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# Occurrence of NDM-1-producing *Morganella morganii* and *Proteus mirabilis* in a single patient in Portugal: probable *in vivo* transfer by conjugation

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**Objectives:** To decipher the genetics of acquisition of carbapenemase-encoding genes identified in two carbapenem-resistant Enterobacteriaceae recovered from a single patient in Portugal.

**Methods:** Carbapenemase genes were searched by PCR assays and mating-out assays were performed to further characterize the plasmid support of the carbapenemase genes. Genetic characterization of the plasmid supports was performed by whole-plasmid sequencing using the Illumina technology.

**Results:** We identified here two NDM-1-producing isolates, namely a *Morganella morganii* and a *Proteus mirabilis*, sharing the same $bla_{NDM-1}$-positive plasmid. This 154 kb plasmid belonged to the IncA/$C_2$ type, recently renamed IncC, and co-harboured two AmpC β-lactamase genes, namely $bla_{CMY-4}$ and $bla_{DHA-1}$, in addition to the 16S rRNA methylase gene *armA* encoding high-level resistance to aminoglycosides. In addition, the *M. morganii* isolate produced the CTX-M-33 extended-spectrum β-lactamase possessing weak carbapenemase activity, encoded by another plasmid.

**Conclusions:** We showed here that, in addition to KPC-type and OXA-181 carbapenemases, which have been identified as widespread in this country, another concern is the emergence of NDM-1-producing enterobacterial isolates in Portugal. We demonstrated here the *in vivo* plasmid transfer of a $bla_{NDM-1}$-positive plasmid leading to dissemination of this carbapenemase gene within different enterobacterial species in a single patient.

## Introduction

The occurrence of carbapenemase-producing bacteria, especially those producing NDM-1 and its variants, is a major public health concern worldwide.[1] NDM-1 hydrolyses a wide range of β-lactam antibiotics, including carbapenems, and its activity is not inhibited by clinically available β-lactamase inhibitors. Considering that the $bla_{NDM-1}$ gene is most often located on plasmid scaffolds encoding co-resistance to other antibiotic families, and that clinical isolates bearing these $bla_{NDM-1}$-positive plasmids also possess other MDR plasmids, very few options remain available for the treatment of infections caused by these resistant isolates. The $bla_{NDM-1}$ carbapenemase gene is spreading increasingly and rapidly worldwide, particularly in several European countries where it has been identified among different enterobacterial species, among different strain backgrounds within each species, and on many plasmid backbones, including IncA/C-type, IncN and IncF.[2,3] The first of these backbones, which possesses a broad host range, has particularly been frequently identified in association with $bla_{NDM-1}$, in human and animal isolates.

We report here the isolation of two subsequent NDM-1-producing enterobacterial isolates (*Morganella morganii* and *Proteus mirabilis*) from a single patient in Portugal.

## Materials and methods

### *Identification and susceptibility testing*

Identification of the isolates at the species level was performed using the API-20E system (bioMérieux, La Balme-les-Grottes, France). Antimicrobial

susceptibility testing was performed and interpreted as recommended by EUCAST (www.eucast.org). Carbapenemase production was detected by using the Carba NP test.[4]

### Molecular analysis

A series of acquired resistance genes were searched by PCR, including those encoding ESBLs ($bla_{TEM}$-, $bla_{SHV}$- and $bla_{CTX-M}$-like genes), carbapenemases ($bla_{KPC}$, $bla_{NDM}$, $bla_{VIM}$, $bla_{IMP}$, $bla_{OXA-48}$ and $bla_{OXA-181}$) and 16S rRNA methylases genes (*armA*, *rmtA* to *rmtH* and *npmA*).[3,5] The obtained amplicons were sent for sequencing (Microsynth®, Balgach, Switzerland).

### Plasmid analysis and mating-out assays

Transferability of the carbapenem resistance determinant was assessed by mating-out assay. Briefly, the donors as well as the *Escherichia coli* strain J53 as recipient were separately inoculated overnight in LB broth. The samples were then mixed at a ratio of 10:1 (recipient:donor) for 5 h and plated onto LB agar plates supplemented with azide (100 mg/L) and ceftazidime (30 mg/L). Antimicrobial susceptibility testing was performed on the *E. coli* transconjugant in order to identify putative co-resistances. Kieser extraction[6] followed by gel electrophoresis analysis was performed for the resulting *E. coli* transconjugant strains in order to estimate the size of the transferred plasmid. *E. coli* strain 50192, carrying four plasmids with known sizes (7, 48, 66 and 154 kb), was used as a molecular marker.

PCR-based replicon typing was performed to identify specific replicase genes eventually differentiating plasmid incompatibility groups.[7] Replicon typing of the *in silico*-identified plasmid was performed using the web-based tool PlasmidFinder version 2.1 (https://cge.cbs.dtu.dk/services/PlasmidFinder/).

### Plasmid sequencing and bioinformatic analysis

Sequencing of plasmid DNAs was performed by using Illumina technology. Templates used corresponded to plasmid extracts obtained by using the Qiagen Large Plasmid Construct kit (Qiagen, Hilden, Germany) for the *M. morganii* and *P. mirabilis* isolates. Genomic libraries were assessed using a Nextera XT library preparation kit (Illumina Inc., San Diego, CA, USA), and sequencing was performed using an Illumina MiniSeq system with 150 bp paired-end reads and a coverage of ×50. The generated FastQ data were compiled and analysed using the CLC Genomic Workbench (version 7.5.1; CLC Bio, Aarhus, Denmark). Reads were *de novo* assembled with automatic bubble and word size, and contigs with a minimum contig length of 800 nt were generated using the back to contigs mapping map mode. The resulting contigs were uploaded to the Center for Genomic Epidemiology server (http://www.genomicepidemiology.org/). The plasmid replicon type and MLST according to the Warwick typing scheme were determined using the PlasmidFinder (version 1.3) and MLST (version 1.8) programs, respectively.

### Sequence database

The full sequence of plasmid pPM154 has been deposited in the GenBank database under accession number MN550958.

### Ethics

The publication of this case report complies with national and institutional regulations in Portugal.

## Results and discussion

In January 2019, a patient institutionalized at a nursing home in the region of Lisbon, Portugal, attended a plastic surgery consultation at a private hospital in Lisbon. The patient had an infected calcaneal pressure ulcer and was at that time under amoxicillin/clavulanic acid therapy due to a presumed respiratory infection. The ulcer was likely caused by traumatic immobilization after femoral neck fractures that happened in 2015 and then in 2018. Each time, the patient was hospitalized and underwent orthopaedic surgery with cefazolin prophylaxis for 24 h. In 2018, after hospital discharge, the patient was finally admitted to a nursing home.

**Table 1.** MICs of β-lactams and other antibiotics for *P. mirabilis* and *M. morganii* isolates

| | MIC (mg/L) | | |
|---|---|---|---|
| β-Lactam(s)[a] | *M. morganii* | *P. mirabilis* | *E. coli* J53 (pPM154) |
| Amoxicillin | >512 | >512 | >512 |
| Amoxicillin/clavulanic acid | >512 | >512 | >512 |
| Ticarcillin | >512 | 64 | >512 |
| Ticarcillin/clavulanic acid | >512 | 32 | >512 |
| Piperacillin | 16 | 8 | 16 |
| Piperacillin/tazobactam | 8 | 4 | 8 |
| Cefoxitin | >32 | >32 | >32 |
| Ceftazidime | >32 | >32 | >32 |
| Ceftazidime/avibactam | >256 | >256 | >256 |
| Cefotaxime | 2 | 4 | 8 |
| Cefepime | 1 | 4 | 8 |
| Aztreonam | <0.12 | <0.12 | 1.5 |
| Imipenem | 16 | 8 | 8 |
| Meropenem | 2 | 2 | 4 |
| Ertapenem | 1 | 2 | 2 |
| Ciprofloxacin | <0.12 | 0.5 | <0.12 |
| Gentamicin | >32 | >32 | >32 |
| Tobramycin | >32 | >32 | >32 |
| Kanamycin | >32 | >32 | >32 |
| Chloramphenicol | <0.12 | 16 | <0.12 |
| Trimethoprim/sulfamethoxazole | <0.12 | >32 | <0.12 |

[a]Clavulanic acid was added at 2 mg/L; tazobactam was added at 4 mg/L.

At the time of consultation, an ulcer swab was withdrawn and an MDR *M. morganii* was recovered. The enterobacterial isolate showed resistance to expanded-spectrum cephalosporins but remained susceptible to meropenem (Table 1). In addition, it was resistant to all aminoglycosides and fosfomycin. Moreover, the isolate showed high-level resistance to ceftazidime/avibactam (MIC >256 mg/L). Conversely, it remained susceptible to aztreonam, piperacillin and piperacillin/tazobactam, and to ciprofloxacin, chloramphenicol and trimethoprim/sulfamethoxazole. Since no systemic infection was observed, the patient did not receive antibiotics.

Results of the Rapid Carba NP test together with PCR amplification and sequencing showed that the isolate produced an NDM-1 MBL. Identification of this enzyme was in accordance with the susceptibility pattern observed, even though susceptibility to piperacillin and piperacillin/tazobactam is indeed uncommon for an NDM-1-producing isolate. Actually, susceptibility to piperacillin/tazobactam has already been reported for some NDM-1-producing Enterobacteriaceae.[2] In addition, the *M. morganii* isolate harboured the $bla_{CTX-M-33}$ ESBL gene, known to possess weak

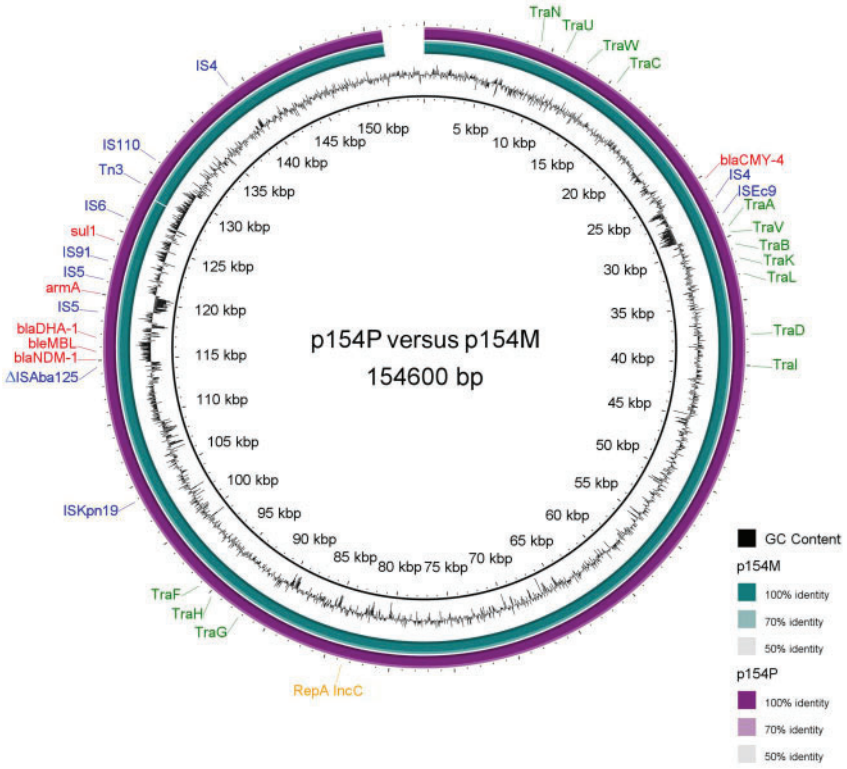


**Figure 1.** Circular representation of the sequence comparison between p154P (*M. morganii*) and p154M (*P. mirabilis*). GC content is represented on the distance scale (in kbp) on the inner map. p154P was selected as the reference sequence. Resistance genes (red), ISs (blue), replication gene of IncC (yellow) and conjugative transfer genes (green) are also indicated on the external ring. The map was drawn using BLAST Ring Image Generator (BRIG) (http://sourceforge.net/projects/brig/). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

carbapenemase activity,[8] the $bla_{TEM-181}$ ESBL gene, two plasmid-encoded AmpC β-lactamases, namely $bla_{CMY-4}$ and $bla_{DHA-1}$ genes, in addition to its intrinsic $bla_{DHA}$-like AmpC gene. Since the isolate was resistant to all aminoglycosides, 16S rRNA methyltransferase genes were searched by PCR amplification and the *armA* gene was identified, which is commonly observed among NDM-1 producers.[2,9]

Mating-out assays gave *E. coli* transconjugants possessing the $bla_{NDM-1}$ gene. This plasmid, named p154P, was fully sequenced, showing that it was 154 kb in size and belonged to the IncC (formerly IncA/$C_2$)[10] incompatibility group, co-harbouring the $bla_{DHA-1}$, $bla_{CMY-4}$ and *armA* genes (Figure 1). Analysis of its *repA* replicase gene using PlasmidFinder revealed a perfect match with the *repA* gene of plasmid pNDM-KN encoding the NDM-1 carbapenemase in *Klebsiella pneumoniae*.[11]

In May 2019, another swab was withdrawn from the infected calcaneal ulcer of the same patient and an ESBL-producing and carbapenem-resistant *P. mirabilis* was recovered (Table 1). This isolate also possessed the $bla_{NDM-1}$ gene; therefore, a mating-out assay was also performed and the corresponding plasmid was also submitted to complete sequencing. Detailed analysis by complete sequencing showed that this plasmid was almost identical to p154P from *M. morganii* (Figure 1). It differed by only 54 bp out of 154 600 bp, and also carried the $bla_{DHA-1}$, $bla_{CMY-4}$ and *armA* genes, strongly suggesting an *in vivo* transfer by conjugation of this IncA/C-type plasmid between the *M. morganii* and *P. mirabilis* isolates. Compared with the *M. morganii* isolate, the *P. mirabilis* isolate showed additional resistance to chloramphenicol and trimethoprim/sulfamethoxazole, but remained susceptible to fosfomycin, and harboured the narrow-spectrum $bla_{TEM-1}$ instead of the $bla_{TEM-181}$ ESBL gene.

After the first and only report of five NDM-1-producing *Providencia stuartii* isolates of 2015 causing an outbreak in a hospital in Lisbon (a different hospital from this report), no other case of NDM producers has been reported from Portugal. Interestingly, a BLAST analysis of the whole sequence of plasmid p154P against the draft genome of an NDM-1-producing *P. stuartii* isolate recovered in Portugal in 2015[12] showed a perfect match with contigs of this *P. stuartii* strain, therefore indicating that the plasmid structure of this latter strain was either identical with or very similar to p154P.

Here, we showed that the NDM-1 carbapenemase may also be an additional source of carbapenem resistance in Portugal, where KPC producers to a major extent, and OXA-181 to a lesser extent, have been extensively reported.[13,14] Notably, these two NDM-1-producing isolates were recovered while the patient was not receiving an antibiotic regimen, thus in the absence of obvious selective pressure. Interspecies transfer of NDM-1-encoding plasmids has been previously reported in clinical institutions.[15] Interestingly, the *in vivo* transfer of an NDM-1-encoding IncC

plasmid within different enterobacterial species was recently reported in infected chickens, but also in hospitalized patients, with a single IncC plasmid (published as IncA/$C_2$) being identified in *Citrobacter freundii*, *E. coli*, *K. pneumoniae* and *Klebsiella oxytoca*.[16,17]

NDM-1-producing *P. mirabilis* have been previously reported in different parts of the world, but usually the $bla_{NDM-1}$ gene was identified within an SGI-type genomic island, hence at a chromosomal location.[18] Here, we showed it may also be found as plasmid located, on a broad host range IncC plasmid co-encoding β-lactamases CMY and TEM, in addition to the 16S rRNA methylase ArmA, as previously found.[9]

The main reservoirs of NDM producers are considered to be the Indian subcontinent, the Balkans regions, North Africa and the Middle East.[1] Of note, we recently showed that NDM-1-producing Enterobacteriaceae are widespread in Portuguese-speaking African countries, which might represent putative sources of MDR strain importations in Portugal, due to the close demographic relationship.[19] However, this patient and his close relatives did not have a history of recent international travelling. There is also no information regarding colonization or infection with carbapenemase producers among individuals living in the same nursing home.


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## Transparency declarations

None to declare.

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