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MCR-1 and MCR-1.5 Producing *Escherichia coli* Clinical Isolates from Argentina

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Abstract

Due to the paucity of remaining antibiotics for treating infections caused by carbapenem-resistant *Enterobacteriaceae*, polymyxins have become the last resort antibiotics. As a consequence, colistin resistance is increasingly reported worldwide. The aim of this study was to analyze colistin-resistant *E. coli* clinical isolates, recovered between 2014 and 2016 at the University Hospital of Buenos Aires, Argentina. Nine clinical colistin resistant *E. coli* isolates were studied. These isolates were recovered from urine samples of 5 inpatients and 4 outpatients. Whole genome sequencing was performed using Illumina technology. Plasmid characterization and mating-out assay was done using *E. coli* J53 as receptor strain. Antibiotic susceptibility (MIC) of clinical isolates and their transconjugants was determined using broth microdilution method. WGS analysis revealed the presence of mcr-1 gene in six out of the 9 isolates: 4 isolates carried mcr-1 and 2 carried mcr-1.5 alleles. All the clinical isolates had MIC values for colistin in the range of 4-16 mg/L. The three isolates lacking any mcr variant, presented point mutations in the chromosomal pmrA or pmrB genes. The mcr-1 gene were located on plasmids similar to the prototypical Incl2-type (KY471308, pMCR-M15049) differing only by little deletions. Until this date mcr-1.5 allele was reported once in Argentina and in Japan, suggested a transcontinental dissemination of this variant.

Keywords: Colistin; *Enterobacteriaceae*; Plasmid-encoded; Resistance

Introduction

Colistin has become one of the last antibacterials to retain activity against Carbapenem-Resistant Gram-Negative Bacilli (CR-GNB) [1]. As a consequence, increased use of colistin has led to colistin resistance in GNB by modifications of the lipopolysaccharide [2]. These modifications consist in addition(s) of cationic groups such as 4-Amino-L-arabinose (L-Ara4N) and/or Phosphoethanolamine (pETN), to the lipid A of the outer-membrane of the GNB, which results in electrostatic repulsion between the added cationic group and the polymyxin. These modifications of the lipid A may be due to mutations in chromosome-encoded genes of the two-component systems PhoP/PhoQ or PmrA/PmrB, or of their regulator MgrB, which results in high MICs of colistin (8 to >32 mg/L) associated to a high-fitness cost for the bacteria [2,3].

The first plasmid-encoded resistance to polymyxin, named MCR-1, has been described late 2015 [4]. MCR-1 catalyzes the addition of a pETN to lipid A resulting in MICs of colistin

ranging from 2 to 8 mg/L [2]. Mcr-1 genes have now been reported worldwide in *Enterobacteriaceae* (mostly *Escherichia coli*), recovered from human and animal samples [2,5]. On top of its ability to be transferred between *enterobacterial* species, recent reports indicate its very low (or lack) fitness cost for the bacteria fearing a rapid dissemination of this mechanism [6]. Currently, nine families of *mcr* genes have been assigned and seven reported in Enterobacteriaceae [7,8]. They form a very heterogeneous family of enzymes sharing only 29-82% amino-acid sequence identity with MCR-1. *Mcr*-1 gene and its variants (*mcr*-1.2 to *mcr*-1.7) are the most prevalent *mcr*-genes found in human enterobacterial isolates and are located on plasmids (pMCRs) belonging to replicon types IncI2, IncHI2 and IncX4 [2,5,9]. MCR-1-producing *E. coli* isolates have rarely been reported from the Americas [10,11]. Very recently mcr-1.5 variant was reported in Argentina and in Japan [10,12]. The aim of our study was to characterize the molecular determinant of colistin-resistance in nine *E. coli* isolates recovered from a University Hospital in Buenos Aires, Argentina. Six of them were MCR-1- or MCR-1.5-producing *E. coli* isolates. We also characterized their plasmidic location and compared them with the previously described IncL2 *mcr*-harboring plasmid from Argentina [10].

Materials and Methods

Bacterial Strains

E. coli 6383, 2336, 1724, 1670, 979, 789, 4070, 94427 and 4222 clinical isolates were identified using Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry (MALDI Biotyper CA system, Bruker Daltonics, Billerica, MA, USA). Azide-resistant *E. coli* J53 was used for conjugation assays.

Antimicrobial Agents, Susceptibility Testing and Microbiological Techniques

The susceptibility of the nine *E. coli* isolates was determined by BD Phoenix™ 100 ID/AST system (Becton Dickinson, Sparks Glencoe, MD, USA) using the Phoenix NMC-406 panel according to the manufacturer's recommendations. At the same time, the nine isolates were evaluated for colistin resistance by the In-house colorimetric Andrade Screening Antimicrobial Test (ASAT) [13]. Colistin Minimal Inhibitory Concentration (MIC) values were determined using Sensititre® broth microdilution method (Thermo Scientific, Villebon sur Yvette, France) and interpreted according to the EUCAST breakpoints, updated in 2016 (http://www.eucast.org). MALDI-TOF based technology, MALDIxin test (Bruker Daltonics, Billerica, MA, USA) was performed following the published recommendations, as previously reported [14].

PCR

Whole-cell DNAs of all the different *E. coli* clinical isolates were used as a template for PCR using *mcr*-1 specific primers, CLR5F 5'CGGTCAGTCCGTTTGTTC 3' and CLR5R 5' CTTGGTCGGTCTGTAGGG 3' [4].

Whole Genome Sequencing (WGS)

Colistin-resistant *E. coli* isolates were sequenced using Illumina technology as previously described [15], in order to determine their resistome and Multi Locus Sequence Type (MLST), by uploading assembled genomes using CLC Genomics Workbench software (CLC bio, Qiagen, Les Ulis, France) to the Resfinder v3.1 (https://cge.cbs.dtu.dk/services/ResFinder/) and MLST v2.0 (https://cge.cbs.dtu.dk/services/MLST/), respectively [16,17].

For the reconstruction of *mcr*-1- and *mcr*-1.5-carrying plasmids the obtained contigs were mapped against KY471308 (pMCR-M15049) [10], KY471309 (pMCR-M15224) [10] using CLC Genomics Workbench software (CLC bio, Qiagen). Gaps were filled by PCR amplification and Sanger sequencing. Open reading frames (ORFs) were annotated using the RAST server (rast.nmpdr.org) followed by manual comparative curation and determination of sequence similarity using the BLAST web server. Alignments with other IncI2 pMCRs were performed by using the BRIG tool [18].

Plasmid Characterization and Mating-Out Assay

Plasmid DNA from the different clinical isolates were extracted using the Kieser method [19]. To perform the filter mating-out assay, *E. coli* 6383, 1724, 1670, 979, 4070 and 4222 clinical isolates used as donors and recipient *E. coli* J53 were each grown overnight in BHI (brain heart infusion) broth supplemented with colistin (2 mg/L) for plasmid maintenance in donor cells. 0.25-ml donor culture was mixed with 4.75 ml BHI broth and incubated at 37°C for 5 h without shaking. Recipient cultures of *E. coli* J53 grown overnight were diluted 1:50 in BHI broth and incubated at 37°C for 5 h without shaking. After incubation, 200 µl of the donor culture was gently mixed with 800 µl of the recipient culture, and 200 µl of this mating mix was filtered through a 0.45-µm filter (Millipore). Filters were incubated on prewarmed plates at 37°C for 3 h. Mating assays were ended by placing the filters into 4ml of an ice-cold 0.9% NaCl solution, followed by vigorous agitation for 30s. Transconjugants were selected on Mueller-Hinton agar supplemented with colistin (2 mg/L) and azide (100 mg/ L). From the transconjugants harboring *mcr*-1 and *mcr*-1.5 genes, plasmid DNA was extracted using Kieser method and subsequently analyzed on a 0.7% agarose gel stained with ethidium bromide.

Nucleotide Sequence Accession Number

The nucleotide sequence of *mcr*-1.5 gene has been submitted to the EMBL/Genbank nucleotide sequence database under the accession number KY271416.1. The sequences of the plasmids reported here have been deposited in GenBank under accession MG594798 (p6383), MG594799 (p4222), MG594800 (p1724),

MG598814 (p1670), MG598815 (p4070) and MG598816 (p979). The Whole Genome Shotguns have been deposited at DDBJ/ENA/GenBank under the accession PIJR00000000 (6383), PIIZ00000000 (1670), PIJA00000000 (4070), PIJB00000000 (979), PIJY00000000 (4222) and PIJS00000000 (1724).

Results and Discussion

Escherichia coli Isolates

9 colistin resistant *E. coli* isolates were recovered at a University Hospital in Buenos Aires, Argentina, between 2014 to 2016 from urine samples of 6 hospitalized- and 3 external-patients. All of them were resistant according to Phoenix system and were positive for the ASAT test [13], indicating that this 9 isolates could growth in the presence of colistin. They displayed MIC values for colistin ranging from 4 mg/L to 16 mg/L (Table 1). PCR using *mcr*-1-like gene specific primers, revealed that six isolates were positive for *mcr*-1-like genes. Moreover, all the strains were analyzed by MALDIxin test giving a positive result for colistin resistance. Six of the isolates were positive for plasmid encoded resistance mechanism [14].

Strain	Patient	MIC colistin (mg/L)	WGS #[1]	MLST	Resistance genes for								
					Aminoglycoside	β-lactams	Sulphonamide	Tetracycline	Trimethoprim	Quinolone	Phenicol	Macrolide	Polymyxin
979	External	4	PIJB00000000	ST-410	aadA1	$bla_{CTX-M-2}$	*sul*1	*tet*(A)	-	-	*cat*A1	-	*mcr*-1
1724	External	4	PIJS00000000	ST-2722	-	-	-	-	-	*qnr*B19	-	-	*mcr*-1
4070	External	4	PIJA00000000	ST-744	aph(3')-Ia strA, strB, aadA5	bla_{TEM-1B}	-	-	-	-	-	*mph*(A)	*mcr*-1
4222	Hospitalized	4	PIJY00000000	ST-101	aadA1, aadB	$bla_{CTX-M-2}$	*sul*1	*tet*(A)	*drf*A1	-	-	-	*mcr*-1
6383*	Hospitalized	4	PIJR00000000	ST-602	aac(3)-IId, aadA1, strB, strA	bla_{TEM-1B}	*sul*1, *sul*2	*tet*(A)	*dfr*A1	-	-	-	*mcr*-1.5
1670	Hospitalized	4	PIIZ00000000	ST-602	aadA1, aadA2, aadB	$bla_{CTX-M-2}$	*sul*1	*tet*(A)	*dfr*A12	-	-	*mph*(A)	*mcr*-1.5
789	Hospitalized	16	RAHE00000000	ST-1193	aph(3'')-Ib aph(6)-Id	bla_{TEM-1B}	-	-	-	-	-	*mph*(A) *mdf*(A)	-
2336*	Hospitalized	16	RAHF00000000	ST-345	aadA2 aph(6)-Id aph(3'')-Ib	bla_{TEM-1B} bla_{KPC-2}	-	-	-	-	-	*mdf*(A)	-
94427	Hospitalized	16	RAHD00000000	ST-131	aac(3)-IIa	$bla_{CTX-M-15}$	*sul*1	*tet* (A)	*dfr* A17	-	-	*mdf*(A)	-

*isolates recovered in 2014. The remaining isolates were from 2016.
[1] DDBJ/ENA/GenBank accession numbers of Whole Genome Shotgun Sequences.

Table 1: Characteristics of colistin-resistant *E. coli* clinical isolates.

Genomic Analysis

The genome of the 9 *E. coli* isolates was analyzed by searching for acquired resistance genes and for point mutations involved in resistance. Only contigs bigger than 300-bp were retained for further analysis. All the *E. coli* isolates presented with the exception of *E. coli* 1724, resistance genes to β-lactams (bla_{TEM-1} or $bla_{CTX-M-2}$) and aminoglycosides (Table 1). Four of the six isolates *mcr*-1 positive presented genes that conferred resistance to tetracyclines and sulphonamides. In *E. coli* 1724 only *qnr*B19, a plasmid-encoded quinolone resistance gene was found. An *mcr*-1 allele was identified in *E. coli* isolates 4070, 979, 4222 and 1724 (Table 1). In isolates 6383 and 1670 a novel *mcr*-1-variant was identified that presents a single nucleotide substitution (C1354T) resulting in an amino acid change of H452Y. Upon submission to GenBank nucleotide sequence database under the accession number KY271416.1 it was assigned as *mcr*-1.5 variant.

In the isolates negative for any known *mcr*-gene, point mutations in chromosomal genes involved in colistin resistance mechanisms were found. Isolate 2336 harbored a mutated *pmr*A gene that led to a G53W substitution, which has not yet been reported in *pmr*A gene of *E. coli.* However, mutations at position 53 of PmrA in *K. pneumoniae* (G53C and G53S) and in *Salmonella enterica* (G53E, G53R) have been shown to be responsible for colistin resistance [20,21]. The isolate 789 harbored a mutated *pmr*B gene that led to a P94L substitution, which has not been described in *E. coli* but a substitution P94Q has already been identified in *S. enterica* [21]. The isolate 94427 harbored a mutation in *pmrB* gene that resulted in a substitution V88E in PmrB that has never been described so far in colistin resistance. However, mutations close by (L82R, S85R) have already been incriminated in colistin resistance, thus, suggesting the importance of this region in the functionality of PmrB [20,22].

The MLST analysis revealed that the clinical *E. coli* isolates belonged to different sequence types (ST), except for 6383 and 1670 isolates that belonged to the same ST (ST-602) (Table 1). According to *E. coli* MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli), colistin-resistant *E. coli* of ST-744 and ST-101, have been reported in South America (Colombia and Brazil), from human, poultry or livestock samples, but not from Argentina. ST-602, was reported once from a companion animal in Brazil, and ST-2722 from reptiles in Australia and from poultry in Denmark, but never from human samples or from South America. *E. coli* 979 belongs to ST-410, a hyper epidemic clone and founder of the widely disseminated clonal complex 23 (CC23) [23]. There is evidence that *E. coli* ST-410 has been successful for interspecies transmission between wild animals, food-producing animals, companion animals, humans, and the environment, increasing the risk of becoming a successful pandemic clone [24]. ST-410 carrying *mcr*-1 gene and bla_{CTX-M} genes have been recovered from turkey meat in Germany [25] and from a human blood culture in Brazil [26].

Plasmid Analysis

Whole genome sequence analysis and mating-out assay results indicated that *mcr*-1 gene or its variant were inserted onto a IncI2 type plasmid of c.a 60 kb (Figure 1) and were closely related to pMCR-M15049 (KY471308) [10]. The transconjugants showed the same MIC values for colistin (4 mg/L) than the clinical strains, revealing the possibility of dissemination through the conjugative mechanism.

Comparative sequence analysis between the previously described pMCR-M15049 and the plasmids harbored by the six colistin resistant *E. coli*, revealed a conserved backbone length, responsible for its replication, maintenance, and transfer, and similar overall genetic arrangements (Figure 2). The comparison of the immediate genetic environment of *mcr*-1.5 genes showed that *mcr*-1.5 and *pap2* genes, were bracketed by two copies of ISApI1, likely forming a composite transposon, Tn6330, in plasmids p6383 and p1670 as in pMCR-M15049 [10], while in mcr-1 gene environment IS*ApI1* were absent (Figure 2). It has been proposed that the presence or absence of this IS correlates with the adaptation of *mcr*-1 to a new host. A composite transposon indicates a recent acquisition of this marker whereas the absence of IS*Apl1* or the presence of a single copy suggests that it has been already adapted [27]. Nevertheless, a recent report demonstrated that the composite transposon Tn*6330*, can translocate to other plasmids [28], which notoriously increases, the possibility of dissemination to plasmids from other incompatibility groups. *Mcr* genes are commonly located in plasmid of the incompatibility groups IncI2, HI1, HI2, P, X1/X2, X3/X4, X4, FI, FIP and FII, which are self-transferable plasmids responsible for inter-species dissemination worldwide [27] In south America only a few reports identified the *mcr*-1 carried plasmids. In these, plasmids belong to the incompatibility groups X4 and I2 carry and an initial analysis of the plasmid backbone suggests that these elements are highly similar to those reported in China, Europe or North America [27]. The plasmid sequence comparison of the p6383, p1670 and pMTY17668-MCR1.5 (AP018110) which harbored the only reported *mcr*-1.5 in Japan [12], showed a conserved backbone, with only *mcr*-1.5 as resistance gene, indicating that this plasmid is disseminated also in Japan.

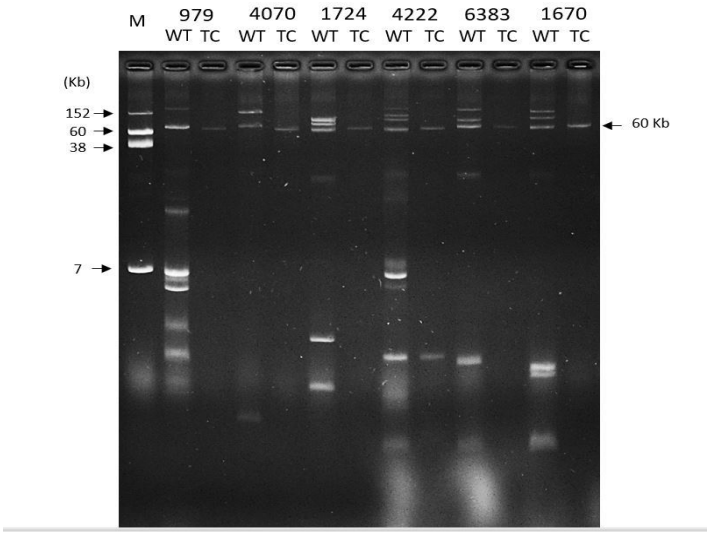

Figure 1: Plasmid extractions and analysis by Kieser method of the MCR-1-producing isolates [19].

WT: *E. coli* clinical isolates and; TC: the respective colistin resistant *E. coli* J53 transconjugants carrying pMCR plasmid. M: marker.

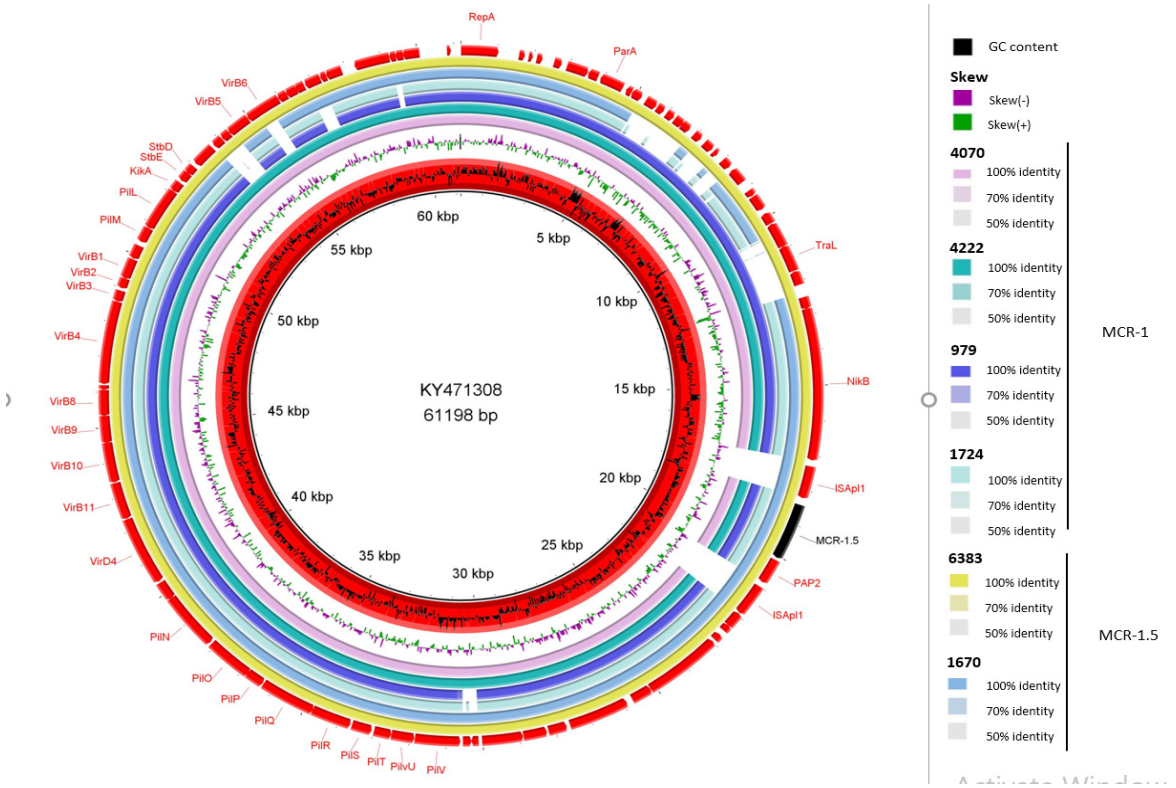

Figure 2: Sequence alignment of IncI2-type *mcr*-carrying plasmids. pMCR-M15049 (KY471308) was used as a reference to align and compare plasmids obtained from the clinical *E. coli* isolates of this study. The outer circle with red arrows indicates annotations from the reference sequence. Gaps in the inner circles are missing regions when compared with the reference. The sequences of the plasmids reported here have been deposited in GenBank under accession MG594798 (p6383), MG594799 (p4222), MG594800 (p1724), MG598814 (p1670), MG598815 (p4070) and MG598816 (p979).

Conclusions

We have characterized 4 MCR-1 and 2 MCR-1.5 producing *E. coli* isolates recovered from a University hospital in Argentina. These isolates were not clonally-related and belonged to hospitalized or external patients. All the *mcr*-carrying plasmids belonged to the IncI2 incompatibility group, demonstrating the role of this plasmid-type in the dissemination of the *mcr* genes worldwide. Carbapenem-resistance rates among Enterobacteriaceae are very high in Argentina and is mainly due to KPC-2 dissemination [29]. Therefore, the use of colistin has drastically increased, being in many instances the last active therapeutic option. As a consequence, carbapenem-resistant bacteria that become colistin-resistant may rise, as exemplified by isolate 2336, that became colistin-resistant due to a chromosomal mutation in PmrA. The finding that plasmid-encoded colistin resistance is now also spreading in Argentinian hospitals and in the community, portrays a very scary scenario.

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Transparency Declaration

None to declare

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