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1 **Genetic and functional characterization of an MCR-3-like producing**

2 ***Escherichia coli* recovered from swine, Brazil**

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23

24 **A collection of 126 pigs were screened for carriage of colistin-resistant**

25 **Enterobacteriaceae in a farm in Minas Gerais, Brazil. Out of this collection, eigth**

26 **colistin-resistant *Escherichia coli* isolates were recovered, including one from Minas**

27 **Gerais State, producing a new MCR-3 variant (MCR-3.12). Analysis of the**

28 **lipopolysaccharide revealed that MCR-3.12 had a similar function as MCR-1 and MCR-**

29 **2 by adding a phosphoethanolamine group to the lipid A. Genetic analysis showed that**

30 **the *mcr-3.12* gene was carried by an IncA/C_2 plasmid and was embedded in an original**

31 **genetic environment. This study reports the occurrence of the MCR-3-like determinant**

32 **in South America and firstly demonstrates the functionality of this group of enzymes as**

33 **a phosphoethanolamine transferase.**

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36 **INTRODUCTION**

37 The increasing occurrence of colistin-resistant Enterobacteriaceae is of great concern since
38 colistin represents one of the last-resort treatments for infections caused by carbapenem-
39 resistant Enterobacteriaceae (CRE). In addition to chromosomally-encoded resistance
40 mechanisms corresponding to mutations or deletions in genes involved in the biosynthesis of
41 the lipopolysaccharide (LPS), acquired resistance through horizontal gene transfer has been
42 recently described (1). Five different plasmid-mediated colistin resistance genes have been
43 identified so far in Enterobacteriaceae, including *mcr-1, mcr-2, mcr-3, mcr-4 and mcr-5* (2-6).
44 They code for enzymes that modify the lipid A moiety of the LPS of Gram-negative bacteria
45 and consequently conferring resistance to polymyxin B and colistin (1). To date, only MCR-1
46 and MCR-2 have been shown to function as phosphoethanolamine transferases (7). The *mcr-1*
47 and *mcr-2* genes likely originate from *Moraxella* species (8), with *Moraxella pluranimalium*
48 being the progenitor of *mcr-2* (9), *Aeromonas* spp. that of *mcr-3*-like genes (4), and
49 *Shewanella* spp. that of *mcr-4*-like genes (5). The origin of the newly discovered *mcr-5* gene
50 remains unknown (6). The high prevalence of MCR-1-producing *E. coli* isolates in food-
51 producing animals, and therefore the high rate of colistin-resistant isolates may be explained
52 by the constant use of colistin in veterinary medicine, in particular in livestock for the

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53 treatment of poultry, swine and cattle (1). To date, six *mcr-3* variants have been reported since
54 the discovery of *mcr-3.1* in June 2017, identified from an *Echerichia coli* isolate from a
55 healthy pig in China (4) and in a *Salmonella* isolate from human infections in Denmark (10).
56 The *mcr-3.2* variant was identified in *E. coli* from cattle in Spain (11). The *mcr-3.3* to *-3.9*
57 variants were identified in *Aeromonas* spp. (12-15), and the *mcr-3.10* in *E. coli* from duck in
58 China (15). Finally, the *mcr-3.11* gene was from an *E. coli* isolate recovered from chicken in
59 China (unpublished, Genbank accession number MG489958.1). Even if *Aeromonas* spp. was
60 described as the progenitor of the *mcr-3* genes, this gene might also be found as an acquired
61 determinant in that species (13).

62 Here we report a novel *mcr-3* variant detected in an *E. coli* isolate recovered from a post-
63 weaning diarrhea of a pig that was previously treated by colistin in Brazil.

64 RESULTS

65 **Characterization of a new *mcr-3* variant and susceptibility testing.** Out of the 126
66 pig samples, eight samples were found to contain colistin-resistant *E. coli* isolates. All the
67 animals received treatment including colistin for 15 days after the weaning period. Out of the
68 8 colistin-resistant *E. coli* isolates, only a single isolate (I112) was positive by PCR for the
69 *mcr-3* gene. The other colistin-resistant *E. coli* isolates remaining negative for the other *mcr-*

70 like genes. Sequencing of the PCR products revealed that the *mcr-3*-like gene corresponded to
71 a new variant named *mcr-3.12* (Genbank accession number: MG564491) encoding for a
72 MCR-3.12 enzyme sharing 97% of amino-acid identity with the original MCR-3 variant and
73 between 97% and 99% of amino-acid identity with the other MCR-3-like variants (Figure 1).
74 Isolate I112 showed resistance to broad-spectrum cephalosporins, tetracycline,
75 chloramphenicol, florfenicol, nalidixic acid, sulfonamides, sulfomethoxazole/trimethoprim
76 and kanamycin. It was found positive with the Rapid Polymyxin NP test and showed an MIC
77 of colistin at 4 µg/ml using broth microdilution method. MLST analysis showed that isolate
78 I112 belonged to the ST641 and to the phylogroup A. Analysis with Serotypefinder1.1
79 indicated that it belonged to the O160:H25 serotype. Phylogenetic analysis of the known *mcr-*
80 *3* showed a significative diversity among the variants. Three major subgroups could be
81 identified including, (i) MCR-3.5, MCR-3.6 and MCR-3.8, (ii) MCR-3.4 and the MCR-3.11,
82 (iii) MCR-3.1, MCR-3.2, MCR-3.3, MCR-3.7 and MCR-3.11, respectively. The MCR-3.9
83 and MCR-3.10 enzymes were found to be both close to MCR-3.12 and MCR-3.1 variants
84 (Figure 1).

85 **MCR-3 is a phosphoethanolamine transferase conferring resistance to colistin.**
86 Mass spectrometry analysis of the LPS showed that unlike the J53 negative control showing a

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87 single *m/z* 1798 peak corresponding to the bis-phosphorylated hexa acylated lipid A, the
88 MCR-1 and MCR-3 producers showed an identical additional peak at *m/z* 1921 (Δ*m/z* 123)
89 corresponding to an addition of a phosphoethanolamine (PEtN) groupment to the lipid A as it
90 was previously described (7, 16) (Figure 2). Induction of the $pBAD_b$-*mcr-3-like* plasmid
91 allowed to obtain an MIC of colistin at 4 µg/ml whereas the non-induced clone presented an
92 MIC at 0.03 µg/ml showing that the production of MCR-3-12 conferred a 130-fold increase of
93 colistin MIC. Altogether, these results showed the phosphoethanolamine transferase activity
94 of the MCR-3.12 enzyme and its impact on the colistin susceptibility.

95 **Plasmid analysis.** Mating-out assays were successful with *E. coli* J53 and *Klebsiella*
96 *pneumoniae* CIP53153 as recipients, but also with *Aeromonas punctata* CIP102629,
97 highlighting its broad host range property. By contrast, no transconjugant was obtained using
98 *P. aeruginosa* PaO1 as recipient. Conjugation followed by PCR showed that *mcr-3.12* was
99 located onto a conjugative plasmid named p112. That latter plasmid encoded resistance to
100 tetracyclines, sulfonamides, chloramphenicol and florfenicol. PBRT analysis showed that
101 plasmid p112 belonged to the IncA/C_2 incompatibility group. Kieser extraction followed by
102 gel electrophoresis identified its size to be ca. 140-kb in size. MICs of colistin of the *E. coli*
103 and *K. pneumoniae* transconjugants were at 4 and 8 µg/ml, respectively, being therefore

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104 categorized as resistant according to the EUCAST breakpoint (original MICs of the bacterial
105 hosts being at 0.25 and 0.12 µg/ml, respectively) (http://www.eucast.org). Interestingly, MICs
106 of colistin of the *A. punctata* transconjugant was at 16 µg/ml (original MIC at 0.12 µg/ml),
107 indicating a very significant impact of MCR-3.12 on colistin susceptibility in that species.

108 **Bioinformatic analysis and genetic context of the *mcr-3-12 gene.*** Whole genome
109 sequencing of *E. coli* I112 data identified a series of resistance determinants including genes
110 encoding resistance to β-lactams (*bla*$_{\text{TEM-1B}}$ and *bla*$_{\text{CTX-M-8}}$ genes), aminoglycosides (*aph[3']-*
111 *Ia, strA* and *strB*), tetracyclines (*tetA*), phenicols (*catA1* and *floR*), sulphonamides (*sul2*) and
112 trimethoprim (*dfr18*). The *mcr-3*-like gene was found in association with a gene encoding for
113 a diacylglycerol kinase *dgkA*-like sharing 98% of nucleotide identity with the *dgkA* gene
114 identified in association with the first *mcr-3* described on plasmid pWJ1 (4).

115 The *mcr-3.12* gene was located between two insertion sequences belonging to the IS*66*
116 and IS*30* families, respectively (Figure 3). Interestingly, 90-bp after the end of the inverted
117 repeat right (IRR) of the IS*30*-like, an IRL-like of the IS*66* was detected, sharing 100% of
118 nucleotide identity with the first 24-nt of the IRL of IS*66* (Figure 3). The presence of this
119 IRL-like downstream the IS*30*-like could form a putative transposon with the IS*66*.

120 Further analysis showed that this putative transposon was embedded in a longer structure that
121 was inserted between nucleotides 1,049 and 1,050 of a DNA methyltransferase gene located
122 on the IncA/C_2 backbone. This structure was 20,376-bp long and is represented in Figure 3F.
123 It could be defined into three different regions, (i) a 5' region characterized by a 7,666-bp
124 region with a GC content of 39% containing three putative open reading frames including two
125 encoding for putative site-specific integrases, (ii) the putative transposon containing the *mcr-3*
126 variant and three ORFs (α, β and γ) presenting a GC content of 49% and (iii) a 3' region of
127 526-bp with a similar %GC as the first 7,666-bp region (Figure 3F). The ORF α, β and γ
128 encoded for a reverse transcriptase, a transcriptional regulator and a diguanylate cyclase,
129 respectively. Their products showed strong amino acid identity (98%) with putative proteins
130 from *Aeromonas dhakensis*.

131 **DISCUSSION**

132 We report here the identification of a novel variant of the *mcr-3* gene, detected in an *E.*
133 *coli* isolate recovered from a pig in Brazil. Interestingly, previous studies also described
134 MCR-3 producers recovered from animal samples (11, 13), suggesting the same link between
135 animal and colistin resistance as it has been established for the *mcr-1*gene. The pigs screened
136 in this study were treated previously with colistin for fifteen days after the weaning period.

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137 This suggests the possible selection of colistin-resistant strain during this period as we showed
138 in our previous study describing a high prevalence of MCR-1-producers in a pig farm in
139 Portugal where animals had received colistin (17). There have been many reports of MCR
140 producers in Brazil, with MCR-1 being the only variant systematically identified. These
141 isolates were a single *Salmonella enterica* serotype Typhymurium was recovered from retail
142 meat (18), *E. coli* isolates recovered from chicken meat (19), from migratory penguins (20),
143 recovered on public beaches (21), or recovered from patients with bloodstream infections (22,
144 23). Also, KPC-2-producing *E. coli* (24), and KPC-2-producing *Klebsiella pneumoniae*
145 belonging to ST392 and ST437 (25, 26) were identified. A quite extensive study identified a
146 series of 59 MCR-1-producing *E. coli* isolates recovered from humans, chicken, chicken
147 meat, bovine, turkey, swine and penguin (27). However, we might speculate that most studies
148 were designed to detect only the *mcr-1* gene so far, and few investigating the occurrence of
149 the most recently-identified other variants.

150 Isolate I112 carried a novel *mcr-3* variant named *mcr-3.12*. It belonged to ST641
151 which was previously found to carry the *mcr-1* gene, corresponding to isolates recovered from
152 pigs in Germany in 2016 (28). It belongs to the phylogroup A of *E. coli* therefore
153 corresponding to a commensal strain. Sequence alignment analysis showed that *mcr-3.12*

154 shares 99% of nucleotide identity with a sequence from *Aeromonas veronii.* This suggests that
155 this new variant may have originated from that particular species or may have widely
156 dissiminated as an acquired resistance trait within that species. Noteworthy, we showed here
157 that the IncA/C_2-type plasmid bearing the *mcr-3.12* gene could replicate in *Aeromonas* sp. We
158 may therefore speculate that such plasmid type might have been involved in the original
159 spread of *mcr-3*-like genes from their progenitors to other bacterial species, including
160 members of the *Enterobacteriaceae* family.

161 Induction experiments and analysis of the lipid A of the isolate strongly indicates that
162 the MCR-3 enzyme confers colistin resistance the same way as MCR-1 and MCR-2 enzymes
163 by adding a phosphoethanolamine group to the lipid A although this enzyme only shared 45
164 and 47% of amino-acid identity with MCR-1 and MCR-2, respectively. The fact that MCR-1,
165 -2, and -3 share similar functions was previously hypothesized through an in-silico protein
166 structure analysis (4).

167 The *mcr-3* gene was previously described onto IncHI2 and IncX4 plasmids which are
168 commonly found in association with the *mcr-1* and *mcr-2* genes. Here, we described the first
169 IncA/C_2 plasmid carrying a plasmid-mediated colistin resistance determinant. This plasmid
170 backbone is commonly identified as a support of many different antibiotic resistance genes.

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171 Here, the determinants *tetA*, *sul2* and *floR* encoding for resistance to tetracycline, 172 sulfonamides and phenicols respectively, were also detected on this same plasmid. The broad 173 host range of this plasmid was demonstrated, by evidencing its ability to replicate not only in 174 *E. coli* and *K. pneumoniae*, but also in *A. punctata*.

175 The *mcr-3.12* is located into a putative transposon including the IS*66* upstream of the 176 *mcr-3* gene and an IS*30-like* downstream. Interestingly, a 24-nt region found 90-bp 177 downstream the IS*30-like* was found identical to the IRL of the IS*66*. Further experiments will 178 be conducted to confirm whether IS*66* could have played a role in the acquisition of this 179 phosphoethanolamine transferase gene by a mechanism similar to a one-handed transposition 180 as it has been described for IS*Ecp1* in the mobilization of $bla_{\text{CTX-M-15}}$ (29).

181 The genetic context of the putative *mcr-3* transposon is complex and the chronology of 182 acquisition of this structure into the IncA/C2 plasmid can hardly be explained. One hypothesis 183 is summarized in the Figure 3. The IS*66* might have been involved in the original 184 mobilization of the *mcr-3.12* gene from *Aeromonas* spp. (Figures 3A-D). Then, a second 185 mobilization event may have occurred involving an unknown mechanism between the genetic 186 structure containing the putative integrases (Figure 3E) and the *mcr-3*-carrying structure 187 forming an 20,376-bp integron-like genetic complex. Finally, this whole structure may have

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188 been mobilized and inserted between the nt 1,049 and nt 1,050 of a DNA methyl transferase

189 gene located on an IncA/C_2 plasmid backbone (Figure 3F). The resulting resistance plasmid is

190 at the end one of those responsible for the spread of *mcr* genes among Enterobacteriaceae.

191 **MATERIAL AND METHODS**

192 **Bacterial isolate and susceptibility testing**. Screening of colistin-resistant isolates

193 was performed from 126 different pigs in ten swine herds in different states of the state of

194 Minas Gerais in Brazil, all pigs presenting post-weaning diarrhea. The isolates were initially

195 tested for colistin resistance using agar dilution methods. All colonies growing on plates

196 supplemented with >2µg/ml of colistin were confirmed by the commercialized Rapid

197 Polymyxin NP test (ELITech Microbiology, France) (30) and minimal inhibitory

198 concentrations (MICs) were determined by broth microdilution method using cation-adjusted

199 MH broth. Antimicrobial susceptibility testing for other antibiotics families was performed

200 according to the standard disk diffusion method on Mueller-Hinton (MH) agar plates

201 following the CLSI recommendations (31).

202 **WGS and molecular analysis.** PCR screening for *mcr* genes was performed using

203 primers designed to detect all known variants of MCR-3. Primers MCR-3allF (5'-GCA TTT

204 ATG CTG AAC TGG CG-3') and MCR-3allR (5'-AGC GGC TTT CTG CTG CAA AC -3')

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205 were used, and corresponding amplicons were subsequently sequenced (Microsynth, Balgach,
206 Switzerland). Whole genomic DNA of the MCR-3-positive isolate was extracted with the
207 Sigma-Aldrich GenElute™ Bacterial Genomic DNA Kit. Genomic libraries were assessed
208 using the NexteraXT library preparation kit (Illumina Inc., San Diego, CA) and sequencing
209 was performed using the Illumina MiniSeq system with 300-bp paired-end reads and a
210 coverage of 50X. Generated FastQ data were compiled and analyzed using the CLC genomic
211 workbench 7.5.1 (CLC bio, Aarthus, Denmark). Reads were de novo assembled with
212 automatic bubble and word size and contigs were generated using the mapping mode "map
213 reads back to contigs" with a minimum contig length of 800 nucleotides.

214 The resulting contigs were uploaded into the Center for Genomic Epidemiology server
215 (http://www.genomicepidemiology.org/). Plasmid replicon typing, multilocus sequence
216 typing, serotype and antimicrobial resistance determinants were determined using
217 PlasmidFinder 1.3, MLST 1.8, SerotypeFinder 1.1 and ResFinder 3.0, respectively (32-34).
218 Phylogroup analysis was performed by using the Clermont method (35). Sequence
219 alignements and construction of phylogenetic trees were performed with the Seaview
220 alignment tool version 4 (Prabi, La Doua, Lyon, France) (36).

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221 Plasmid analysis was performed using Kieser extraction method (37) followed by gel
222 electrophoresis in order to estimate the size of the plasmid containing the *mcr-3* gene using
223 the *E. coli* strain 50192 harboring four plasmids of 154, 66, 48 and 7 kb, respectively, as
224 plasmid size marker. The determination of the incompatibility group was confirmed by PCR-
225 based replicon typing (PBRT) (38).

226 Conjugation experiments were performed using the azide-resistant *E. coli* J53 strain. In
227 addition, conjugation were also performed in the temocillin-resistant *Pseudomonas*
228 *aeruginosa* PAO1, in the azide-resistant *Klebsiella pneumoniae* CIP53153 and in the azide-
229 resistant *Aeromonas punctate* CIP102629 recipient strains to test the broad host range of the
230 plasmid coding for the *mcr-3.12* variant. Both donor and recipient strains were cultured in
231 exponential phase, then mixed on solid LB agar using filters at a 1:10 donor:recipient ratio.
232 After 5 h of incubation, filters were resuspended in NaCl 0.85% and bacterial mixture were
233 plated onto agar plates supplemented with colistin (1 µg/ml) and sodium azide (100 µg/ml)
234 for *E. coli* or with temocillin (50 µg/ml) and sodium azide (100 µg/ml) for *P. aeruginosa*.
235 Since the plasmid bearing the *mcr-3.12* gene conferred resistance to tetracycline, conjugations
236 using *K. penumoniae* and *A. punctata* as recipients were attempted using tetracycline (100
237 µg/ml) and sodium azide (100 µg/ml) as selective molecules. Susceptibility of all

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238 transconjugants to antibiotics was confirmed by antibiogram followed by PCR for the *mcr-3*-

239 like gene.

240 **Analysis of the LPS modification**. The LPS of *E. coli* J53 (unmodified lipid A),

241 TCAf24 (J53-*mcr-1* transconjugant) and I112 (MCR-3-like producers) were analyzed by mass

242 spectrometry (MS). The lipid A was obtained by the hydrolysis of 3 mg of lyophilized

243 bacteria in 120 µl of isobutyric acid and 1 M ammonium hydroxide (5:3; v:v), heated for 1 h

244 at 100°C and cooled at 4°C before centrifugation, as previously described (39). The

245 supernatant was then diluted with water and lyophilized before wash with methanol. The

246 insoluble lipid A obtained was finally extracted in a chloroform:methanol:water (3:1:0.25,

247 v:v:v) mixture. MALDI-MS analysis was performed using a PerSeptive Voyager STR (PE

248 Biosystems, France) time-of-flight mass spectrometer in linear negative ion mode.

249 Dihydroxybenzoic acid (DHB) at 10 mg/ml in 0.1 M citric acid in chloroform:methanol:water

250 (3:1.5:0.25;v:v:v) was used as matrix.

251 **Cloning and overexpression of the *mcr-3.12* gene.** The new *mcr-3* variant was

252 cloned into the arabinose-inducible $pBAD_b$ vector in order to determine the impact of the

253 expression of the MCR-3-12 phosphoethanolamine transferase on colistin susceptibility.

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254 Induction of $pBAD_b$ vector was performed using MH broth supplemented with L-arabinose
255 1% as previously described (8).

256

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398 **Figure Legends**

399 Figure 1. Phylogenetic tree obtained for all the identified MCR-like enzymes including all
400 MCR-3 variants by distance method using Neighbor-Joining algorythm (SeaView version 4
401 software). Branch lengths are drawn to scale and are proportional to the number of amino
402 acids substitutions with 500 bootstrap replications. The distance along the vertical axis has no
403 significance. Percentage of amino acids identity shared between the MCR-3.12 variant and
404 the other MCR-like enzymes is indicated in brackets.

405

406 Figure 2. Mass spectrometry analysis of lipid A from strain *E. coli* J53 (A), its transconjugant
407 carrying the *mcr-1* gene (B) and the clinical isolate I112 expressing the *mcr-3.12* gene (C).
408 The addition of a PEtN group is indicated by a black arrow.

409

410 Figure 3. Proposed model of the chronology of the acquisition of the *mcr-3.12* gene into the
411 IncA/C2 plasmid. The genes *eamA* and *dgkA* encode for a metabolite transporter and a
412 diacylglycerol kinase , respectively. *intA* and *intB* represent putative integrases ; α, β and γ are
413 the ORF encoding for a reverse transcriptase, a transcriptional regulator and a diguanylate
414 cyclase, respectively ; δ corresponds to the ORF encoding for a DNA methyltransferase
415 located onto the IncA/C2 plasmid backbone.

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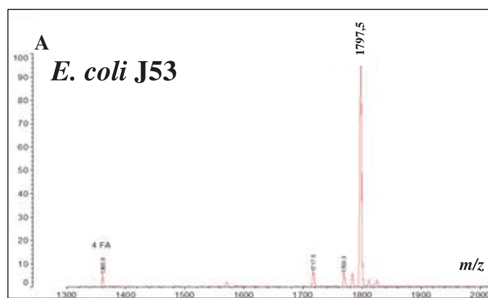

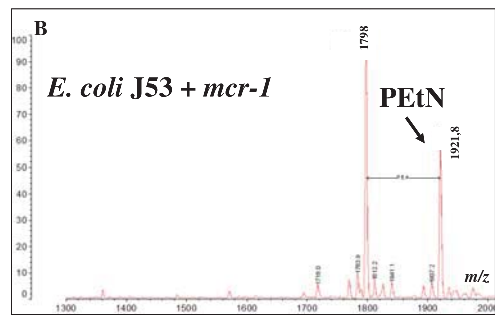

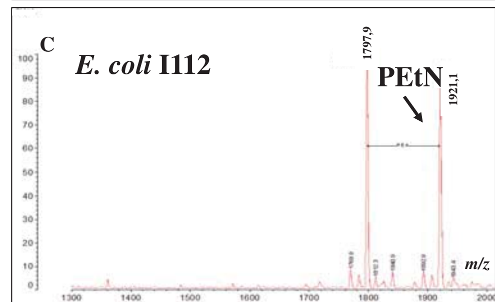

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