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1 **Genomic characterisation of an *mcr-1* and *mcr-3*-producing *Escherichia coli* strain**  
2 **isolated from pigs in France**

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4 Running title: ***mcr-1/mcr-3* in *Escherichia coli* from pigs, in France**

5

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14

15 **Keywords:** *mcr* genes, *mcr-1*, *mcr-3*, colistin resistance, whole-genome sequencing.

16 **Abstract**

17 **Introduction:** Colistin is considered last resort antibiotic against carbapenem-resistant  
18 isolates. Nowadays, this antibiotic is facing the emergence of mobilised colistin resistance  
19 (*mcr*) genes which confer colistin resistance.

20 **Objective:** Genomic characterisation of an atypical multidrug-resistant *Escherichia coli*  
21 harbouring two *mcr* genes in France. Samples collected from a pig farm in Avignon  
22 (Vaucluse department) were subjected to molecular screening targeting *mcr* variants.

23 **Materials and methods:** Samples were cultured on selective Lucie-Bardet-Jean-Marc-Rolain  
24 medium (LBJMR). Growing bacteria were identified using MALDI-TOF, followed by  
25 antibiotic susceptibility testing (AST). Whole genome sequencing (WGS) and bioinformatic  
26 genome analysis was performed.

27 **Results:** Selective culture of stools revealed the presence of an *E. coli* strain named Q4552  
28 harbouring *mcr*-1.1 and *mcr*-3.5 genes which is also resistant to fourteen antibiotics. Genome  
29 sequencing and assembly resulted to a complete and circular chromosome and eight different  
30 plasmids. Sequence analysis demonstrated an integration of a mobile genetic element (MGE)  
31 carrying *mcr*-1.1 in the chromosome while *mcr*-3.5 gene was in the plasmid and its resistome  
32 was composed of twenty-two resistance genes. Q4552 strain was identified as an ST-843  
33 clone which belonged to the clonal complex Cplx-568 and is the only ST type of this cplx-  
34 568 which has been isolated from animals, humans, and the environment.

35 **Conclusion:** We report the first co-occurrence of *mcr*-1 and *mcr*-3 genes in France from a  
36 pathogenic *E. coli* isolated from a pig. Since this clone (ST-843) has been reported in  
37 zoonotic transmissions, programs to monitor such bacterium are urgently required to avoid its  
38 spread and zoonotic transmission to humans.

## 39 **Introduction**

40 Colistin (polymyxin E) is a polycationic antibiotic that interacts with the lipid A of  
41 lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria (GNB) leading to  
42 cell lysis [1]. This antibiotic is considered as one of the last-line antimicrobial agents to treat  
43 infections caused by multidrug resistant (MDR) Gram-negative bacteria, especially  
44 *Enterobacteriaceae* such as carbapenemase-producing *Escherichia coli* and *Klebsiella*  
45 *pneumoniae* [2]. Furthermore, the use of polymyxins in veterinary medicine has become very  
46 common within the European Union and European Economic Area (EU/EEA) [3]. The  
47 widespread use of this antibiotic has caused a significant increase in colistin resistance,  
48 especially in *E. coli* from pigs. Consequently, as reported, the emergence of colistin resistant  
49 bacteria in animals and humans is increasing [4]. Previously, it has been shown that colistin  
50 resistance is mediated by chromosomal gene substitutions. Gram-negative bacteria, especially  
51 *Enterobacteriaceae*, have several mechanisms against colistin including amino acid mutations  
52 in the two-component system PhoP/PhoQ and PmrA/PmrB, leading to the acquisition of high  
53 level of colistin resistance [5]. Other mechanisms, such as the inactivation of the *phoP/phoQ*  
54 negative feedback regulator (*mgrB*) or overexpression efflux pumps have also been reported  
55 [6]. Liu *et al.* reported the first *E. coli* harbouring the plasmid bearing mobile colistin  
56 resistance *mcr-1* gene, which they isolated from pigs in China [7]. Several methods and  
57 efforts were performed to discover other *mcr* genes variant from *mcr-2* until *mcr-10* [8]

58 Further studies have already reported the co-occurrence of colistin resistance genes in  
59 Asiatic countries, this remains rare in Europe. In this study, we report the first co-occurrence  
60 of two mobile colistin resistance genes, *mcr-1*, and *mcr-3*, in an *E. coli* Q4552 strain isolated  
61 from pigs in France.

62 **Materials and Methods:**

63 **Decree and law authorising the use of animal products for research and**  
64 **diagnosis:** Prefectorial authorisation (Bouches-du-Rhône) No.13 205 107 of 4 September  
65 2014 authorising the IHU Méditerranée Infection to use unprocessed animal by-products in  
66 categories 1, 2 and 3 for research and diagnostic purposes. Order of 8 December 2011 laying  
67 down health rules concerning animal by-products and derived products in application of  
68 Regulation (EC) No. 1069/2009 and Regulation (EU) No. 142/2011. In 2020, pig faecal  
69 samples (n=80) were collected in the Vaucluse department, France. Stool samples are  
70 collected at the time of defecation. At this time the stool is carefully collected with a sterile  
71 wooden spatula then put it in sterile laboratory tubes. The pigs (2 to 8 months old) are  
72 crossbreeds (mother: Large White X Landrace cross - father: Pietrain X Duroc cross) and  
73 divided in several batches. After fattening, they are intended for human consumption in  
74 Corsica, France.

75 **Screening of colistin resistant Gram-negative bacteria:** Faecal samples were  
76 enriched in TSB for 72 hours then cultured on a selective LBJRM (Lucie Bardet Jean-Marc  
77 Rolain) medium, containing colistin sulphate salt (4 µg/ml) and vancomycin (50 µg/ml), with  
78 glucose as the fermentative substrate, on a Purple Agar Base [10]. When bacterial growth is  
79 observed, colonies with different morphologies were picked and identified by MALDI-TOF  
80 MS using a Microflex LS spectrometer (Bruker Daltonics, Bremen, Germany). The strain was  
81 properly identified at the species level when the score is  $\leq 2$ .

82 **Molecular characterization :** DNA was extracted from the pig faecal samples. 1g of  
83 faeces, Buffer G2 and proteinase K were incubated at 56°C overnight and extracted using the  
84 EZ1 DNeasy Blood Tissue Kit (Qiagen GmbH, Hilden, Germany) in line with the  
85 manufacturer's protocol. The presence of colistin resistance genes (*mcr-1*, *mcr-2*, *mcr-3*, *mcr-*  
86 *4*, *mcr-5*, *mcr-8*) was investigated by Real-Time PCR assay using the CFX96 TM Real time

87 System / C1000™ Touch thermal Cycler (Bio-Rad, Singapore). Targeted genes were  
88 detected using specific primers and probes (**Suppl. Table S1**) [9]. Standard PCR was then  
89 performed to confirm positive qPCR results, followed by sequencing.

90

91 **Antibiotic susceptibility testing (AST):** The Disk diffusion, microdilution  
92 (Biocentric, Bandol, France), and E-test (BioMérieux, France) methods were performed to  
93 evaluate the bacterial susceptibility against sixteen antibiotics including ampicillin,  
94 amoxicillin-clavulanic acid, ceftazidime, cefotaxime, cefepime, cefoxitin, piperacillin-  
95 tazobactam, aztreonam, meropenem, ertapenem, imipenem, tigecycline, ciprofloxacin,  
96 gentamicin, trimethoprim-sulfamethoxazole, and colistin (Bio-Rad, Marne-la-Coquette,  
97 France).

98 **Genomic and sequence analysis :** Whole genome sequencing was performed using MiSeq  
99 Illumina (Illumina Inc., San Diego, CA, United states) and Nanopore MINION technologies.  
100 Raw sequence data were assembled using “Canu” assembler, which combines Illumina and  
101 Nanopore sequence reads, and genome annotation was performed using Prokka. Resistome  
102 analysis was investigated using different databases including Resfinder, ARG-ANNOT,  
103 CARD, and Plasmid Finder. CGview software was then used for circular representation of  
104 chromosome and plasmid sequences. Finally, the genotyping of isolated strains was carried  
105 out from genomic sequences and the set of close typing sequences was determined by  
106 PubMLST then compared with the 11,808 ST of *Escherichia* spp (<https://pubmlst.org/>) using  
107 Cytoscape and layout software.

## 108 **Results**

109 **Gene screening, culture, and bacterial identification from stool samples:** The RT-  
110 PCR screening on stool samples revealed one positive result. This sample was positive for the  
111 *mcr-1* and *mcr-3* genes, with CT values of 24 and 21, respectively. The selective culture of  
112 this sample on the LBJMR medium and MALDI-TOF identification reveals the presence of  
113 an *E. coli* strain named Q4552 strain. As shown in (**Suppl. Figure S1A and S1B**), the *E. coli*  
114 Q4552 strain was resistant to colistin with MIC<sub>COL</sub>= 8µg/ml. The performed standard PCR  
115 sequencing confirmed the presence of both *mcr-1.1* and *mcr-3.5* gene variants (**Suppl. Figure**  
116 **S1C**). The AST results revealed that the *E. coli* Q4552 strain was resistant amoxicillin,  
117 amoxicillin/clavulanic acid, doxycycline, clindamycin, erythromycin, and sulphamides but  
118 remained susceptible to cephalosporins, to carbapenems, and to tigecycline (**Table 1**).

119 **Genomic analysis:** genome assembly using combined raw reads from the Illumina  
120 and Nanopore technologies resulted in the circular chromosome with 4,778,049-bp with  
121 80.87% GC content and eight circular plasmids with sizes ranged from 84,331-bp to 3,607-bp  
122 and a %GC content ranging from 59.94% to 43.39% (**Suppl. Table S2**). As presented in  
123 **Table 1**, resistome analysis reveals the presence of several genes encoding for resistance to β-  
124 lactams (*bla*<sub>TEM</sub>), to aminoglycosides, especially streptomycin (*aadA1* and *aadA2*), to  
125 macrolides, especially clindamycin and erythromycin (*ermB* and *mphA*), and to quinolones  
126 (ciprofloxacin) by nucleotide deletion in the topoisomerase IV *parC* gene (A186-) leading to  
127 truncated protein.

128 Interestingly, sequence analysis shown that the *mcr-1.1* gene was in a mobile genomic  
129 element (MGE) with size of 51,089 bps from the Q4552 chromosome. As shown in **Figure 1**,  
130 the *E. coli* Q4552 chromosome was compared with published sequences, including the  
131 chromosome sequence of *E. coli* K-12 MG1655, *E. coli* CP66\_6, and *E. coli* plasmid pPN43.  
132 Our analysis reveals that the MGE: *mcr-1.1* was integrated within a specific integration site

133 upstream of a tRNA-Met gene and mediated by a phage integrase *intA*, as compared to the *E.*  
134 *coli* K-12 MG1655 chromosome (**Fig. 1B**). Moreover, the *mcr-1* gene is flanked on both sides  
135 by the IS30, which is suggestive of a transposition event. Interestingly, this MGE is also  
136 identified within the chromosome of the *E. coli* CP66\_6 strain isolated from pigs in Sichuan  
137 (China) and in a plasmid *E. coli* pPN43 strain from a human sample in Thailand with a slight  
138 difference (**Fig. 1B**). In addition to the *mcr-1* gene, a restriction-modification system type 1  
139 (RMS type-1) and toxin/antitoxin system were identified within this MGE (**Fig. 1B**)  
140 suggestive of a stabilization of MGE within the chromosome and thus promoting its vertical  
141 transmission. Regarding *mcr-3.5*, our genomic analysis revealed its localization in one of the  
142 eight identified plasmids (pQ4552\_*mcr-3*, an IncFII type) (**Fig. 2**). As shown in **Figure 2**, this  
143 plasmid pQ4552\_*mcr-3* (84,331 bps; 52,61%CG) was compared with six similar plasmids  
144 with an alignment coverage between 78% and 98% and identity percentage from 98.60 to  
145 99.96%. This analysis shows that *mcr-3* is only present in two out of the six plasmids,  
146 including the *E. coli* plasmid pVE769 (AP018353.1) from pigs and the unnamed *K.*  
147 *pneumoniae* plasmid (CP041102.1) also isolated from pigs. A toxin/antitoxin (*pemI/pemK*)  
148 system allows this plasmid to be maintained in *E. coli* Q4552 strain (**Fig. 2**).

149 **Multilocus sequence typing analysis:** MLST analysis classified the Q4552 strain  
150 into ST-843, belonging to the clonal complex Cplx-568. As presented in **Suppl. Figure S2**,  
151 the Cplx-568 complex is far composed of 14 ST types according to the Institut Pasteur  
152 database. Interestingly, only the ST-843 (our Q4552) was isolated from the three different  
153 sources including animal, human, and the environment.

## 154 **Discussion**

155 According to the literature, the main source of the strains with a co-occurrence of *mcr-*  
156 1 and *mcr-3* are animals. Here, the ecosystem of *E. coli* Q4552 is also farm animals.  
157 Although, pigs meat is consumed around the entire world in various forms, and humans are



158 consequently exposed to contamination by strains with the *mcr* genes, as already reported in  
159 farm workers and the surrounding environment being largely contaminated by the *mcr-1* gene  
160 [11]. So far, few studies have reported the co-occurrence of two *mcr* genes within a  
161 bacterium. This co-occurrence has been reported in an *E. coli* strain isolated from cattle [12]  
162 in Spain, from pigs in Thailand [13], and dog faeces in China [14]. Moreover, the co-  
163 occurrence of *mcr-1* and *mcr-3* has also been reported in humans, such as the *E. coli* strain  
164 isolated from a human urine sample in New Zealand [15], and a *K. pneumoniae* strain isolated  
165 from a stool sample from a healthy volunteer in Thailand [16]. The identified MGE  
166 harbouring the *mcr-1.1* gene from the Q4552 chromosome appears to be extremely rare in  
167 available genomes from the NCBI database, and has only been detected in an *E. coli* CP66-6  
168 strain with *mcr-1* which was isolated from a pig in China [17], but also from a plasmid of an  
169 *E. coli* strain isolated from a human in Thailand [18]. However, both *E. coli* strains are not  
170 ST-843 but share this MGE. The two *mcr* genes have different genetic localization which  
171 suggests the risk of vertical and horizontal transmission favoring their emergence.

172 *E. coli* Q4552 is an atypical bacterium which belongs to the clonal complex CcpX-568  
173 and has an ST-843. ST-843 appears closely related to ST-80 and ST-1946 which are two *E.*  
174 *coli* clones well reported in zoonotic transmissions from animals and humans. Indeed, ST-80  
175 has well been reported as a uropathogenic *E. coli* clone from retail food such as chicken and is  
176 able to cause community acquired infections [19]. The investigation of the isolation sources  
177 of these ST types revealed diverse sources, including humans, animals, and the environment.  
178 Interestingly, the three ST types, namely ST-80, ST-1946, and ST-843, were isolated from  
179 humans and animals, suggesting the ability of this clone to be transferred from animals to  
180 humans and only the ST-80 (our strain) is isolated from the three different sources.

181 In conclusion, *E. coli* Q4552 exhibited a multidrug resistance phenotype mediated by  
182 various resistance genes. The most unusual co-occurrence is the fact that *mcr-1.1* gene is in

183 the chromosome, with more than 4 IS transposases whereas *mcr-3.5* gene is in the plasmid.  
184 As reported in the literature, *mcr* genes encode for phosphoethanolamine transferase proteins  
185 and could serve as defensive enzymes against phage attacks [20]. These results give rise to  
186 questions about the real functions of these *mcr* genes within this Q4552 strain, which was not  
187 more resistant to colistin, despite this co-occurrence.

188           **Sequence accession number.** Complete genome sequence including the chromosome  
189 and the eight plasmids of the *E. coli* Q4552 strain have been deposited in the NCBI GenBank  
190 database under accession numbers: CP077063, CP077064, CP077065, CP077066, CP077067,  
191 CP077068, CP077069, CP077070, and CP077071.

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196           **Conflicts of interest.** The authors declare that they have no competing interests.

197           **Authors’ contributions.** SMD, BD, and JMR designed the study; AF performed  
198 microbiology analyses; AF and SMD drafted the manuscript; AF, BD, JMR, and SMD made  
199 corrections and critical revisions. All the authors have read and approved the final version of  
200 the manuscript.

201

## Figure legends

202 **Figure 1: A.** Circular maps of the chromosomal genome with *mcr-1*. **B.** Zoom on the genetic  
203 environment of the transposon carrying the *mcr-1.1* gene compared with the top best hit blast  
204 *E. coli* CP66-6 and plasmid *E. coli* PN43.

205 **Figure 2:** Circular map of the plasmid carrying *mcr-3.5* compared to six homologous  
206 plasmids with considerable query coverage.

207

208 **Table 1:** Phenotypic and genotypic comparison of *E. coli* Q4552 according to antibiotic family

Antibiotic family	Antibiotics	Inhibition diameters (mm)	MIC (mg/ml)	ARGs	Encoding gene for	% COVERAGE ARG ANNOT	% COVERAGE Resfinder
<b>Penicillins</b>	Amoxicillin	R (0)	R ( $\geq 256$ )	(Bla) <i>ampC2</i>	Class C $\beta$ -lactamase	99%	100%
	Amoxicillin + Clavulanic acid	R (12)	R ( $\geq 256$ )	(Bla) <i>ampC1</i>	Class C $\beta$ -lactamase	99.88%	100%
	Piperacillin	S (24)	S (2)				
	Penicillin G	R (0)	R ( $\geq 32$ )	(Bla)TEM	Class A $\beta$ -lactamase	100%	100%
	Piperacillin- tazobactam	S (24)	S (0.38)				
<b>Monobactams</b>	Aztreonam	S (30)	S (0.46)				
<b>Cephalosporins</b>	Cephalosporin G1: cefalotin	I (16)	I (0.32)				
	Cephalosporin G2: cefotaxime	S (25)	S ( $\leq 0.25$ )				
	Cephalosporin G3: ceftriaxone	S (38)	S ( $\leq 1$ )				
	Cephalosporin G4: cefepime	S (30)	S ( $\leq 1$ )				
<b>Carbapenems</b>	Meropenem	S (24)	S ( $\leq 0.125$ )				
	Ertapenem	S (39)	S (0.03)				
	Imipenem	S (35)	S ( $\leq 0.32$ )				
<b>Macrolides</b>	Clindamycin	R (0)	R ( $\geq 256$ )	<i>mphA</i>	Macrolide 2'-phosphotransferase I	100%	100%
	Erythromycin	R (0)	R ( $\geq 256$ )	<i>ermA</i> , <i>ermB</i> *, <i>ermR</i>	rRNA adenine N-6-methyltransferase	100%	100%
<b>Quinolones</b>	Ciprofloxacin	R (2)	R (1024)	<i>parC</i> truncated (A186-)	DNA topoisomerase <i>parC</i>	97.87%	99%
	Levofloxacin	S (27)	S (0.125)				
<b>Tetracyclines</b>	Doxycycline	R (10)	R (32)	(Tet) <i>tetA</i> <sup><math>\delta</math></sup> ,	Tetracycline efflux pump	94.12%	99,92%
	Minocycline	R (2)	R (12)	(Tet) <i>tetA</i> <sup><math>\delta</math></sup> ,	Tetracycline efflux pump	100%	100%
<b>Sulphonamides</b>	Trimethoprim	R (0)	R ( $\geq 256$ )	<i>dfrA12_8</i>	Dihydrofolate reductase	98.5%	100%
	Sulfamethoxazole						
	Sulphamide	R (1)	R (64)	(Sul) <i>sul3</i>	Sulfonamide resistance gene	100%	100%

<b>Aminoglycosides</b>	Streptomycin	R (2)	R ( $\geq 32$ )	(AGly) <i>aadA1-pm</i> (AGly) <i>aadA2</i>	Aminoglycoside-6-acetyltransferase	93.86% 100%	98.48% 95.24%
	Gentamicin	S (37)	S ( $\leq 0.64$ )				
	Netilmicin	S (35)	S (0.25)				
	Kanamycin	S (28)	S (1.5)				
	Amikacin	S (25)	S (1.5)				
<b>Phenicol</b>	Chloramphenicol	R (13)	R ( $\geq 8$ )	(Phe) <i>cmlA1</i>	Chloramphenicol efflux transporter	100%	98%
<b>Polymyxin E</b>	Colistin	R (13)	R (4)	(Col) <i>mcr-1.1</i>	Phosphoethanolamine Transferase	100%	100%
				(Col) <i>mcr-3.5*</i>		100%	100%
<b>OTHERS</b>	Fosfomicin	R (9)	R ( $\geq 32$ )	<i>mdtG</i>	Multidrug efflux pump	99.27 %	100%
	Metronidazole	R (0)	R ( $\geq 256$ )				
	Rifampicin	R (0)	R ( $\geq 32$ )				
	Tigecycline	S (31)	S (0.5)				

209 \* These genes were identified from the plasmid pECQ4552\_*mcr-3* ;  $\delta$ , this gene was detected from the plasmid pECQ4552\_IHU04.

210

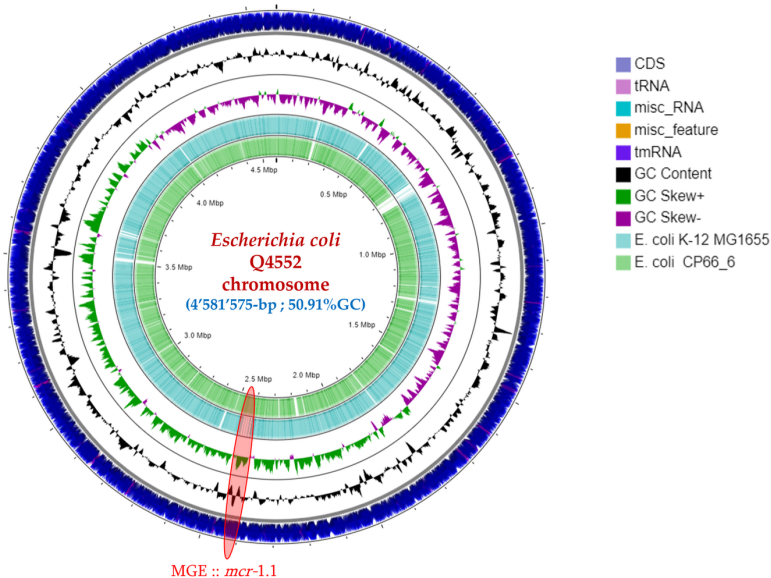
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