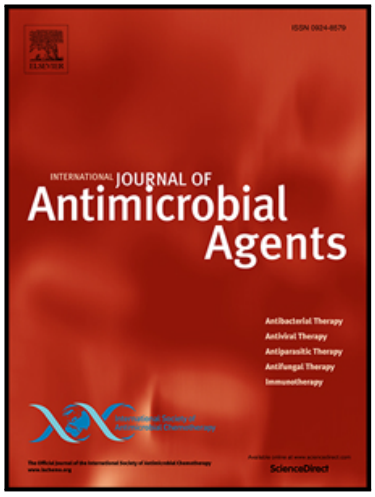
Editor name: Dr L. Loucif

Journal Pre-proof



An outbreak sustained by ST15 Klebsiella pneumoniae carrying 16S rRNA methyltransferases and bla$_{NDM}$: evaluation of the global dissemination of these resistance determinants

Federica Sacco , Giammarco Raponi , Alessandra Oliva , Giulia Bibbolino , Vera Mauro , Federica Maria Di Lella , Lorenzo Volpicelli , Guido Antonelli , Mario Venditti , Alessandra Carattoli , Gabriele Arcari

PII: S0924-8579(22)00127-3
DOI: https://doi.org/10.1016/j.ijantimicag.2022.106615
Reference: ANTAGE 106615

To appear in: *International Journal of Antimicrobial Agents*

Received date: 19 October 2021
Accepted date: 30 May 2022

Please cite this article as: Federica Sacco , Giammarco Raponi , Alessandra Oliva , Giulia Bibbolino , Vera Mauro , Federica Maria Di Lella , Lorenzo Volpicelli , Guido Antonelli , Mario Venditti , Alessandra Carattoli , Gabriele Arcari , An outbreak sustained by ST15 Klebsiella pneumoniae carrying 16S rRNA methyltransferases and bla$_{NDM}$: evaluation of the global dissemination of these resistance determinants, *International Journal of Antimicrobial Agents* (2022), doi: https://doi.org/10.1016/j.ijantimicag.2022.106615

An outbreak sustained by ST15 *Klebsiella pneumoniae* carrying 16S rRNA methyltransferases and *bla*$_{NDM}$: evaluation of the global dissemination of these resistance determinants

Federica Sacco[1], Giammarco Raponi[2,4], Alessandra Oliva[2], Giulia Bibbolino[3], Vera Mauro[2], Federica Maria Di Lella[4], Lorenzo Volpicelli[2], Guido Antonelli[1], Mario Venditti[2], Alessandra Carattoli[1]*, and Gabriele Arcari[1]

[1]Department of Molecular Medicine, Sapienza University of Rome, Rome, Italy

[2]Department of Public Health and Infectious Diseases, Sapienza University of Rome

[3]Department of Molecular Medicine and Medical Biotechnologies, University of Naples Federico II, Naples

[4]Microbiology and Virology Unit, University Hospital Policlinico Umberto I, Rome, Italy

* Corresponding author, alessandra.carattoli@uniroma1.it

Abstract

The spread of extremely-drug resistant *Klebsiella pneumoniae* has become a major health threat worldwide. This is largely mediated by certain lineages, recognized as high-risk clones dispersed in all the world. The analysis of an outbreak of nine ST15, NDM-1 metallo-β-lactamase producing *K. pneumoniae* was performed. An IncC plasmid carrying the $bla_{\text{NDM-1}}$ gene also carried the rare *rmtC* gene, encoding for a 16S rRNA methyltransferases (16RMTases), conferring resistance to all aminoglycosides. We studied the global spread of NDM variants and their association with the 16RMTases among *K. pneumoniae* complete genomes available in GenBank, producing a complete overview of the association of 16RMTases and NDM in *K. pneumoniae* genomics.

NDM is more and more often associated with16RMTases and both are spreading in *K. pneumoniae*, conferring resistance to every beta-lactam and aminoglycoside. Our analysis suggest that aminoglycosides have limited future as second line treatment against NDM-producing *K. pneumoniae*.

Highlights

- An outbreak of NDM-1-ST15 producing *Klebsiella pneumoniae* was for the first time reported in Italy
- The outbreak clone carried the rarely reported 16S RNA methylase *rmtC* gene
- A global genomic analysis indicates that 16S RNA methylase genes are very often associated with bla_{NDM} genes *K. pneumoniae*

1. Introduction

With the prevalence of carbapenem-resistant Enterobacterales that has increased since the early 2000s, the spread of antimicrobial resistant Gram-negative bacteria confirms itself as one of the most worrying global health threats [1,2]. The New Delhi Metallo (NDM) enzyme, initially described in patients receiving healthcare in India, is now worldwide heterogeneously distributed, with areas of high endemicity such as the Indian sub-continent and the Balkan region [3–5]. However, even in the rest of Europe, its spread is on the rise: this metallo-β-lactamase is swiftly passing the borders of the nations, and Italy in the last years reported an increment of its detection [6–9].

The NDM carbapenemases have been mainly detected in *Klebsiella pneumoniae*, *Escherichia coli* and, with less frequency, in other bacterial species. The bla_{NDM} genes are carried on different plasmid types, IncX3, IncFII and IncC being the most frequent ones [4]. In Italy great emphasis has been given to several outbreaks, mainly caused by *K. pneumoniae* belonging to the ST147 producing NDM-5 and -9 [7,8].

Aminoglycosides are an important second line therapeutic choice for infections caused by carbapenem resistant Enterobacterales [10]. Above all, great hopes lie in the development of new generation aminoglycosides (neoglycosides), which presumably will be not affected by the most diffused resistance mechanisms to aminoglycosides (i.e. Aminoglycosides Modifying Enzymes, AMEs) [11]. Yet, even these molecules, could be rendered ineffective by 16S rRNA methyltransferases (16RMTases), which act by modifying the active site on which aminoglycosides operate [12]. Every aminoglycoside employed in clinical practice (except apramycin, which is used in the veterinary field [13]) is useless against 16RMTases.

Yet, the diffusion of 16RMTases too is growing at a fast pace [12,14] and it might even be underestimated, owing to the fact their detection is troublesome with microbiological methods, even if some systems have been developed (e.g. the use of arbekacin disks [15]). For these reasons the spread and diffusion of 16RMTases is hard to track without resorting to molecular methods.

The fact that the epidemiology of 16RMTases is not well known, both from a local and a global point of view, poses a great threat to the use of all aminoglycosides, especially neoglycosides.

This study started with the characterization of a Sequence Type (ST) 15 *K. pneumoniae* outbreak clone identified in the hospital, carrying the bla_{NDM} and the *rmtC* 16RMTase genes. The clone was placed within a wider global view of the distribution of *K. pneumoniae* high-risk clones showing association of NDM and 16RMTase resistant determinant.

2. Materials and methods

2.1 Strains isolation and antimicrobial susceptibility testing

The isolation of the strains took place between June and October 2020 from clinical samples obtained during routine testing from hospitalized patients at the University Hospital Policlinico Umberto I in Rome, Italy [16].

K. pneumoniae strains were identified using the MALDI-TOF MS system (Bruker Daltonik GmbH, Bremen, Germany); antimicrobial susceptibility testing was performed by either Vitek2 (bioMérieux, Inc., Marcy l'Etoile, France) or MiscroScan (Beckman and Coulter, Brea, California, USA) systems.

Strains showing a carbapenem-resistant phenotype (according to EUCAST criteria; http://www.eucast.org/clinical_breakpoints/) were tested using the lateral flow immunoassay NG-test CARBA 5 (NG biotech, Guipry, France) to evaluate the presence of the VIM, IMP, KPC, OXA-48-like and NDM carbapenemase enzymes.

2.2 Whole Genome Sequencing

The Illumina MiSeq instrument (Illumina Inc., San Diego, CA, USA) was used to obtain Whole-Genome Sequencing (WGS) of the four strains isolated from respiratory tract samples of patients 1, 6, 7 and 8, named 0831, 1009, 1021 and 1027, respectively. Bacteria were grown overnight at 37°C on LB agar with ampicillin (50 mg/mL). Genomic DNA was purified following the MachereyNagel DNA extraction kit procedures (Düren, Germany) directly from the LB plate.

The strains 0831 and 1027 were also subjected to Oxford Nanopore Technologies sequencing. To obtain high molecular weight DNA, the bacterial pellet of 7 mL LB

liquid incubated overnight was resuspended in TE with 2% SDS and 20 µL of 25 mg/ml proteinase K. Protein digestion was performed for 1h at 55°C followed by phenol (pH 8.0) extraction and isopropanol precipitation. After washing with 70% Et-OH, DNA was resuspended overnight in TE at +4°C and purified using AMPure XP beads in a 0.5/1 ratio. Libraries were prepared by the rapid barcoding sequencing kit (SQK-RBK004). Pooled libraries were cleaned up using AMPure XP beads and loaded into the MinION Flow Cell (R9.4.1) following SQK-RBK004 sequencing procedures. Sequencing was performed on an Mk1C MinION platform.

2.3 Bioinformatics

2.3.1 Assembly

Paired-end libraries generated using the Nextera XT DNA sample preparation kit with the 2x300PE protocol (Illumina, Inc.) and the *de novo* assembly of Illumina reads was performed using Galaxy version 3.14.1 of the SPAdes pipeline through the ARIES public Galaxy server (https://w3.iss.it/site/aries/).

The assembly of the raw nanopore reads was performed at the Europe Galaxy Server (https://usegalaxy.eu/) using Flye version 2.6 with and estimated genome size of 5 megabases.

The Unicycler tool version 0.4.8.0 was used for the hybrid assembly of short and long reads, using a normal bridging mode [17].

2.3.2 Genotyping

The exact species (*Klebsiella pneumoniae "sensu stricto"*) of the four strains subjected to WGS was confirmed by the Kleborate tool, which has also been used to identify the MultiLocus Sequence Type (MLST), for the analysis of the K (capsule) and O (Lipopolysaccharides, LPS) antigen locus and of the genes encoding virulence determinants associated with hypervirulence (yersiniabactin, colibactin, aerobactin, salmochelin and regulators of the mucoid phenotype) [18].

The analyses of the antimicrobial resistance genes and of the replicon genes were carried out at the Center for Genomic Epidemiology using the ResFinder and PlasmidFinder online tools (https://cge.cbs.dtu.dk/services/), respectively. Insertion sequences were identified by ISFinder (https://isfinder.biotoul.fr). Phage prediction was performed at the PHASTER website (https://phaster.ca/).

Complete plasmid sequences were annotated using the RAST server (http://rast.nmpdr.org/)

2.3.3 Genomes and plasmids selection from the GenBank database

To obtain the 16RMTases-harboring genome sequences, BLASTN was performed with the *armA*, *npmA*, *rmtA, rmtB, rmtB*$_2$, *rmtC, rmtD, rmtD*$_2$, *rmtE, rmtF, rmtG* and *rmtH* 16RMTases genes nucleotide sequences (GenBank Acc. Nos AY220558, AB261016, AB120321, AB103506, JN968578, AB194779, DQ914960, HQ401565, GU201947, JQ808129, JX486113, KC544262, respectively) as queries, against maximum 1000 target *K. pneumoniae* sequences from the NCBI GenBank database (November 2020).

All sequences matching with a full-length 16RMTases query with 100% coverage and 100% identity were selected. Those associated with BioProjects, including whole and accessory genomes were selected for further analysis. Complete plasmids and chromosomes sequences were exported. Geographic distribution and isolation source of the 16RMTases positive genomes were reported in supplementary dataset 1, when available.

The same procedure has been replicated to obtain all the *K. pneumoniae* strains harbouring *bla*$_{NDM}$, using *bla*$_{NDM-1/-24}$ genes as queries in the BLASTN analysis.

In the case of presence of one or more 16RMTases or NDM encoding genes in the same strain, this was taken into consideration only once.

All the downloaded *K. pneumoniae* strains were analysed by the Kleborate tool to ascertain the correctness of the species and to obtain their MSLT, K typing and their "virulence score" (https://github.com/katholt/Kleborate/wiki/Scores-and-counts)

The sequences of 145 *K. pneumoniae* plasmids belonging to Incompatibility group C (IncC) included in a phylogeny tree were obtained by BLASTN of the IncC probe downloaded from the Center for Genomic Epidemiology website (https://cge.cbs.dtu.dk/services/PlasmidFinder/) in the GenBank database using *K. pneumoniae* as target organism.

2.3.4 Phylogenesis and synteny

The sequences of genomes and plasmids were annotated using Prokka, and the resulting General Feature Formats (GFFs) were analysed using Roary v3.11.3 to identify core and accessory genes and to obtain a pangenome alignment.
Recombining regions removal was carried out by Gubbins algorithm generating a Maximum Likelihood (ML) phylogenetic tree using RAxML. The visualisation of the tree, metadata and pangenome was performed with MicroReact [19] and then adjusted using the open source InkScape software.
A synteny analysis was performed on the two sequenced IncC plasmids of the 1027 and 0831 strains and other sixteen highly related IncC plasmids. The 1027 IncC plasmid was used as a reference for the BLASTN-based comparison between the plasmids; visualisation has been carried out using the Circos tool [20] at the public Europe Galaxy server (https://usegalaxy.eu/)

2.4 Data availability

Genomes have been submitted under BioProject no. PRJNA746265 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA746265?reviewer=nb0o7v8jsrg6gj86rh21eoir6c). Complete plasmid sequences of strains 0831 and 1027 were released under EMBL accession nos MZ606383 and MZ606384, respectively.

2.5 PCR typing

PCR assays were adopted to type the NDM-producing *K. pneumoniae* isolated in the same period of our study which were not subjected to WGS, using the *mtC* primers of the already described 16RMTases multiplex [21] (rmtC forward 5' CAGGGGTTCCAACAAGT 3' and rmtC reverse 5' AGAGTATATAGCTTGAACATAAGTAGA 3') and the IncC *repA* primers of the PCR-based replicon typing (IncA/C FW 5' GAGAACCAAAGACAAAGACCTGGA 3' and IncA/C RV 5' ACGACAAACCTGAATTGCCTCCTT 3') [22].

3. Results and discussion

3.1 An outbreak of bla_{NDM} positive *K. pneumoniae*

In the period between 24th August to 27th October 2020 at the Policlinico Umberto I University Hospital, Rome, Italy, there were 9 cases of colonization or infections sustained by NDM-producing *K. pneumoniae*

The 9 patients (7 females, 2 males) had a median age of 70 years (IQR 52-81.5) and the median length of hospital stay was 38 days (IQR 20.5-89). Four patients were hospitalized and 7 received carbapenems in the previous 90 days (Table 1). Most patients were hospitalized in Intensive Care Unit (ICU, n=5), 3 were hospitalized in pneumology wards and only one patient was followed after lung transplantation as outpatient. In-hospital mortality was observed in 7 subjects.

Antimicrobial-susceptibility testing revealed that these strains were resistant to all tested beta-lactams (amoxicillin/clavulanic acid, cefepime, cefotaxime, ceftazidime, piperacillin/tazobactam, imipenem, meropenem ceftolozane/tazobactam and ceftazidime-avibactam). . All strains were susceptible to colistin, tigecycline and fosfomycin with the exception of strain 0831, which resulted resistant to fosfomycin (MIC= 64 mg/L). Among them, the four *bla*$_{\text{NDM}}$-positive strains (namely 0831, 1021, 1027, and 1009) causing respiratory tract infections underwent Whole Genome Sequencing (WGS). The strains 1021 and 1009 were subjected to Illumina, while the 1027 and 0831 to both Illumina and Nanopore sequencing.

In silico MLST assigned all strains to the high-risk ST15 clone and detected the KL112 capsule locus type (*wzi* 93), O2v1 O-antigen locus and ybt16 yersiniabactin siderophore gene cluster, associated with an Integrative Conjugative Element ICE*Kp12*. None of the strains carried other virulence determinants (such as colibactin, aerobactin and salmochelin siderophores, or the *rmpA* and *rmpA2* genes). The *aac(6')-Ib3, sul1,* Δ*qacE, rmtC, bla*$_{\text{NDM}}$, *ble*$_{\text{MBL}}$ and *bla*$_{\text{CMY-6}}$ resistance genes were found in all genomes, located on incompatibility type C (IncC) plasmids [23]. Furthermore, these strains presented a ColRNAI and a IncFIB/FII(K) [K7: A-: B-] plasmids. Strain 0831 presented a frameshift mutation in the glycerol-3-phosphate transporter GlpT protein (ΔGlpT[pE286fs]).

To the best of our knowledge NDM-producing ST15 *K. pneumoniae* were not previously reported in Italy.

3.2 The IncC plasmid carrying *bla*$_{\text{NDM}}$ and *rmtC* genes

By assembling with the Unicycler tool [17] the short Illumina reads and the Oxford Nanopore Technologies long reads, we obtained the 137600 bp complete, circular sequence of two highly related plasmids assigned to the IncC group by pPlasmidfinder, from strains 1027 and 0831, respectively [24].

The *bla*$_{NDM}$, *aac(6')-Ib-3, sul1* and Δ*qacE* genes were all distributed in the same ARI-A resistance island on the IncC plasmid, while *bla*$_{CMY-6}$ was found, as already described, between an IS*Ecp1* and the *blc-sugE* genes (encoding for lipocalin, an outer membrane protein, and for a small multidrug resistance efflux transporter, respectively) [25].

To assign these IncC plasmids to types identified by Ambrose and colleagues [23], we performed a phylogenetic study based on a SNPs analysis on 20 conserved genes, using IncC plasmid already assigned to types as references. The phylogenesis allocated IncC plasmids identified in 1027 and 0831 strains into the type 1 group (data not shown). In these plasmids, like in all the other type 1 IncC plasmids the ARI-A region was located between the two *tra* loci [26].

A synteny study of the sequenced 1027 and 0831 plasmids was obtained, including sixteen type 1 IncC plasmids from the global epidemiology, demonstrating that all type 1 were phylogenetically related (0-6 SNPs) and showed tight evolutive correlation based on the *bla*$_{NDM}$ and the *rmtC* gene presence and location (Supplementary Figure 1). The synteny study also demonstrated that there was at least one of these IncC plasmids with no *bla*$_{NDM}$ and *rmtC* genes (FDAARGOS_445), and at least one plasmid showing the presence of the *bla*$_{NDM}$ gene alone with no *rmtC* (p12085-Ct1). Despite it cannot be demonstrated, this observation is presumptive of an evolutionary sequence of acquisition of resistance modules, which indicates the *bla*$_{NDM}$ gene arriving as first in the IncC plasmid. However, it has been reported how, even very closely related plasmids, may have acquired *bla*$_{NDM}$ genes by different mechanisms [27].

The archetype of the ARI-A island has been described in the pRMH760 IncC plasmid as a complex mosaic structure composed of a class 1 integron and multiple transposons. In our plasmids, the structure of the ARI-A is bounded by 38-bp inverted repeats (IR) interrupted by integration of an IS4321, which as described for other IncC "secures" the resistance island in place [26]. The bleomycin resistance gene, *ble*$_{MBL}$, *trpF* (encoding a phosphoribosylanthranilate isomerase), *dsbD* (encoding a twin-arginine translocation pathway signal

sequence domain protein), *cutA* (encoding a periplasmic divalent cation tolerance protein), and *groES-groEL* (encoding chaperonin), and the TnAs3 from *Aeromonas salmonicida* followed the 3'-end of the bla_{NDM} gene in the ARI-A.

The *rmtC* gene has initially been described flanked by the 3'-end of an IS*Ecp1*-like element (which provides the promoter sequence for the expression) in a *Proteus mirabilis* strain of clinical origin [28,29]. Here it can be found in the ARI-A of the IncC plasmids closely linked to the bla_{NDM} gene, bracketed within a gene encoding for a type III endonuclease and an IS*Kpn14*. This insertion sequence interrupted the IS*Aba125* upstream of bla_{NDM}.

3.3 Phylogenesis of the Italian ST15 against global 16RMTases-producing *K. pneumoniae*

The strains sequenced in this study were compared with a total of 126 genomes carrying one of the ten 16RMTase genes available from the NCBI GenBank database (Supplementary dataset). Fifty genomes were submitted to NCBI from China, 23 from USA, 13 from Republic of Korea and 7 from Europe (United Kingdom, Czech Republic, Italy and Germany). Despite these data were biased by the NCBI collection that has not epidemiological value, there were few reports about 16RMTase genes in K. pneumoniae isolates from Europe. The genomes of strains isolated at the Policlinico Umberto I were compared with the 126 genomes from GenBank generating a Maximum Likelihood (ML)-phylogenetic tree based on the SNPs present in the 3304 genes of the core genome (Figure 1 A). The 0831, 1009, 1021 and 1027 strains were highly related to each other, differing for 0-6 SNPs on the core genome (Supplementary dataset 2).

The analysis of the phylogenetic tree of the 16RMTases depicts a peculiar epidemiology of these genes; the *rmtC* gene was always associated to bla_{NDM-1}, even if the spread among *K. pneumoniae* isolates was limited to a handful of described cases.

Yet, except the firm tie that bonds *rmtC* and bla_{NDM} on IncC plasmids, already been described worldwide even in species different from *K. pneumoniae* (e.g. [30–32]), there still is no univocal relationship between these two classes of genes.

The 16RMTase of preference in *K. pneumoniae* is *armA* gene located on diverse plasmids, in various STs (Figure 1 A). Despite it has been demonstrated how in *E.*

coli the presence of *armA* impacts on the fitness of the bacteria [33], the *armA* diffusion in *K. pneumoniae* may in explained by the physical association to *bla*$_{NDM}$ on the same plasmid. This can be demonstrated for 44 strains from GenBank harbouring *armA*, 22 (50%) also carried *bla*$_{NDM,}$ in 19 cases located on the same plasmid (in 5 genomes *armA* was associated with *rmtB* or *rmtF*, suppl. Dataset 1). Conversely, the *rmtB* gene (detected in 61/126 genomes Genbank) was not linked to *bla*$_{NDM}$ but was strictly related with the high-risk ST11 clone (mainly on IncFII/IncR plasmids) (Suppl. dataset).

No *rmtA, rmtD*, *rmtD*$_2$, *rmtE, rmtH* and *nmpA* genes were found *K. pneumoniae* in the GenBank database.

3.4 Phylogenesis of the Italian ST15 against global NDM-producing *K. pneumoniae* population

Phylogenesis comparing the four ST15 *K. pneumoniae* sequenced in this study with 91 strains of the same species carrying the *bla*$_{NDM}$ gene showed how our strains fit into a well-described ST15 cluster of NDM-1-producing *K. pneumoniae* isolated worldwide, co-producing RmtC or other 16RMTases [34–36].

Considering all STs, the diffusion of the 16RMTases genes among NDM-producing strains is high: 48,3% of the analysed strains (44/91) co-harboured at least one of these genes, 9 strains carried two or three 16RMTases (Figure 1 B). These findings reveal that the worldwide spread of NDM-16RMTases-producing *K. pneumoniae* is not limited to a few high-risk clones but involves many different *K. pneumoniae* STs.

4. Conclusions

The association between *bla*$_{NDM}$ and 16RMTases is, as time passes by, more and more tangible, and this fact puts in great risk the use of aminoglycosides against the most diffused metallo-β-lactamase.

Our data suggest that, against NDM-producing *K. pneumoniae*, the use of every aminoglycoside, even of neoglycosides, should be limited to the cases in which there is a proven susceptibility but cannot be used as a support empiric treatment.

This study, though being limited by the fact that most of the analysed strains come from the GenBank database and not from impartial collection performed worldwide, supplies a complete insight on the association between the 16RMTases and NDM.

Further studies are needed to assess the relationship between 16RMTases and the other major carbapenemases (i.e. KPC, which in our epidemiology is strictly associated to *armA* in *K. pneumoniae* ST101, OXA-48, and VIM), in order to evaluate the usefulness of new generation aminoglycosides against Enterobacterales carrying these specific resistance genes.

5 Ethical approval

The study received written consent and was approved by the local Ethics Committee (approval no. 449/19)

6 Funding information

This work was supported by Sapienza University of Rome, Top Scientist 1% Funds 2019 and Ateneo Funds 2021 no. RM12117A70BE8124 to Alessandra Carattoli.and Ateneo Funds 2019 no. RM11916B6A8B5F8D to Giammarco Raponi.

[1] Bonomo RA, Burd EM, Conly J, Limbago BM, Poirel L, Segre JA, et al. Carbapenemase-Producing Organisms: A Global Scourge. Clin Infect Dis 2018;66:1290–7. https://doi.org/10.1093/cid/cix893.

[2] Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. Discovery , research , and development of new antibiotics : the WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis 2018;18. https://doi.org/10.1016/S1473-3099(17)30753-3.

[3] Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, et al. Characterization of a new metallo-β-lactamase gene, bla NDM-1, and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India. Antimicrob Agents Chemother 2009;53:5046–54. https://doi.org/10.1128/AAC.00774-09.

[4] Wu W, Feng Y, Tang G, Qiao F, McNally A, Zong Z. NDM metallo-β-lactamases and their bacterial producers in health care settings. Clin Microbiol Rev 2019;32:1–45. https://doi.org/10.1128/CMR.00115-18.

[5] Bush K, Bradford PA. Epidemiology of β-lactamase-producing pathogens. Clin Microbiol Rev 2020;33:1–37. https://doi.org/10.1128/CMR.00047-19.

[6] Conte V, Monaco M, Giani T, D'Ancona F, Moro ML, D'Andrea MM, et al. Molecular epidemiology of KPC-producing Klebsiella pneumoniae from invasive infections in Italy: increasing diversity with predominance of the ST512 clade II sublineage. J Antimicrob Chemother 2016;71:3386–91. https://doi.org/10.1093/jac/dkw337.

[7] Tavoschi L, Forni S, Porretta A, Righi L, Pieralli F, Menichetti F, et al. Prolonged outbreak of New Delhi metallo-beta-lactamase-producing carbapenem-resistant Enterobacterales (NDM-CRE), Tuscany, Italy, 2018 to 2019. Euro Surveill 2020;25. https://doi.org/10.2807/1560-7917.ES.2020.25.6.2000085.

[8] Falcone M, Giordano C, Barnini S, Tiseo G, Leonildi A, Malacarne P, et al. Extremely drug-resistant NDM-9-producing ST147 Klebsiella pneumoniae causing infections in Italy , May 2020. Eurosurveillance 2020:3–8.

[9] Di Pilato V, Errico G, Monaco M, Giani T, Del Grosso M, Antonelli A, et al. The changing epidemiology of carbapenemase-producing Klebsiella pneumoniae in Italy: Toward polyclonal evolution with emergence of high-risk lineages. J Antimicrob Chemother 2021;76:355–61. https://doi.org/10.1093/jac/dkaa431.

[10] Karaiskos I, Lagou S, Pontikis K, Rapti V, Poulakou G. The "Old" and the "New" antibiotics for MDR Gram-negative pathogens: For whom, when, and how. Front Public Heal 2019;7:1–25. https://doi.org/10.3389/fpubh.2019.00151.

[11] Aggen JB, Armstrong ES, Goldblum AA, Dozzo P, Linsell MS, Gliedt MJ, et al. Synthesis and spectrum of the neoglycoside ACHN-490. Antimicrob Agents Chemother 2010;54:4636–42. https://doi.org/10.1128/AAC.00572-10.

[12] Doi Y, Wachino J, Arakawa Y. Aminoglycoside Resistance: The Emergence of Acquired 16S Ribosomal RNA Methyltransferases. Infect Dis Clin North Am 2017;176:139–48. https://doi.org/10.1016/j.idc.2016.02.011.Aminoglycoside.

[13] Livermore DM, Mushtaq S, Warner M, Zhang JC, Maharjan S, Doumith M, et al. Activity of aminoglycosides, including ACHN-490, against carbapenemresistant Enterobacteriaceae isolates. J Antimicrob Chemother 2011;66:48–53. https://doi.org/10.1093/jac/dkq408.

[14] Wachino JI, Doi Y, Arakawa Y. Aminoglycoside Resistance: Updates with a Focus on Acquired 16S Ribosomal RNA Methyltransferases. Infect Dis Clin North Am 2020;34:887–902. https://doi.org/10.1016/j.idc.2020.06.002.

[15] McGann P, Chahine S, Okafor D, Ong AC, Maybank R, Kwak YI, et al. Detecting 16S rRNA

methyltransferases in enterobacteriaceae by use of Arbekacin. J Clin Microbiol 2016;54:208–11. https://doi.org/10.1128/JCM.02642-15.

[16] Ministero della Salute U 5 P delle malattie trasmissinili e profilassi internazionale. 2019 - Aggiornamento delle indicazioni per la sorveglianza e il controllo delle infezioni da Enterobatteri resistenti ai carbapenemi (CRE) 2019.

[17] Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol 2017;13:1–22. https://doi.org/10.1371/journal.pcbi.1005595.

[18] Lam MMC, Wick RR, Watts SC, Cerdeira LT, Wyres KL, Holt KE. A genomic surveillance framework and genotyping tool for Klebsiella pneumoniae and its related species complex. Nat Commun 2021;12. https://doi.org/10.1038/s41467-021-24448-3.

[19] Argimón S, Abudahab K, Goater RJE, Fedosejev A, Bhai J, Glasner C, et al. Microreact: visualizing and sharing data for genomic epidemiology and phylogeography. Microb Genomics 2016;2:e000093. https://doi.org/10.1099/mgen.0.000093.

[20] Krzywinski M, Schein J, Birol I, Connors J, Gascoyne R, Horsman D, et al. Circos: An information aesthetic for comparative genomics. Genome Res 2009;19:1639–45. https://doi.org/10.1101/gr.092759.109.

[21] Bercot B, Poirel L, Nordmann P. Updated multiplex polymerase chain reaction for detection of 16S rRNA methylases: High prevalence among NDM-1 producers. Diagn Microbiol Infect Dis 2011;71:442–5. https://doi.org/10.1016/j.diagmicrobio.2011.08.016.

[22] Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfall EJ. Identification of plasmids by PCR-based replicon typing. J Microbiol Methods 2005;63:219–28. https://doi.org/10.1016/j.mimet.2005.03.018.

[23] Ambrose SJ, Harmer CJ, Hall RM. Evolution and typing of IncC plasmids contributing to antibiotic resistance in Gram-negative bacteria. Plasmid 2018;99:40–55. https://doi.org/10.1016/j.plasmid.2018.08.001.

[24] Carattoli A, Zankari E, García-Fernández A, Larsen MV, Lund O, Villa L, et al. In Silico detection and typing of plasmids using plasmidfinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother 2014;58:3895–903. https://doi.org/10.1128/AAC.02412-14.

[25] Pietsch M, Irrgang A, Roschanski N, Brenner Michael G, Hamprecht A, Rieber H, et al. Whole genome analyses of CMY-2-producing Escherichia coli isolates from humans, animals and food in Germany. BMC Genomics 2018;19:1–18. https://doi.org/10.1186/s12864-018-4976-3.

[26] Harmer CJ, Hall RM. The A to Z of A/C plasmids. Plasmid 2015;80:63–82. https://doi.org/10.1016/j.plasmid.2015.04.003.

[27] Wailan AM, Sidjabat HE, Yam WK, Alikhan N, Petty NK, Sartor AL, et al. Mechanisms Involved in Acquisition of bla NDM Genes by IncA / C 2 and. Antimicrob Agents Chemother 2016;60:4082–8. https://doi.org/10.1128/AAC.00368-16.Address.

[28] Wachino JI, Yamane K, Kimura K, Shibata N, Suzuki S, Ike Y, et al. Mode of transposition and expression of 16S rRNA methyltransferase gene rmtC accompanied by ISEcp1. Antimicrob Agents Chemother 2006;50:3212–5. https://doi.org/10.1128/AAC.00550-06.

[29] Wachino JI, Yamane K, Shibayama K, Kurokawa H, Shibata N, Suzuki S, et al. Novel plasmid-mediated 16S rRNA methylase, RmtC, found in a Proteus mirabilis isolate demonstrating extraordinary high-level resistance against various aminoglycosides. Antimicrob Agents Chemother 2006;50:178–84. https://doi.org/10.1128/AAC.50.1.178-184.2006.

[30] Shen X, Liu L, Yu J, Cao X, Zhan Q, Guo Y, et al. Coexistence of blaNDM-1 and rmtC on a

transferrable plasmid of a novel ST192 Klebsiella aerogenes clinical isolate. Infect Drug Resist 2019;12:3883–91. https://doi.org/10.2147/IDR.S228130.

[31] Al-Agamy MH, El-Mahdy TS, Radwan HH, Poirel L, Garcia-Perdomo HA. Cooccurrence of NDM-1, ESBL, RmtC, AAC(6′)-Ib, and QnrB in Clonally Related Klebsiella pneumoniae Isolates Together with Coexistence of CMY-4 and AAC(6′)-Ib in Enterobacter cloacae Isolates from Saudi Arabia. Biomed Res Int 2019;2019. https://doi.org/10.1155/2019/6736897.

[32] Caméléna F, Morel F, Merimèche M, Decousser JW, Jacquier H, Clermont O, et al. Genomic characterization of 16S rRNA methyltransferase-producing Escherichia coli isolates from the Parisian area, France. J Antimicrob Chemother 2020;75:1726–35. https://doi.org/10.1093/jac/dkaa105.

[33] Lioy VS, Goussard S, Guerineau V, Yoon EJ, Courvalin P, Galimand M, et al. Aminoglycoside resistance 16S rRNA methyltransferases block endogenous methylation, affect translation efficiency and fitness of the host. Rna 2014;20:382–91. https://doi.org/10.1261/rna.042572.113.

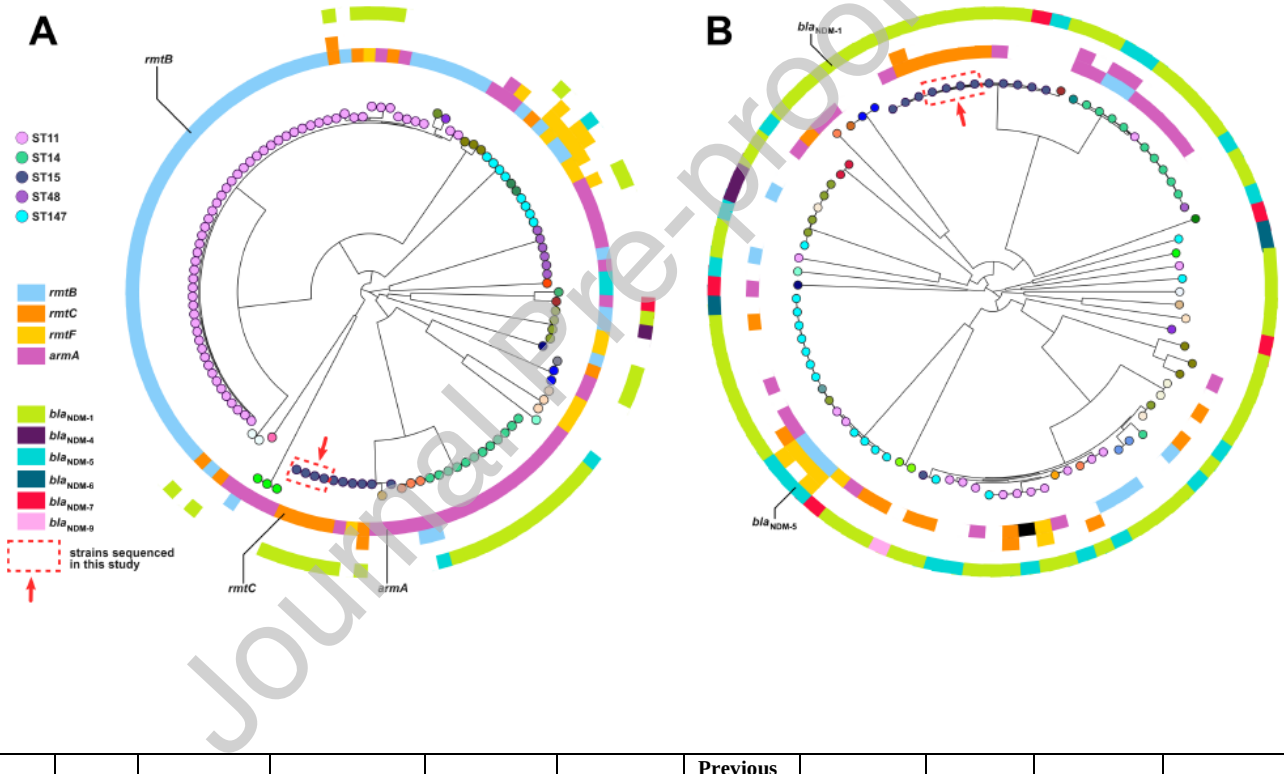
[34] Stoesser N, Giess A, Batty EM, Sheppard AE, Walker AS, Wilson DJ, et al. Genome sequencing of an extended series of NDM-producing Klebsiella pneumoniae isolates from neonatal infections in a Nepali hospital characterizes the extent of community- Versus hospital- associated transmission in an endemic setting. Antimicrob Agents Chemother 2014;58:7347–57. https://doi.org/10.1128/AAC.03900-14.

[35] Gutiérrez-Gutiérrez B, Salamanca E, de Cueto M, Hsueh PR, Viale P, Paño-Pardo JR, et al. Effect of appropriate combination therapy on mortality of patients with bloodstream infections due to carbapenemase-producing Enterobacteriaceae (INCREMENT): a retrospective cohort study. Lancet Infect Dis 2017;17:726–34. https://doi.org/10.1016/S1473-3099(17)30228-1.

[36] de Man TJB, Lutgring J, Lonsway D, Anderson K, Kiehlbauch J, Chen L, et al. Genomic Analysis of a Pan-Resistant Isolate of Klebsiella pneumoniae, United States 2016. MBio 2018;9.

Figure 1. Phylogenetic analysis of several K. pneumoniae downloaded from the GenBank database compared with the ones sequenced in this study

Panel A: Unrooted ML phylogenetic tree comparing 130 K. pneumoniae belonging to several different STs harbouring at least one 16S rRNA methyltransferases (16RMTases) and (panel B) 95 K. pneumoniae carrying one of the blaNDM genes. Nodes are colour coded according to their ST, with the main STs listed in the upper left corner. The three inner metadata rings represent the presence of one (or more) 16RMTases and the outer one represents (colour coded according to the legend in the middle part of the left hand of the figure).



	Age/sex	Days of hospitalization	Comorbidities	Previous (90-d) hospitalization	Previous (90-d) antibiotic therapy	Previous (90-d) carbapenem therapy	Ward of isolation	Type of samples	Infection/ colonization	Type of microorganism
Pt# 1	40/F	13	None	yes	yes	yes	ICU	Bronchial aspirate	Infection	*K. pneumoniae*
Pt# 2	74/F	57	COPD, tetraparesis	no	yes	yes	Pneumology	Rectal swab	Colonization	*K. pneumoni*

										ae
Pt# 3	79/F	109	DM, AH, COPD, CAD, Obesity, PH	yes	yes	yes	ICU	Rectal swab	Colonization	*K. pneumoniae*
Pt# 4	51/ M	28	CAD, AH, obesity	no	yes	yes	ICU	Rectal swab	Colonization	*K. pneumoniae*
Pt# 5	58/F	92	CAD, AH, obesity	yes	yes	yes	Pneumology	Rectal swab	Colonization	*K. pneumoniae*
Pt# 6	70/F	38	Lung transplantation	no	yes	yes	ICU	BAL	Infection	*K. pneumoniae*
Pt# 7	53/F	1	CF, lung transplantation	no	no	no	CF Day hospital	BAL	Infection	*K. pneumoniae*
Pt# 8	89/F	32	None	no	yes	yes	Pneumology	Bronchial Aspirate	Infection	*K. pneumoniae*
Pt# 9	50/ M	27	AH	yes	yes (piperacillin tazobactam)	No	ICU	Rectal swab	Colonization	*K. pneumoniae*