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Genetic and functional characterization of an MCR-3-like producing *Escherichia coli* recovered from swine, Brazil

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A collection of 126 pigs were screened for carriage of colistin-resistant Enterobacteriaceae in a farm in Minas Gerais, Brazil. Out of this collection, eigth colistin-resistant *Escherichia coli* isolates were recovered, including one from Minas Gerais State, producing a new MCR-3 variant (MCR-3.12). Analysis of the lipopolysaccharide revealed that MCR-3.12 had a similar function as MCR-1 and MCR-2 by adding a phosphoethanolamine group to the lipid A. Genetic analysis showed that the *mcr-3.12* gene was carried by an IncA/C_2 plasmid and was embedded in an original genetic environment. This study reports the occurrence of the MCR-3-like determinant in South America and firstly demonstrates the functionality of this group of enzymes as a phosphoethanolamine transferase.

INTRODUCTION

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

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-*

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

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

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

Plasmid analysis. Mating-out assays were successful with *E. coli* J53 and *Klebsiella pneumoniae* CIP53153 as recipients, but also with *Aeromonas punctata* CIP102629, highlighting its broad host range property. By contrast, no transconjugant was obtained using *P. aeruginosa* PaO1 as recipient. Conjugation followed by PCR showed that *mcr-3.12* was located onto a conjugative plasmid named p112. That latter plasmid encoded resistance to tetracyclines, sulfonamides, chloramphenicol and florfenicol. PBRT analysis showed that plasmid p112 belonged to the IncA/C$_2$ incompatibility group. Kieser extraction followed by gel electrophoresis identified its size to be ca. 140-kb in size. MICs of colistin of the *E. coli* and *K. pneumoniae* transconjugants were at 4 and 8 µg/ml, respectively, being therefore

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*.

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

DISCUSSION

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

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

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

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

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

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

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

been mobilized and inserted between the nt 1,049 and nt 1,050 of a DNA methyl transferase gene located on an IncA/C_2 plasmid backbone (Figure 3F). The resulting resistance plasmid is at the end one of those responsible for the spread of *mcr* genes among Enterobacteriaceae.

MATERIAL AND METHODS

Bacterial isolate and susceptibility testing. Screening of colistin-resistant isolates was performed from 126 different pigs in ten swine herds in different states of the state of Minas Gerais in Brazil, all pigs presenting post-weaning diarrhea. The isolates were initially tested for colistin resistance using agar dilution methods. All colonies growing on plates supplemented with >2µg/ml of colistin were confirmed by the commercialized Rapid Polymyxin NP test (ELITech Microbiology, France) (30) and minimal inhibitory concentrations (MICs) were determined by broth microdilution method using cation-adjusted MH broth. Antimicrobial susceptibility testing for other antibiotics families was performed according to the standard disk diffusion method on Mueller-Hinton (MH) agar plates following the CLSI recommendations (31).

WGS and molecular analysis. PCR screening for *mcr* genes was performed using primers designed to detect all known variants of MCR-3. Primers MCR-3allF (5'-GCA TTT ATG CTG AAC TGG CG-3') and MCR-3allR (5'-AGC GGC TTT CTG CTG CAA AC -3')

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

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

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

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

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.

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

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Figure Legends

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

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

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