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Short communication

# Characterization of a multidrug resistant *Citrobacter amalonaticus* clinical isolate harboring $bla_{NDM-1}$ and *mcr-1.5* genes

Diego Faccone[a,b], Ezequiel Albornoz[a], Nathalie Tijet[c], Estefania Biondi[d], Sonia Gomez[a,b], Fernando Pasterán[a], Miryam Vazquez[d], Roberto G. Melano[c,e], Alejandra Corso[a,*]

[a] Servicio Antimicrobianos, National and Regional Reference Laboratory in Antimicrobial Resistance, Instituto Nacional de Enfermedades Infecciosas (INEI)-ANLIS "Dr. C. Malbrán", Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina
[c] Public Health Ontario Laboratory, Toronto, Ontario, Canada
[d] Hospital de Niños Dr. Ricardo Gutiérrez, Buenos Aires, Argentina
[e] Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada

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ABSTRACT

A multidrug resistant isolate, identified as *Citrobacter amalonaticus* using MALDI-TOF MS and confirmed by genomic analysis, was recovered from a pediatric patient in a hospital from Buenos Aires, Argentina. By whole-genome sequencing a total of 16 resistance genes were detected, including $bla_{NDM-1}$ and *mcr-1.5*. To the best of our knowledge this is the first description of these two genes together in a clinical isolate of the *Citrobacter* genus.

## 1. Introduction

*Citrobacter* is a gram-negative coliform bacterial genus comprising 14 species that have been isolated from water, soil, food and the gastrointestinal tract of humans and animals (Clermont et al., 2015). *C. freundii* and *C. koseri* are the most common species recovered from human infections while *C. amalonaticus* is seldom isolated from faecal and urine samples and only occasionally associated with invasive infections, like osteomyelitis, arthritis and peritonitis (Garcia et al., 2016; Maraki et al., 2017; Wong et al., 2012). *Citrobacter* spp. are usually susceptible to third-generation cephalosporins, carbapenem and colistin (Maraki et al., 2017). However, carbapenem resistant *Citrobacter* spp. isolates have been reported due to the acquisition of worldwide disseminated carbapenemases, such as New Delhi Metallo-β-lactamase (NDM) (Arana et al., 2017; Hammerum et al., 2016; Pasteran et al., 2016). In the last few years, a transferable colistin-resistant mechanism mediated by *mcr* genes has been recently described (8). *mcr-1* gene has been found in several *Enterobacteriaceae* genus and species but it has mainly been detected in *Escherichia coli* from animal, food, environmental and human sources (Al-Tawfiq et al., 2017; Rapoport et al., 2016; Saavedra et al., 2017). Here, we report the characterization of a clinical multidrug resistant (MDR) *C. amalonaticus* isolate carrying $bla_{NDM-1}$ and *mcr-1.5* genes.

## 2. Materials and methods

On February 2016, isolate M21015 was recovered in a pediatric hospital in Buenos Aires from an oncologic four-year old patient, during an active carbapenemase surveillance program for asymptomatic rectal colonization, and confirmed as *C. amalonaticus* by Vitek2, VitekMS (bioMérieux) and MALDI-TOF MS Biotyper 3.0 (Bruker Daltonics). The isolate was sent to the Argentinian National Reference Laboratory for Antimicrobial Resistance for further characterization.

Antimicrobial susceptibility was evaluated by Sensititre ARGNF customized plates (Trek Diagnostic Systems, Thermo Fisher Scientific), and interpreted according to the Clinical and Laboratory Standards Institute or the European Committee on Antimicrobial Susceptibility Testing (colistin and tigecycline) criteria (Clinical and Laboratory Standards Institute (CLSI)., 2017; EUCAST, 2017. http://www.eucast.org.). The imipenem-EDTA double-disk synergy test was used to detect metallo-β-lactamase (MBL) activity. The presence of MBL ($bla_{NDM}$, $bla_{IMP}$ and $bla_{VIM}$) and *mcr-1* genes were detected by in house PCR.

*C. amalonaticus* M21015 was analyzed by whole genome sequencing (WGS) using Nextera XT for library preparation and Illumina compact MiSeq system for sequencing. Plasmids containing *mcr* and $bla_{NDM}$ genes were independently transferred by biparental conjugation to *E. coli* J53, and the transconjugants selected with colistin (2 μg/ml) or

* Corresponding author.
*E-mail address:* acorso@anlis.gov.ar (A. Corso).

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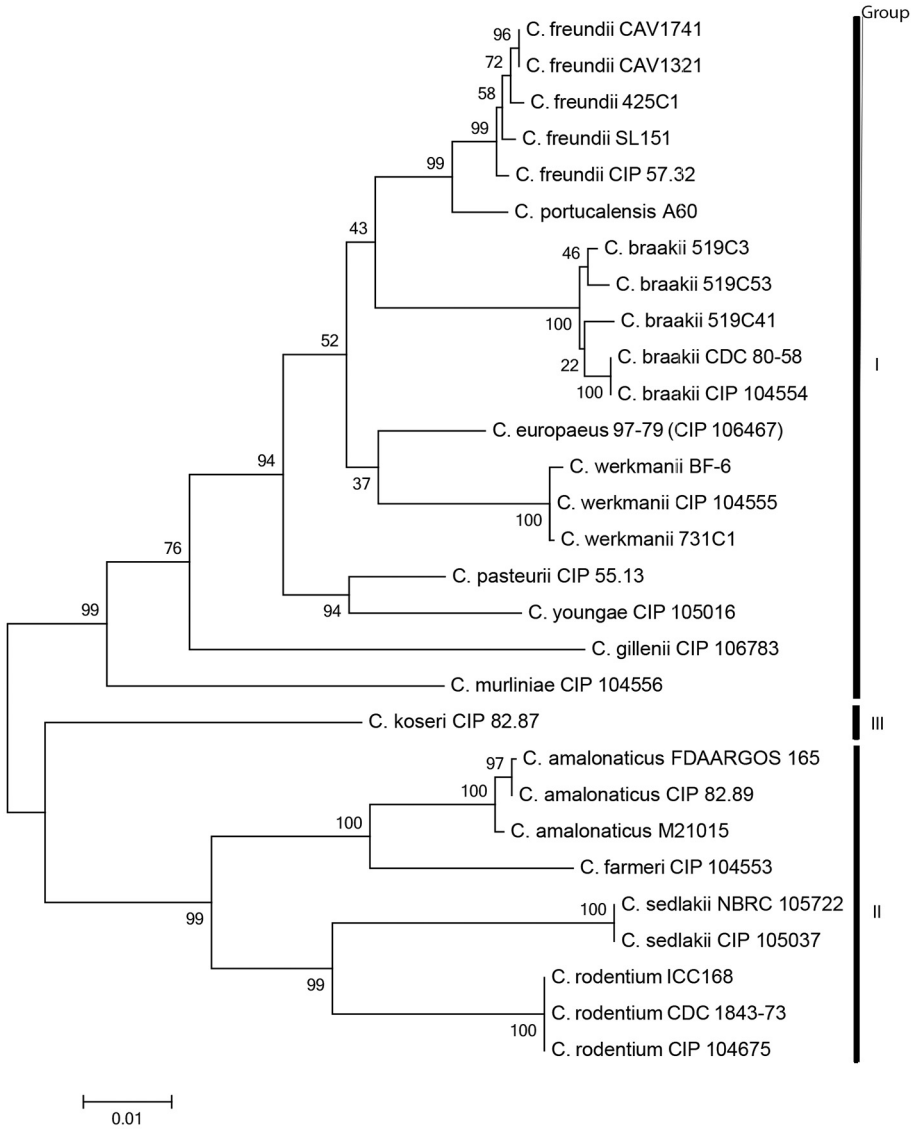


**Fig. 1.** Phylogenetic analysis by maximum likelihood method of *Citrobacter* species.
The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model. Branch lengths measured in the number of substitutions per site. The analysis included 29 nucleotide sequences representing 14 *Citrobacter* species. Groups I, II and III were described in the text. Phylogenetic analyses were conducted in MEGA6 (Tamura et al., 2013).

meropenem (0.2 μg/ml), respectively, and sodium azide (100 μg/ml) (Gomez et al., 2011). Plasmids were extracted and purified for Illumina sequencing, and analyzed as previously described (Tijet et al., 2016). Antimicrobial resistance genes were predicted using the ResFinder, version 3.0 (Zankari et al., 2012).

## 3. Results and discussion

*C. amalonaticus* M21015 was resistant (μg/mL) to imipenem (≥16), meropenem (≥32), cefotaxime (≥64), ceftazidime (≥64), cefepime (≥32), amikacin (≥64), gentamicin (≥16), trimethoprim-sulfamethoxazole (≥4) and colistin (≥8), and susceptible to aztreonam (≤8), ciprofloxacin (≤0.06), fosfomycin (≤32), minocycline (≤4) and tigecycline (≤0.5). MBL inhibition was associated with the presence of $bla_{NDM}$ gene. Resistance to colistin was associated with the presence of *mcr-1*-like gene.

Two plasmids were transferred individually from *C. amalonaticus* M21015 to *E. coli* J53 by conjugation, and subsequently sequenced. One of them, named here pMCR-M21015, harboured the *mcr-1.5* variant (Tijet et al., 2017). pMCR-M21015 (62,891 bp) belonged to the incompatibility group IncI2 sharing > 99.9% identity with pMCR-M15049 plasmid (accession number KY471308) of *E. coli* M15049 also isolated in Argentina(Tijet et al., 2017). *mcr-1.5* and *pap2* genes were identified in a 2749 bp contig. The presence of two copies of IS*ApI1* in the same orientation flanking them was verified by PCR and Sanger sequencing, as well as the 2-bp putative target site duplications, confirming the presence of the composite transposon Tn*6330* (Liu et al., 2016). No other antimicrobial resistance gene was identified in this plasmid (Suppl. Fig. 1). The finding of *mcr-1* in *Citrobacter* spp. is infrequent and to the best of our knowledge has not been described in *C. amalonaticus*.

Sequencing of the second plasmid (named pNDM-M21015) showed that $bla_{NDM-1}$ is located in a type 1 IncC2 plasmid of 138,998 bp with 99% identity with pKP1-NDM-1 (KF992018). $bla_{NDM-1}$ was located within a truncated Tn125 structure previously reported paper de Wailan et al.(Wailan et al., 2015). pNDM-M21015 also contains $bla_{CMY-6}$, *rmtC*, *sul1*, and some small MDR genes encoding for the ethidium bromide-methyl viologen resistance protein EmrE and the quaternary ammonium compound-resistance protein SugE, as well as the bleomycin resistance protein ($ble_{MBL}$) associated to $bla_{NDM}$ (Suppl. Fig. 2).

**Table 1**
Summary of resistance genes detected by plasmid and whole-genome sequencing.

| Antibiotic affected | Resistance gene | Identity (%) | GenBank Accession no. |
|---|---|---|---|
| Polymyxins | *mcr-1.5* | 100 | KP347127 |
| ß-Lactams | $bla_{NDM-1}$ | 100 | FN396876 |
| | $bla_{CMY-6}$ | 100 | AJ011293 |
| | $bla_{TEM-1B}$ | 100 | JF910132 |
| | $bla_{SED-1}$ | 82.64 | AF321608 |
| Aminoglycosides | *rmtC* | 100 | AB194779 |
| | *aadA1* | 100 | JQ414041 |
| | *aadA2* | 100 | JQ364967 |
| | *aacA4* | 100 | KM278199 |
| | *aph(3′)-Ia* | 100 | V00359 |
| Phenicols | *floR* | 98.11 | AF118107 |
| | *cmlA1* | 99.92 | M64556 |
| Sulphonamide | *sul1* | 100 | CP002151 |
| | *sul2* | 100 | GQ421466 |
| | *sul3* | 100 | AJ459418 |
| Trimethoprim | *dfrA12* | 100 | AB571791 |

In addition, other mechanisms of resistance were identified by WGS analysis (Table 1): to aminoglycosides, phenicols, trimethoprim, sulphonamides and β-lactams (including $bla_{CdiA}$, a chromosomally encoded class A β-lactamase similar to Sed-1 from *Citrobacter sedlakii*) (Petrella et al., 2001). An efflux pump similar to OqxA/B (~90% nucleotide identity with OqxA/B found on *K. pneumoniae* chromosomes) was also identified, but the MIC for ciprofloxacin suggests that it is not active against fluoroquinolones.

The identification to the species level of *Citrobacter* is controversial (Ribeiro et al., 2015). The conventional biochemical characterization and MALDI-TOF MS methods yield reliable results with approx. 90% and 95% accuracy for each methodology, respectively (Kolínská et al., 2015). The use of 16S rRNA gene sequences displays limited resolution distinguishing only three groups within the genus, where *C. amalonaticus* is included in group II together with *C. farmeri*, *C. sedlakii* and *C. rodentium* (Clermont et al., 2015). A previously described multilocus sequence analysis of a 2082 bp concatenated sequence of *fusA*, *pyrG*, *leuS* and *rpoB* genes was used to confirm the isolate identity at the species level (Clermont et al., 2015). A phylogenetic analysis was performed including the sequences of 28 isolates representing 14 well defined *Citrobacter* species (Fig. 1). *C. amalonaticus* M21015 grouped together with *C. amalonaticus* FDAARGOS165 and CIP 82.89 in group II (Fig. 1), confirming the identification obtained by biochemical and proteomic methods.

*C. amalonaticus* is an opportunistic pathogen rarely associated with MDR phenotypes (Garcia et al., 2016; Maraki et al., 2017; Wong et al., 2012). To the best of our knowledge this is the first description of both $bla_{NDM}$ and *mcr-1*-like genes in the *Citrobacter* genus, co-existing with other 14 determinants of antimicrobial resistance in this clinical isolate of *C. amalonaticus*. This emergence is coincident with other reports suggesting an increase in the isolation of MDR *Citrobacter* spp. (Arana et al., 2017; Hammerum et al., 2016; Pasteran et al., 2016). Further studies are required to determine its public health significance and the degree of dissemination since *Citrobacter* is not frequently included in surveillance programs for MDR organisms. Remarkably, a highly similar IncI2 pMCR plasmid was now described in two genera in Argentina, suggesting horizontal dissemination not only between *E. coli* isolates(Tijet et al., 2017) but to other *Enterobacteriaceae*. Furthermore, the presence of *mcr-1.5* in an active composite transposon creates the potential for its dissemination to other plasmids, increasing the risk of a more effective spread of polymyxin resistance.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2018.10.020.

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## Transparency declarations

Nothing to declare.

## Knowledge

Raw sequence reads of the whole genome of *C. amalonaticus* M21015 were deposited in the NCBI database under accession number PRJNA495344. Additionally plasmid sequences were submitted individually: pMCR-M21015 (submission ID: 2156778); and pNDM-M21015 (submission ID: 2157761).

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