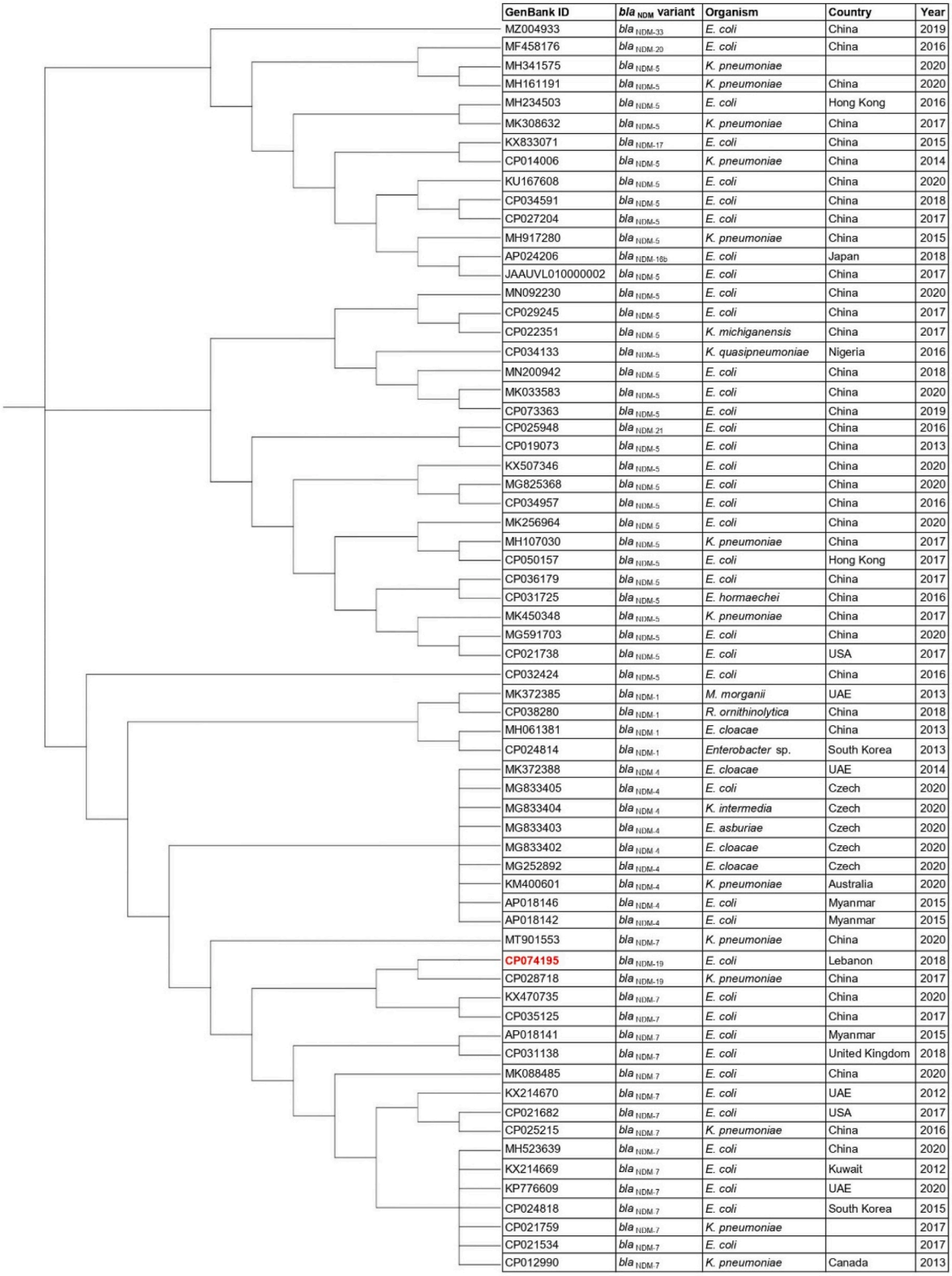


Fig. 2. pLAU-NDM19 sequence details (Genbank accession # CP074195). (A) Six closely related plasmids to pLAU-NDM19 were used for detailed comparison (Genbank accession numbers CP028718 (*bla*$_{NDM-19}$), KT824791 (*bla*$_{NDM-5}$), KX833071 (*bla*$_{NDM-17}$), MF458176 (*bla*$_{NDM-20}$), MK091521 (*bla*$_{NDM-19}$), and MK088485.1 (*bla*$_{NDM-7}$) constructed using BRIG. White spaces represent gaps. The external ring represents the annotation of the plasmids. (B) Genetic environment of the *bla*$_{NDM-19}$ gene from the pLAU-NDM19 plasmid. Downstream of the *bla*$_{NDM-19}$ gene we detected *ble*$_{MBL}$, *trpF*, and *dsbC* respectively coding for bleomycin resistance, phosphoribosylanthranilate isomerase, signal domain protein of the twin-arginine translocation pathway.



GenBank ID	*bla* NDM variant	Organism	Country	Year
MZ004933	*bla* NDM-33	*E. coli*	China	2019
MF458176	*bla* NDM-20	*E. coli*	China	2016
MH341575	*bla* NDM-5	*K. pneumoniae*		2020
MH161191	*bla* NDM-5	*K. pneumoniae*	China	2020
MH234503	*bla* NDM-5	*E. coli*	Hong Kong	2016
MK308632	*bla* NDM-5	*K. pneumoniae*	China	2017
KX833071	*bla* NDM-17	*E. coli*	China	2015
CP014006	*bla* NDM-5	*K. pneumoniae*	China	2014
KU167608	*bla* NDM-5	*E. coli*	China	2020
CP034591	*bla* NDM-5	*E. coli*	China	2018
CP027204	*bla* NDM-5	*E. coli*	China	2017
MH917280	*bla* NDM-5	*K. pneumoniae*	China	2015
AP024206	*bla* NDM-18b	*E. coli*	Japan	2018
JAAUVL010000002	*bla* NDM-5	*E. coli*	China	2017
MN092230	*bla* NDM-5	*E. coli*	China	2020
CP029245	*bla* NDM-5	*E. coli*	China	2017
CP022351	*bla* NDM-5	*K. michiganensis*	China	2017
CP034133	*bla* NDM-5	*K. quasipneumoniae*	Nigeria	2016
MN200942	*bla* NDM-5	*E. coli*	China	2018
MK033583	*bla* NDM-5	*E. coli*	China	2020
CP073363	*bla* NDM-5	*E. coli*	China	2019
CP025948	*bla* NDM-21	*E. coli*	China	2016
CP019073	*bla* NDM-5	*E. coli*	China	2013
KX507346	*bla* NDM-5	*E. coli*	China	2020
MG825368	*bla* NDM-5	*E. coli*	China	2020
CP034957	*bla* NDM-5	*E. coli*	China	2016
MK256964	*bla* NDM-5	*E. coli*	China	2020
MH107030	*bla* NDM-5	*K. pneumoniae*	China	2017
CP050157	*bla* NDM-5	*E. coli*	Hong Kong	2017
CP036179	*bla* NDM-5	*E. coli*	China	2017
CP031725	*bla* NDM-5	*E. hormaechei*	China	2016
MK450348	*bla* NDM-5	*K. pneumoniae*	China	2017
MG591703	*bla* NDM-5	*E. coli*	China	2020
CP021738	*bla* NDM-5	*E. coli*	USA	2017
CP032424	*bla* NDM-5	*E. coli*	China	2016
MK372385	*bla* NDM-1	*M. morganii*	UAE	2013
CP038280	*bla* NDM-1	*R. ornithinolytica*	China	2018
MH061381	*bla* NDM-1	*E. cloacae*	China	2013
CP024814	*bla* NDM-1	*Enterobacter* sp.	South Korea	2013
MK372388	*bla* NDM-4	*E. cloacae*	UAE	2014
MG833405	*bla* NDM-4	*E. coli*	Czech	2020
MG833404	*bla* NDM-4	*K. intermedia*	Czech	2020
MG833403	*bla* NDM-4	*E. asburiae*	Czech	2020
MG833402	*bla* NDM-4	*E. cloacae*	Czech	2020
MG252892	*bla* NDM-4	*E. cloacae*	Czech	2020
KM400601	*bla* NDM-4	*K. pneumoniae*	Australia	2020
AP018146	*bla* NDM-4	*E. coli*	Myanmar	2015
AP018142	*bla* NDM-4	*E. coli*	Myanmar	2015
MT901553	*bla* NDM-7	*K. pneumoniae*	China	2020
CP074195	*bla* NDM-19	*E. coli*	Lebanon	2018
CP028718	*bla* NDM-19	*K. pneumoniae*	China	2017
KX470735	*bla* NDM-7	*E. coli*	China	2020
CP035125	*bla* NDM-7	*E. coli*	China	2017
AP018141	*bla* NDM-7	*E. coli*	Myanmar	2015
CP031138	*bla* NDM-7	*E. coli*	United Kingdom	2018
MK088485	*bla* NDM-7	*E. coli*	China	2020
KX214670	*bla* NDM-7	*E. coli*	UAE	2012
CP021682	*bla* NDM-7	*E. coli*	USA	2017
CP025215	*bla* NDM-7	*K. pneumoniae*	China	2016
MH523639	*bla* NDM-7	*E. coli*	China	2020
KX214669	*bla* NDM-7	*E. coli*	Kuwait	2012
KP776609	*bla* NDM-7	*E. coli*	UAE	2020
CP024818	*bla* NDM-7	*E. coli*	South Korea	2015
CP021759	*bla* NDM-7	*K. pneumoniae*		2017
CP021534	*bla* NDM-7	*E. coli*		2017
CP012990	*bla* NDM-7	*K. pneumoniae*	Canada	2013

Fig. 3. Maximum-likelihood phylogenetic tree sequence-based comparison of the IncX3 plasmid using Roary. GenBank accession numbers, the *bla*$_{NDM}$ variants, the organism, the country, and the year of isolation. Bold red: plasmid recovered from this study (GenBank accession number CP074195). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

carrying many resistance determinants [31]. To our knowledge, this is the first proven occurrence of bla_{NDM-19} on an IncX3 plasmid recovered from a clinical setting in Lebanon. IncX3, are low-prevalence and narrow-host-range plasmids that mediate bla_{NDM} dissemination, and which was in line with the findings of our study.

Many hospitals in Lebanon implement antimicrobial stewardship to improve treatment regimens of infectious diseases and minimize emergence of resistance. Several studies revealed the common resistance mechanisms and profiles within clinical samples recovered from hospitals across Lebanon [8–11,32–41]. This, however, should be coupled with genomic surveillance to track, monitor, and address antibiotic resistance.

This study showed that mobile elements are important and contribute to the variability of IncX3 genomic environment, and which is the case for other plasmids, and highlighted that clonal dissemination in healthcare settings facilitates the spread of resistance determinants. Accordingly, we need to implement effective infection control measures along with antimicrobial stewardship, to reduce the mobilization of resistance genes among nosocomial pathogens and their subsequent clonal dissemination.

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Ethics declaration

Institutional Review Board (IRB) approval was obtained from Makassed General Hospital (MGH) on December 18, 2018. The isolates were collected as part of routine clinical care. Clinical isolates and patient records/information were deidentified prior to analysis. No diagnostic or treatment decisions were affected by the outcomes of this study.

Data availability

Data from this study is available on NCBI Accession number: SAMN18953841.

CRediT authorship contribution statement

Jennifer Moussa: Writing – original draft, Investigation, Formal analysis. **Elie Nassour:** Writing – original draft, Investigation, Formal analysis. **Tamima Jisr:** Supervision, Resources. **Mira El Chaar:** Writing – review & editing, Supervision, Conceptualization. **Sima Tokajian:** Writing – review & editing, Supervision, Resources, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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