



# IncX4 Plasmid Carrying the New *mcr-1.9* Gene Variant in a CTX-M-8-Producing *Escherichia coli* Isolate Recovered From Swine

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We studied a commensal colistin-resistant *Escherichia coli* isolated from a swine cecum sample collected at a slaughter, in Portugal. Antimicrobial susceptibility phenotype of *E. coli* LV23529 showed resistance to colistin at a minimum inhibitory concentration of 4 mg/L. Whole genome of *E. coli* LV23529 was sequenced using a MiSeq system and the assembled contigs were analyzed for the presence of antibiotic resistance and plasmid replicon types using bioinformatics tools. We report a novel *mcr-1* gene variant (*mcr-1.9*), carried by an IncX4 plasmid, where one-point mutation at nucleotide T1238C leads to Val413Ala substitution. The *mcr-1.9* genetic context was characterized by an IS26 element upstream of the *mcr-pap2* element and by the absence of IS*Ap11*. Bioinformatic analysis also revealed genes conferring resistance to  $\beta$ -lactams, sulphamethoxazole, trimethoprim, chloramphenicol and colistin, corresponding to the phenotype noticed. Moreover, we highlight the presence of *mcr-1.9* plus *bla*<sub>CTX-M-8</sub>, a *bla*<sub>ESBL</sub> gene rarely detected in Europe in isolates of animal origin; these two genes were located on different plasmids with 33,303 and 89,458 bp, respectively. MCR-1.9-harboring plasmid showed high identity to other X4-type *mcr-1*-harboring plasmids characterized worldwide, which strongly suggests that the presence of PMCR-encoding genes in food-producing animals, such as MCR-1.9, represent a potential threat to humans, as it is located in mobile genetic elements that have the potential to spread horizontally.

**Keywords:** MCR-1.9, plasmid-mediated colistin resistance, IncX4, CTX-M-8, Portugal

## INTRODUCTION

Since the report of a plasmid-mediated colistin resistance (PMCR) mechanism, designated MCR-1, in *Escherichia coli* and *Klebsiella pneumoniae* isolated from animals, food and humans in China, further reports exposed the global dissemination of *mcr*-type gene in various bacterial species isolated from a wide range of different sources (Caniçaux et al., 2017). In Portugal, PMCR has also

been detected in a wide range of different sources and species, including humans, food-producing animals and meat, and in the environment (Campos et al., 2016; Figueiredo et al., 2016; Jones-Dias et al., 2016; Kieffer et al., 2017; Manageiro et al., 2017; Tacão et al., 2017; Mendes et al., 2018). Noteworthy, are the recent report of two cases presumably associated with the travel of patients from Portugal, one involving animals: a patient repatriated to France after hospitalization for 2 months in Portugal, in 2015 (Beyrouthy et al., 2017), and a New York state patient returning from Portugal in 2016 after staying on a farm with chickens and pigs (Gilrane et al., 2017).

More worrisome is the presence of *mcr* genes in *Enterobacteriaceae* carrying other resistance determinants namely, extended-spectrum  $\beta$ -lactamases (ESBL)- and/or carbapenemase-encoding genes. Since the first report of co-localization of *mcr-1* and ESBL- in 2016 in bovines in France, an increase encoding genes in the proportion of *mcr-1* genes among ESBL-producing *E. coli* in animals has been noticed, suggesting that the use of extended-spectrum cephalosporins may have simultaneously favored the spread of *mcr-1* (Haenni et al., 2016). Here we describe the first detection of a novel *mcr* variant, hereafter-named *mcr-1.9*, identified in a commensal *E. coli* LV23529 isolated from a swine cecum sample collected at a slaughter, in Portugal.

## MATERIALS AND METHODS

### Bacterial Isolate

*Escherichia coli* LV23529 was isolated in 2015 from a swine cecum sample collected at a Portuguese slaughter, during an evaluation study of commensal *E. coli* recovered from swine samples for antimicrobial susceptibility testing.

### Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations (MICs) were determined by microdilution method as previously described (Manageiro et al., 2017). In order to assess decreased susceptibility of the strain, interpretation of the results was done according to the epidemiological cut-off values recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST<sup>1</sup>).

### Screening and Characterization of PMCR- and ESBL-Resistance Mechanisms

#### Molecular Detection of *mcr-1* and *bla*<sub>ESBL</sub>-Encoding Genes

Following phenotypic characteristics, PMCR- and ESBL-resistance mechanisms were searched and identified by molecular methods, as previously described (Manageiro et al., 2017).

#### Transfer Experiments

Conjugation experiments were performed using sodium azide-resistant *E. coli* J53 as a recipient strain. Transconjugants

were selected on McConkey agar supplemented with sodium azide (150 mg/L) and either cefotaxime (2 mg/L) or colistin (2 mg/L). Plasmid DNA was extracted from *E. coli* LV23529 using a NucleoBond Xtra Plus kit (Macherey-Nagel), and transformed into *E. coli* TOP10 OneShot chemically competent cells (Invitrogen), accordingly to manufacturer's protocol. *E. coli* transformants were selected on MacConkey agar supplemented with 2 mg/L of colistin. PCR for *bla*<sub>CTX-M-8</sub> or *mcr-1*-type and MICs of recipients and transformants were determined as mentioned above.

### Genetic Context of *mcr-1.9* Gene

Colistin-resistant *E. coli* LV23529 was genotypically characterized by whole-genome sequencing (WGS), as previously described (Manageiro et al., 2017). Sequence reads were trimmed and filtered according to quality criteria, and de novo assembled into contigs by means of CLC Genomics Workbench 10.0 (Qiagen). The assembled contigs were analyzed and studied for the presence of antibiotic resistance, virulence genes and plasmid replicon types, serotype, multi-locus sequence type (ST) and *fim*-type, using bioinformatics tools<sup>2</sup>. The NCBI prokaryotic genome automatic annotation pipeline (PGAAP) was used for annotation.

Plasmid sequencing was also performed on a MiSeq Illumina platform using 150 bp paired-end reads, after plasmid DNA extraction from TLV23529 (*mcr-1.9*) using a NucleoBond Xtra Plus kit (Macherey-Nagel), and quantification using Qubit 1.0 Fluorometer (Invitrogen), as previously described (Manageiro et al., 2017). Sequence reads were trimmed and filtered according to quality criteria, and mapped against *E. coli* ATCC 25922 genome (NZ\_CP009073). Unmapped reads (80.2%/total reads) were then used for plasmids structure construction by mapping assembly based on the genetic organization of the closest plasmid sequences obtained by BLASTn; this was followed by contig neighbor's prediction from assembly information using CLC Genomics Workbench 10.0 (Qiagen). NCBI Microbial genomes BLAST analysis tool<sup>3</sup> was used to search for plasmid sequences. Plasmid alignments and ORF representations were also done using EasyFig v. 2.2.3 (Sullivan et al., 2011).

### Genomic Epidemiological Analysis

BacWGSTdb database was used for genotyping and source tracking bacterial pathogen (Ruan and Feng, 2016).

### Nucleotide Sequence Accession Number

The pLV23529-MCR-1.9 and pLV23529-CTX-M-8 nucleotide sequences from this study were submitted to the NCBI GenBank Database with accession numbers KY964067 and KY964068, respectively. The new *mcr-1.9* nucleotide sequence was submitted with accession number KY780959.

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession SBIH00000000. The version described in this paper is version SBIH01000000.

<sup>1</sup><http://mic.eucast.org/Eucast2/>

<sup>2</sup><https://cge.cbs.dtu.dk/services/>

<sup>3</sup><https://www.ncbi.nlm.nih.gov/genome/microbes/>

## RESULTS AND DISCUSSION

MIC results showed that LV23529 was non-wild-type to third- and fourth-generation cephalosporins (ceftazidime 2 mg/L, cefotaxime 32 mg/L, cefepime 8 mg/L) with synergy with clavulanic acid; this isolate was also non-wild-type to chloramphenicol (>128 mg/L), sulphamethoxazole (>1024 mg/L), trimethoprim (>32 mg/L), tetracycline (>64 mg/L), and colistin (4 mg/L). LV23529 remained wild-type to carbapenems, fluoroquinolones, aminoglycosides and tigecycline (**Table 1**).

Molecular characterization of the *E. coli* LV23529 isolate allowed the detection of *bla*<sub>CTX-M-8</sub> and *mcr-1*-type genes.

Only the transferability of the *bla*<sub>CTX-M-8</sub> gene was achieved by conjugation, with TcLV23529 (*bla*<sub>CTX-M-8</sub>) exhibiting the ESBL phenotype from LV23529 isolate (cefotaxime 2 mg/L, cefepime 2 mg/L) with synergy with clavulanic acid, and wild-type to colistin ( $\leq 1$  mg/L) (**Table 1**). Although conjugation assays for *mcr-1*-type were negative, the colistin resistance determinant could be transferred to *E. coli* TOP10 competent cells; transformant TLV23529 (*mcr-1*-type) showed the respective resistance to colistin (4 mg/L) (**Table 1**).

The WGS assembly of *E. coli* LV23529 yielded 193 contigs (average 143.7-fold coverage), which together comprised 5,122,415bp, showing a GC content of 50.7%. The largest contig was 320,931 bp long; the N50 statistic, which stands for the minimum contig length of at least 50% of the contigs, was 113,197 bp. The average length of the obtained contigs was 26,541 bp. Overall, the genome sequence comprised 5,124 putative genes, among which 5,037 consisted of protein encoding sequences.

The WGS analysis showed that *E. coli* LV23529 belongs to serotype O8:H19, usually associated with porcine *stx*<sub>2e</sub>-producing *E. coli* (Zweifel et al., 2006; Bai et al., 2015), and to MLST (Achtman scheme) ST201 [clonal complex 469 (CC469)] and to the FimH-type determinant *fimH32*. This ST201 was encountered worldwide mainly in isolates collected from livestock samples (*Escherichia/Shigella* Enterobase database, Alikhan et al., 2018). Three virulence factors were detected: *astA* (heat-stable enterotoxin 1), *lpfA* (long polar fimbriae), and *gad*-type (glutamate decarboxylase).

Further bioinformatics analysis of *E. coli* LV23529 isolate revealed acquired-genes conferring resistance to  $\beta$ -lactams (*bla*<sub>CTX-M-8</sub> and *bla*<sub>TEM-1</sub>), aminoglycosides (*aadA1* and *aadA2*),

**TABLE 1** | Phenotypic and genotypic context of CTX-M-8 and MCR-1.9 producing *E. coli* clinical isolate, transformant, transconjugant, and the respective recipient strains.

Antibiotic	LV23529 <sup>b</sup>	Transformation		Conjugation	
	<i>(bla</i> <sub>CTX-M-8</sub> , <i>mcr-1.9</i> , <i>cmlA1</i> , <i>sul3</i> , <i>tetA</i> , <i>tetM</i> , <i>dfrA12</i> , <i>aadA1</i> , <i>aadA2</i> )	<i>E. coli</i> TOP10 <sup>c</sup>	TLV23529 <sup>d</sup> ( <i>mcr-1.9</i> )	ECJ53AZNa <sup>e</sup>	TcLV23529 <sup>f</sup> ( <i>bla</i> <sub>CTX-M-8</sub> )
Ampicillin	>64	4	8	2	>64
Cefoxitine	4	4	4	4	8
Ceftazidime	2	0.5	0.5	$\leq 0.5$	1
Ceftazidime plus clavulanate <sup>a</sup>	$\leq 0.125/4$	0.5	0.5	$\leq 0.125/4$	$\leq 0.25/4$
Cefotaxime	32	$\leq 0.25$	$\leq 0.25$	$\leq 0.25$	2
Cefotaxime plus clavulanate <sup>a</sup>	$\leq 0.06/4$	0.125	0.125	$\leq 0.06/4$	$\leq 0.06/4$
Cefepime	8	0.125	0.125	$\leq 0.06$	2
Imipenem	0.25	0.5	0.5	0.25	0.25
Meropenem	$\leq 0.03$	0.06	0.06	$\leq 0.03$	$\leq 0.03$
Ertapenem	$\leq 0.015$	$\leq 0.015$	$\leq 0.015$	$\leq 0.015$	$\leq 0.015$
Nalidixic acid	$\leq 4$	$\leq 4$	$\leq 4$	$\leq 4$	$\leq 4$
Ciprofloxacin	$\leq 0.015$	$\leq 0.015$	$\leq 0.015$	$\leq 0.015$	$\leq 0.015$
Chloramphenicol	>128	$\leq 8$	$\leq 8$	$\leq 8$	$\leq 8$
Sulphamethoxazole	>1024	$\leq 8$	$\leq 8$	$\leq 8$	$\leq 8$
Tetracycline	>64	$\leq 2$	$\leq 2$	$\leq 2$	$\leq 2$
Trimethoprim	>32	$\leq 0.25$	$\leq 0.25$	$\leq 0.25$	$\leq 0.25$
Gentamicin	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$
Colistin	4	$\leq 1$	2	$\leq 1$	$\leq 1$
Tigecycline	$\leq 0.25$	$\leq 0.25$	$\leq 0.25$	$\leq 0.25$	$\leq 0.25$

MICs in mg/L.

<sup>a</sup>Clavulanate 4 mg/L.

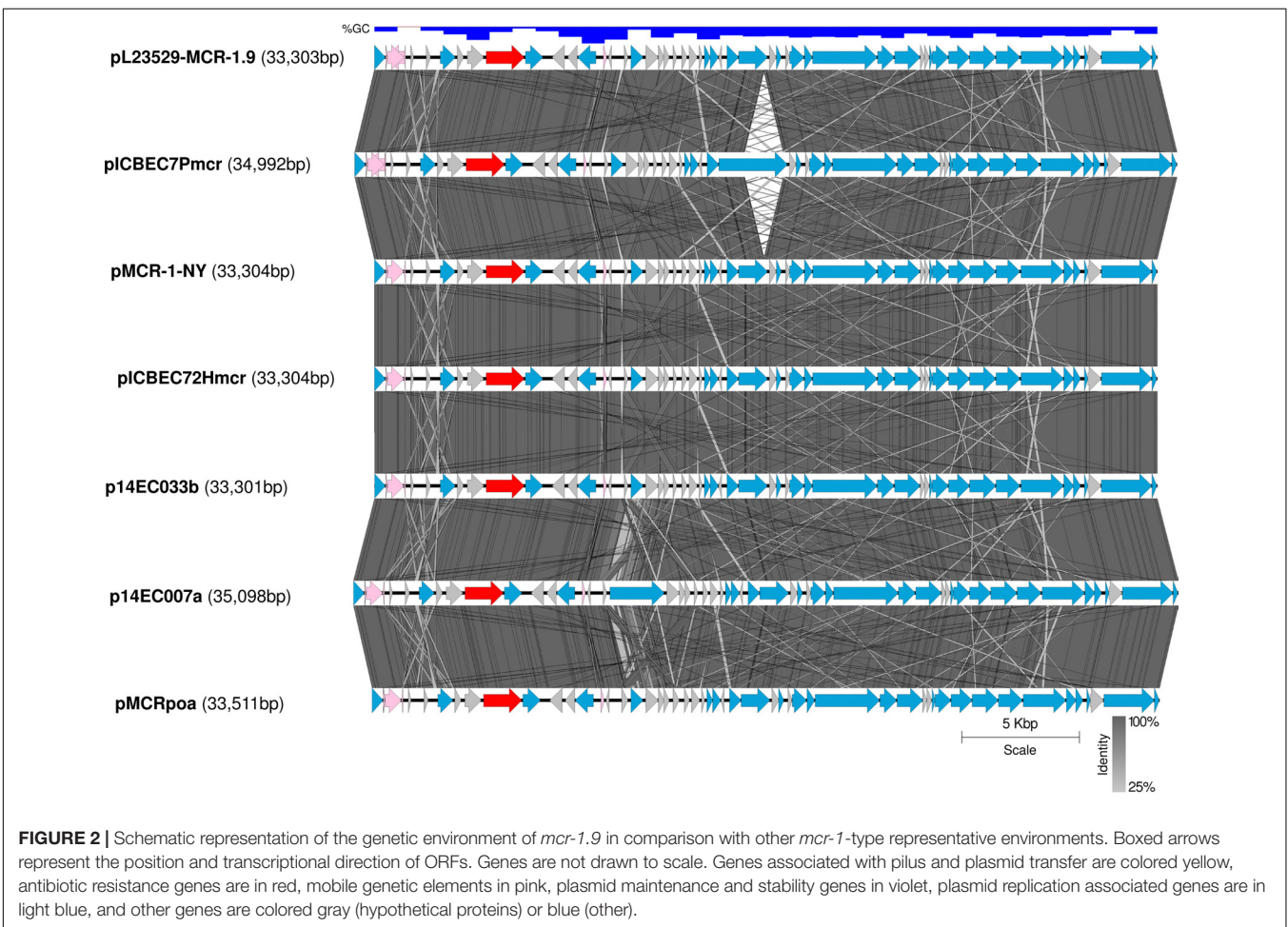
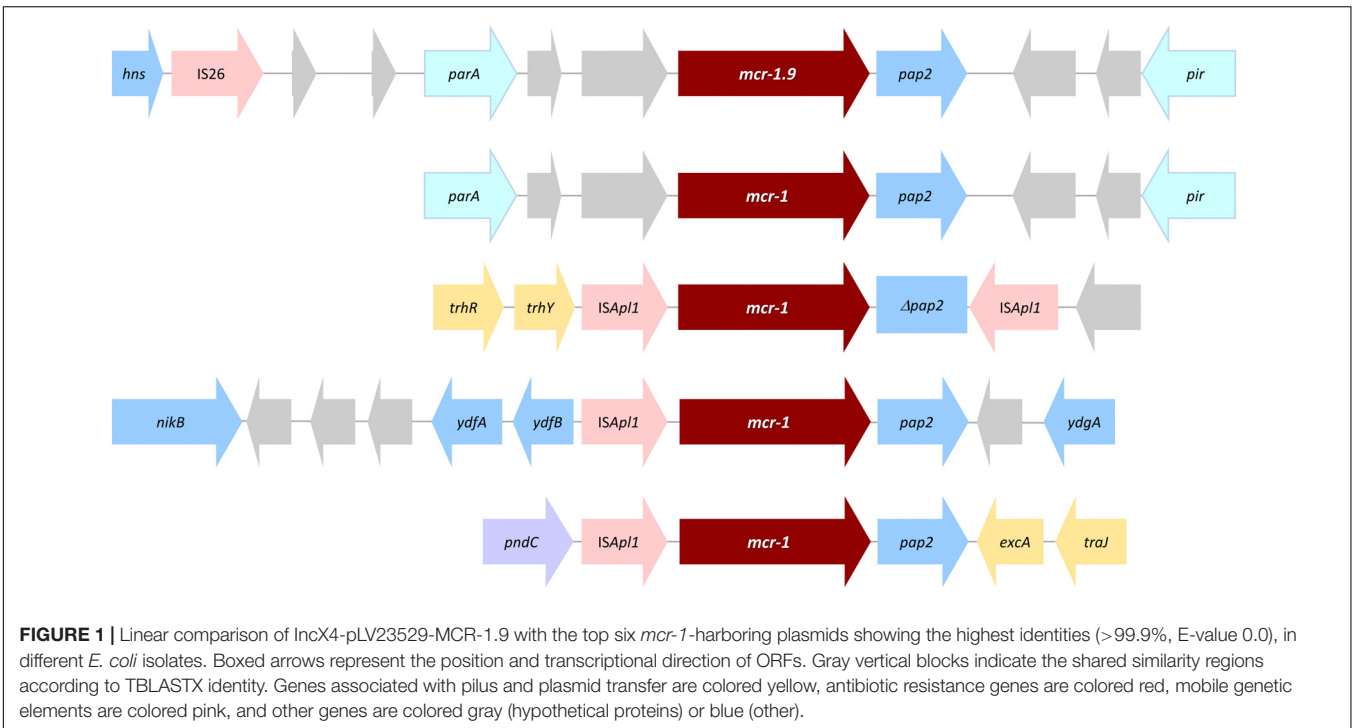
<sup>b</sup>*E. coli* LV23529 was the clinical isolate harboring the acquired antibiotic resistance genes *bla*<sub>CTX-M-8</sub>, *mcr-1.9*, *cmlA1*, *sul3*, *tetA*, *tetM*, *dfrA12*, *aadA1*, and *aadA2*.

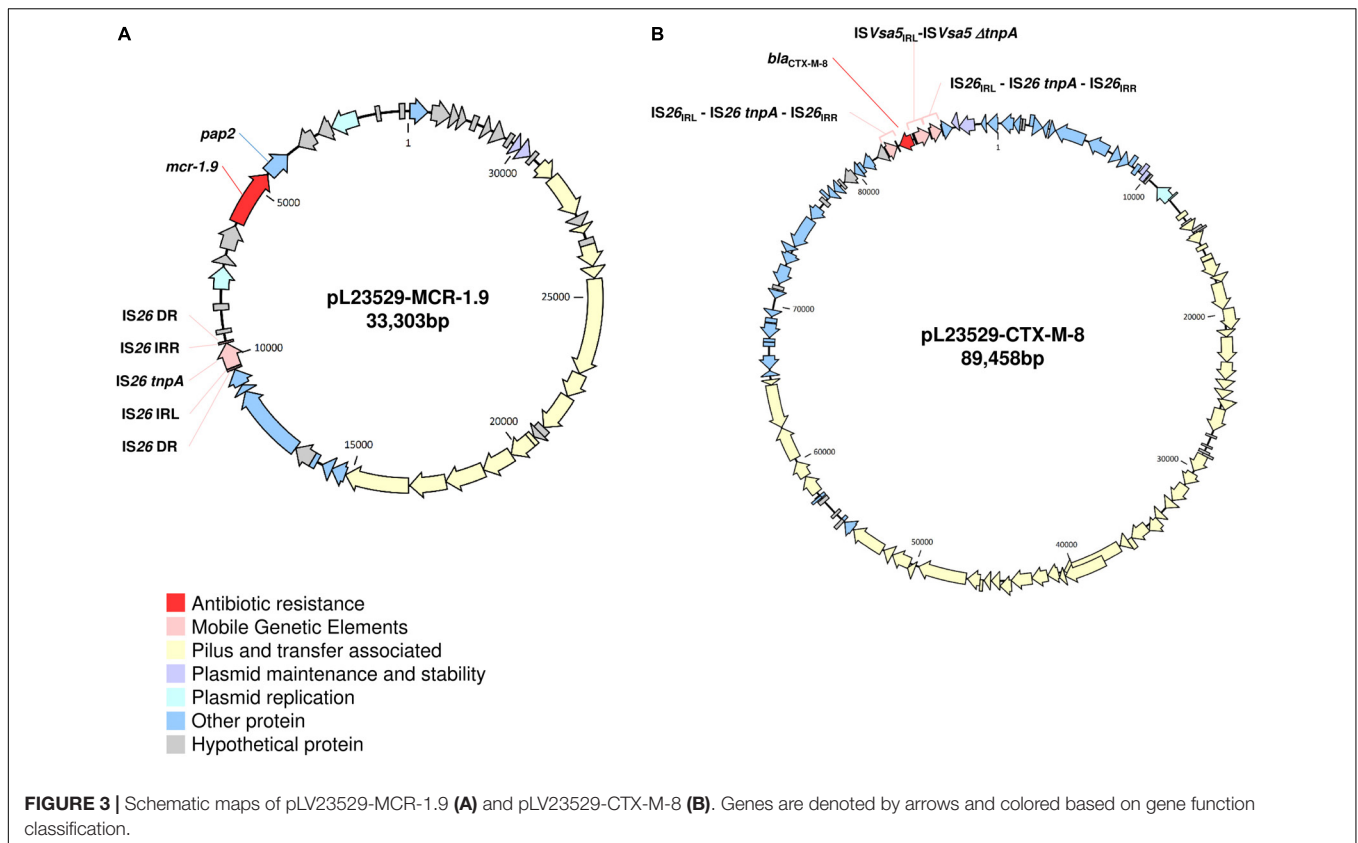
<sup>c</sup>*E. coli* TOP10 was the recipient strain in the transformation experiment.

<sup>d</sup>TLV23529 is a transformant of LV23529 (harboring *mcr-1.9*).

<sup>e</sup>*E. coli* J53AZNa was the recipient strain in the conjugation experiment.

<sup>f</sup>TcLV23529 is a transconjugant of LV23529 (harboring *bla*<sub>CTX-M-8</sub>).





**FIGURE 3 |** Schematic maps of pLV23529-MCR-1.9 (A) and pLV23529-CTX-M-8 (B). Genes are denoted by arrows and colored based on gene function classification.

**TABLE 2 |** Comparison of IncX4-pLV23529-MCR-1.9 with the top six *mcr-1*-harboring plasmids showing the highest identities (>99.9%, E-value 0.0), in different *E. coli* isolates.

IncX4-type Plasmid (bp)	<i>E. coli</i> strain (MLST <sup>a</sup> )	Source/Country/Year	Identity (%)	Mismatches/gap opens (No. of nucleotides)	Query alignment overlap (%)	pLV23529-MCR-1.9 Alignment overlap (%)	Plasmid GenBank Acc. No.
pMCRpoa (33,511)	3431F (ST744)	Human patient/Brazil/2014	99.98	15/1	99.4	100.0	CM007714
pl4EC007a (35,098)	14Ec007 (ST301)	Human patient/China/2014	99.98	15/1	94.9	100.0	CP024132
pMCR-1-NY (33,304)	MDR56 (ST117)	Human patient /United States/2015	99.97	19/1	100.0	100.0	CP019908
pICBEC72Hmcr (33,304)	ICBEC72H (ST101)	Human patient/Brazil/2016	99.95	17/0	100.0	100.0	CP015977
pl4EC033b (33,301)	14EC033 (ST2064)	Human patient/China/2014	99.92	16/3	100.0	100.0	CP024149
pICBEC7Pmcr (34,992)	ICBEC7P (ST10)	Magellanic penguins/Brazil/2013	99.92	16/3	95.2	100.0	CPO17246

<sup>a</sup>MLST accordingly with Warwick scheme (<http://mlst.warwick.ac.uk/mlst/dbs/Ecoli>).

phenicol (*cmlA1*-type and *floR*-type), sulphamethoxazole (*sul3*), tetracycline [*tet(A)*-type and *tet(M)*-type], trimethoprim (*dfrA12*), and colistin (*mcr-1*-type), justifying the phenotype noticed. Additionally, several unknown mutations in the *ampC* (promoter region), *parC*, 16S *rrsB*, 16S *rrsC*, 23S and *pmrB* chromosomal genes were detected, the last gene being described as the primary mechanism for the development of chromosomally encoded resistance to polymyxins (Phan et al., 2017).

The named *mcr-1.9*, differed from *mcr-1* by one-point mutation (T1238C), leading to Val413Ala substitution. The

MCR-1 protein contains a transmembrane domain and a phosphoethanolamine (PEA) transferase domain with 8 $\alpha$ , 12 $\beta$ , and 12 $\eta$  units (Gao et al., 2016). The amino acid substitution of MCR-1.9 occurred in the region between  $\eta$ 7 e  $\eta$ 8 of the PEA transferase domain, which have been found not to influence the function of MCR-1 (Gao et al., 2016).

The *mcr-1.9* genetic context was characterized by an IS26 element upstream of the *mcr-pap2* element and by the absence of IS*AplI* (Figure 1), which is in accordance with other studies about *mcr-1* gene (Veldman et al., 2016; Sun et al., 2017). The *mcr-1.9* gene can be mobilized within an IS*AplI*-flanked

composite transposon (Tn6330), although many sequences have been identified without IS*Apl1* or with just a single copy (Snesrud et al., 2018). Indeed, it has been described that initially IS*Apl1* was presumably involved in the transposition of the *mcr-1* cassette and then was lost, contributing for the stability of *mcr* gene on IncX4 plasmids (Sun et al., 2017; Snesrud et al., 2018).

The PMCR-encoding gene was found in an IncX4 plasmid (pLV23529-MCR-1.9), showing highest identities (>99.9%) with six IncX4-type *mcr-1*-harboring plasmids identified worldwide, in unrelated *E. coli* isolates, mainly collected from human patients (Figure 2 and Table 2). Indeed, all belonged to different MLST, which might suggest a resistance plasmid dissemination across strains (plasmid outbreak) rather than clonal transmission of MCR-1-type-producing strains. Furthermore, no *E. coli* LV23529 closely related isolates were detected among those currently deposited in the public database BacWGSTdb (Ruan and Feng, 2016), which reinforce the importance of the horizontal gene transfer in this study.

Like pLV23529-MCR-1.9, the six plasmids (Table 2) doesn't have the IS*Apl1* element. Hence, similarities may suggest that the one-point mutation (T1238C) in *mcr-1.9* occurred on the X4 plasmid, since mobilization of *mcr-1* occurs as part of a composite transposon (Tn6330) and that structures lacking the downstream IS*Apl1* are not capable of mobilization (Snesrud et al., 2018). The IS26 upstream of the *mcr-pap2* element is flanked by an 8bp direct repeat (Figure 3A), indicating that its insertion wouldn't seem to be related to the *mcr-1.9* context, justifying the differences found with other IncX4 *mcr-1*-harboring plasmids. IncX4 plasmid has been widely implicated in the spread of MCR-1 gene in Europe (Caniaux et al., 2017). In Portugal, this plasmid type is circulating among diverse hosts (humans, pigs, poultry), being responsible for hospital-based outbreak caused by MCR-1 plus KPC-3-producing *K. pneumoniae* (Mendes et al., 2018), as well as for the diffusion of this PMCR at the farm level (Kieffer et al., 2017). Indeed, IncX4 plasmids seem to be efficiently transferred at different temperatures and different lack-of-fitness burdens among bacterial hosts, which may facilitate the transfer of *mcr*-type among *Enterobacteriaceae* (Lo et al., 2014; Wu R. et al., 2018). The pLV23529-MCR-1.9 plasmid backbone contains all the core genes common to IncX plasmids involved in segregation, stability, replication, and conjugative transfer of the plasmid (Figure 3A), namely the IncX-type pilus synthesis operon (*pilX1-pilX11*). However, pLV23529-MCR-1.9 was mobilizable, but not self-transmissible. Of note, we found a

one-point mutation (G64T), leading to Asp22Tyr substitution, in the PilX1, a peptidoglycan hydrolase involved in T-DNA plasmid transfer. This mutation might explain why the attempts to conjugate *mcr-1.9* from *E. coli* LV23529 were unsuccessful (Chen et al., 2009).

Further plasmid analysis revealed the presence of two other plasmids: IncF [F2:A-B-], IncR and the colicinogenic Inc11-ST113-carrying the *bla*<sub>CTX-M-8</sub> (pLV23529-CTX-M-8, Figure 3B). Of note, the *mcr-1.9*-positive isolate, co-harboring *bla*<sub>CTX-M-8</sub> and *bla*<sub>TEM-1</sub> genes, is here reported for the first time in an *E. coli* isolate of animal origin. In fact, *bla*<sub>CTX-M-8</sub> gene is rarely detected in Europe in isolates of animal origin (Börjesson et al., 2016), but in humans seems to be emerging (Eller et al., 2014). Indeed, a recent phylogenetic study suggested an increasing trend of co-existence and transmission of *bla*<sub>CTX-M</sub> and *mcr-1* in both clinical medicine and veterinary medicine (Wu C. et al., 2018).

In conclusion, the presence of PMCR-encoding genes, such as MCR-1.9, in food-producing animals represents a potential threat to humans, as it is located in mobile genetic elements that have the potential to spread horizontally. As mentioned, in Portugal, PMCR is an emerging problem and its international spread is a worrying reality (Beyrouthy et al., 2017; Gilrane et al., 2017).

## AUTHOR CONTRIBUTIONS

VM designed the study, performed the molecular experiments and bioinformatics analysis, interpreted the data, and wrote the manuscript. LC, RR, and EF performed the microbiological and molecular experiments. CS and LV performed the Illumina genome sequencing experiments. MC designed the study, wrote, reviewed and edited the manuscript. All authors read and approved the final manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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