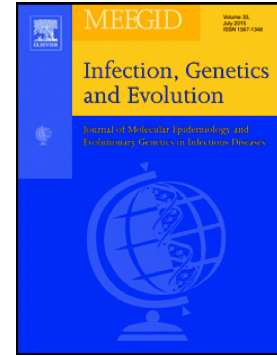


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**Characterization of a Multidrug Resistant *Citrobacter amalonaticus* Clinical Isolate
Harboring *bla*_{NDM-1} and *mcr-1.5* Genes**

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Keywords: *mcr-1*; *bla*_{NDM}; carbapenem; colistin; *Citrobacter amalonaticus*

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.

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.

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; "The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 7.0, 2017. <http://www.eucast.org>," n.d.). 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 analysed 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 μ g/ml) or meropenem (0.2 μ g/ml), respectively, and sodium azide (100 μ g/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).

RESULTS and DISCUSSION

C. amalonaticus M21015 was resistant ($\mu\text{g}/\text{mL}$) 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 2,749 bp contig. The presence of two copies of IS*Ap1* 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 Tn6330 (Liu et al., 2016). No other antimicrobial resistance gene was identified in this plasmid (Suppl. Figure 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. Figure 2).

In addition, other mechanisms of resistance were identified by WGS analysis (Table 1): to aminoglycosides, phenicols, trimethoprim, sulphonamides and β -lactams (including *bla*_{CdiA_r}, 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 2,082 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 (Figure 1). *C. amalonaticus* M21015 grouped together with *C. amalonaticus* FDAARGOS165 and CIP 82.89 in group II (Figure 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.

The following are the supplementary data related to this article.

Supplementary Table 1. Primers used to detect blaNDM, blaIMP, blaVIM and mcr-1 genes.

Suppl. Table 2. Antimicrobial susceptibility of *C. amalonaticus* M21015 (donor), *E. coli* J53 (acceptor), and two *E. coli* transconjugants.

Supplementary figures

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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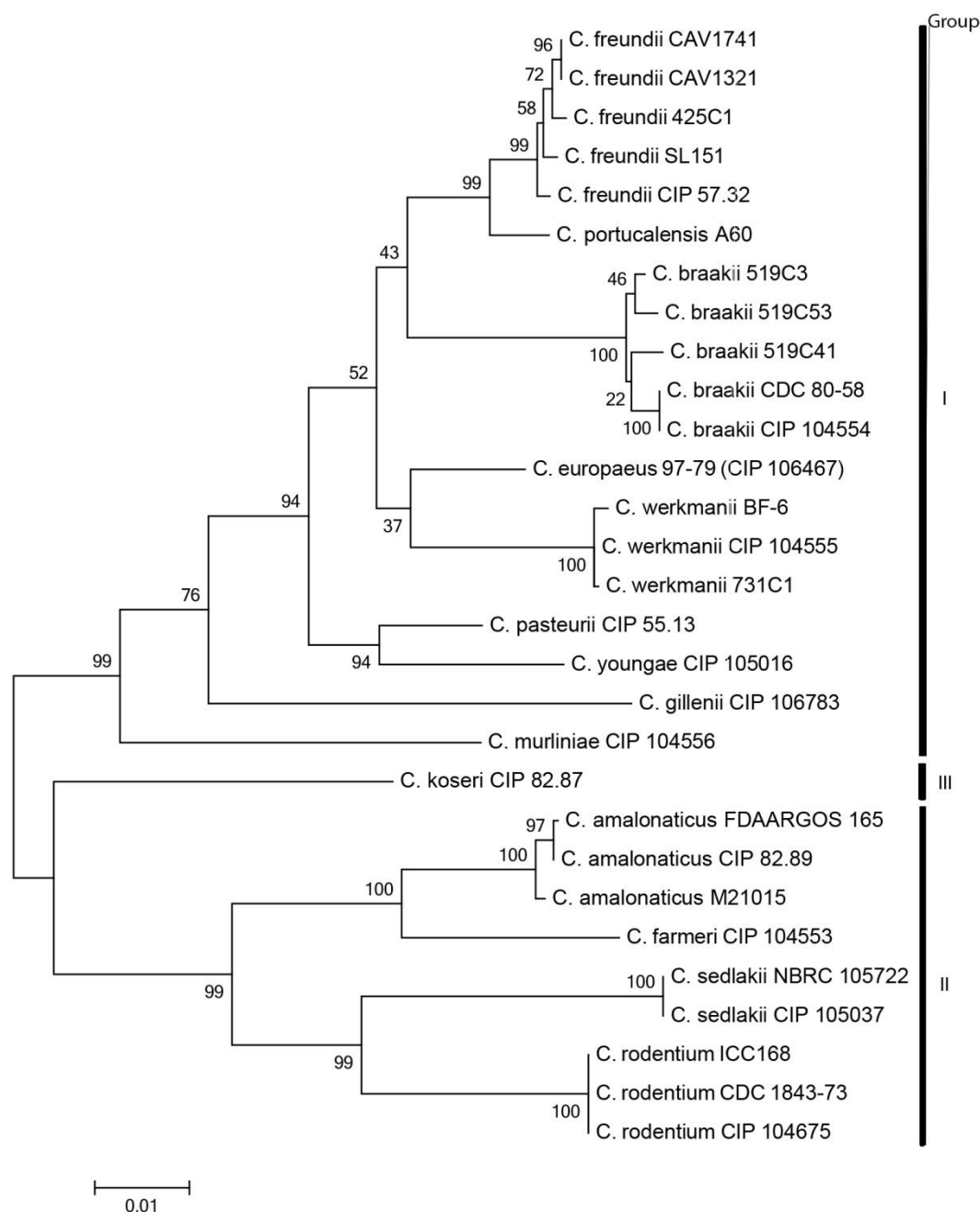
TRANSPARENCY DECLARATIONS

Nothing to declare.

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Figure 1. Phylogenetic analysis by maximum likelihood method of *Citrobacter* species.

FOOTNOTE. 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).

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

Antibiotic affected	Resistance gene	Identity (%)	GenBank Accession no.
Polymyxins	<i>mcr-1.5</i>	100	KP347127
β-Lactams	<i>bla_{NDM-1}</i>	100	FN396876
	<i>bla_{CMY-6}</i>	100	AJ011293
	<i>bla_{TEM-1B}</i>	100	JF910132
	<i>bla_{SED-1}</i>	82.64	AF321608
Aminoglycosides	<i>rmtC</i>	100	AB194779
	<i>aadA1</i>	100	JQ414041
	<i>aadA2</i>	100	JQ364967
	<i>aacA4</i>	100	KM278199
	<i>aph(3')-Ia</i>	100	V00359
Phenicol	<i>floR</i>	98.11	AF118107
	<i>cmIA1</i>	99.92	M64556
Sulphonamide	<i>sul1</i>	100	CP002151
	<i>sul2</i>	100	GQ421466
	<i>sul3</i>	100	AJ459418
Trimethoprim	<i>dfrA12</i>	100	AB571791

Highlights.

1. Multidrug Resistant *Citrobacter amalonaticus* Clinical Isolate
2. *C. amalonaticus* harbouring $bla_{\text{NDM-1}}$ and *mcr-1.5* genes
3. Specie identification was performed using MALDI-TOF MS and confirmed by sequencing

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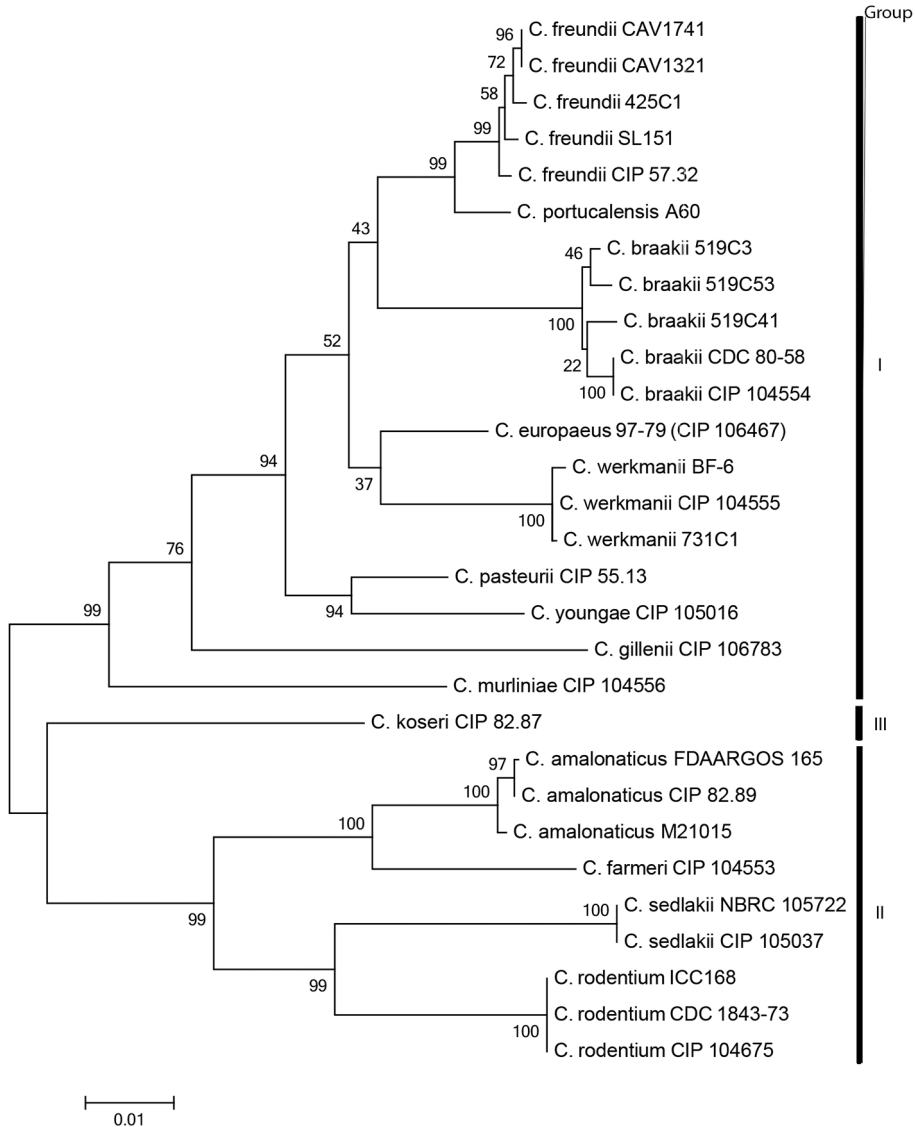


Figure 1