

# MCR-1 in multidrug-resistant and copper-tolerant clinically relevant *Salmonella* 1,4,[5],12:i:- and S. Rissen clones in Portugal, 2011 to 2015

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The *mcr-1* gene was found in 11 isolates from a Portuguese *Salmonella* collection (n = 1,010; 58 serotypes; 2002–15) of clinical samples, foodstuff, food-animals and water. *Mcr-1* has been located on different plasmids (IncX<sub>4</sub>/IncHI<sub>2</sub>) in pig-associated multidrug-resistant, copper-tolerant S.1,4,[5],12:i:-/ST<sub>34</sub> and S. Rissen/ST<sub>469</sub> clones from human and pork products since at least 2011. Our data highlight dissemination of *mcr-1* by successful resistant clones in Europe and raise questions about the efficacy of copper-based interventions to reduce colistin use.

Since the description of plasmid-mediated colistin resistance encoded by the *mcr-1* gene in *Enterobacteriaceae* from multiple sources in China [1] and its worldwide dissemination mostly in animal sources [2], the use of polymyxins (colistin) in food-producing animals has been questioned in Europe because it may have an impact on human health [3]. Nevertheless, data on the transmission of *mcr-1*-mediated colistin resistance particularly by clonal expansion are lacking [3,4]. In fact, the *mcr-1* gene has been found in zoonotic food-borne bacteria such as *Salmonella* [2] but the presence of this gene in particular successful resistant clones has not been demonstrated [3]. In this study, we report the presence of the *mcr-1* gene in pig-associated clinically relevant *Salmonella* serotypes and clones recovered from human clinical samples and pork products in Portugal, collected as early as 2011.

## Laboratory investigation

We analysed a total of 1,010 *Salmonella* isolates of 58 serotypes from several sources (human clinical cases, food products, food-animal production settings and aquatic environments) and regions of Portugal, collected between 2002 and 2015 (Table 1). The isolates were screened for the *mcr-1* gene by PCR and sequencing, using primers CLR5-F (5'-CGGTCCGTTTGTTC-3')

[1] and Mcr1-Rv2 (5'-CCAGCGTATCCAGCACATTT-3') [this study].

The 1,010 isolates comprised the most frequent worldwide *Salmonella* serotypes (n=256 *S. Typhimurium* and n=34 *S. Enteritidis*), but also emerging serotypes (n=436 *S. 1,4,[5],12:i:-* and n=93 *S. Rissen*) or serotypes less frequently detected in European surveillance studies (n=191 isolates from 54 different serotypes). They included all isolates previously characterised [5,6] and recent ones from ongoing surveillance studies (data not shown) covering all serotypes, sampling dates, sources, regions, antibiotic susceptibility phenotypes/genotypes and PFGE types. The isolates positive for *mcr-1* by PCR were further tested for susceptibility to colistin by the proposed broth microdilution method [7] and interpreted according to the European Committee on Antimicrobial Susceptibility Testing [8]. Isolates were also subjected to standard conjugation assays using the recipient strain *Escherichia coli* HB101 [6]. Replicon typing, pMLST, hybridisation experiments (I-Ceul/S1-PFGE nuclease) [5,9] and detection of the insertion sequence element IS*Apl1* was performed in *Salmonella* strains and transconjugants. The presence and location of IS*Apl1* was determined using primers IS*Apl1*-Fw (5'-GTCGCTTTGGACATTGGGAA-3') and IS*Apl1*-Rv (5'-GATTGATGTCTTGGTGCTTCGG-3') designed as part of this study, and CLR5-R (5'-CTTGGTCGGTCTGTAGGG-3') [1]. Clonal relatedness of *Salmonella* strains was assessed by *Xba*I PFGE [5,6] and MLST [10].

## Detection of *mcr-1* gene in pig-associated clinically-relevant clones

The *mcr-1* gene was detected in 11 (1.1%) of the 1,010 Portuguese *Salmonella* isolates, recovered from human clinical sources and pork food products from across the country (Table 1, Table 2). This gene had 100% homology with the first published *mcr-1* sequence in an

TABLE 1

*Salmonella* isolates from different sources by year and presence of the *mcr-1* gene, Portugal, 2002–2015 (n = 1,010)

Source (number of isolates)	Years	Isolates tested for <i>mcr-1</i> (serotype/number of isolates) <sup>a</sup>	<i>mcr-1</i> -positive isolates (serotype/number of isolates) <sup>a</sup>
Human clinical cases (n = 522)	2002–10	258	0
	2011–12	155 ( <i>S.</i> 1,4,[5],12:i:-/n = 75)	4 ( <i>S.</i> 1,4,[5],12:i:-)
	2013–15	109	0
<b>Food products (n = 413)</b>			
Pork (n = 296)	2002–13	44	0
	2014–15	252 ( <i>S.</i> 1,4,[5],12:i:-/n = 130; <i>S.</i> Rissen/n = 23)	7 ( <i>S.</i> 1,4,[5],12:i:-/n = 5; <i>S.</i> Rissen/n = 2)
Other <sup>b</sup> (n = 117)	2002–15	117	0
<b>Food production animals (n = 58)</b>			
Pigs/piggeries (n = 54)	2006–08	54	0
Aquacultures (n = 4)	2010–12	4	0
Aquatic environment (n = 17)	2002–11	17	0

<sup>a</sup> The serotypes of *Salmonella* isolates are presented only for those among which *mcr-1*-positive ones were detected.

<sup>b</sup> Other studied food products comprised: poultry, beef, cow, quail, clam and cooked meals.

*Escherichia coli* strain from China (GenBank accession number: KP347127) [1], which was further described in diverse other *Enterobacteriaceae* including sporadic *Salmonella* isolates from European countries (France, the Netherlands, Spain, the United Kingdom) [2,11–14]. In most of these studies, detection of *mcr-1* gene was only performed in colistin-resistant isolates. This impairs the determination of its real prevalence because the gene may be silent, as described in one *E. coli* strain [15]. All our isolates carrying the *mcr-1* gene presented a minimum inhibitory concentration (MIC) of 4–8 mg/L for resistance to colistin (Table 2).

During the study period (2002 to 2015), *Salmonella* isolates harbouring the *mcr-1* gene were only recovered between 2011 and 2015 and originated from human clinical sources (0.8%, n = 4/522) and pork products, mostly from slaughterhouses, (2.4%, n = 7/296) (Table 1). Colistin has been widely used in veterinary medicine, particularly in food-producing animals, primarily in pigs [16,17]. The available data from 2004 to 2006 had already shown high use of colistin for food-producing animals in Portugal [18], which is one of the European countries with highest consumption of polymyxins that has been increasing in the last years (2011–13) [3,19]. Taking into account the current picture of colistin use in Portugal, the detection of *mcr-1* in the most recent collections and in pork products is of concern. Nevertheless, data on chronology, current prevalence of the *mcr-1* gene and its evolution in bacteria from animals, food and humans are lacking [3].

The 11 *mcr-1*-positive *Salmonella* isolates belonged to the serotypes *S.* 1,4,[5],12:i:- and *S.* Rissen (Table 2), which have been strongly associated with pig production and caused human infections in Europe [5,6,20–22] including in Portugal [23]. In both cases, we found them associated with particular successful

multidrug-resistant (MDR) clonal lineages, either of the *S.* Rissen/ST469 clone or the *S.* 1,4,[5],12:i:-/ST34 European clone that is currently spreading epidemically in European countries [5,20,22] and has been dominant in our *Salmonella* collection for the last years [5,6,20; unpublished data]. A previous report on *mcr-1* in the clinically relevant *Salmonella* serotype *S.* Typhimurium/ST34 was associated with travel to South-East Asia [13].

Of note, all *S.* 1,4,[5],12:i:- and *S.* Rissen *mcr-1*-carrying isolates were co-resistant to antibiotics used in a human and/or veterinary context and carried diverse metal tolerance genes, remarkably those conferring tolerance to copper (all carrying *pcoD*+*silA* on the chromosome) (Table 2), a feed additive mostly used for pigs or piglets in Europe. The fact that these successful clones presented higher tolerance to copper, as previously demonstrated [6,20], can contribute to their selection and wider expansion with potential repercussions for *mcr-1* transmission.

### Location of *mcr-1* gene in diverse plasmid backbones

The *mcr-1* gene was located on two plasmid types, IncX4 (n = 5; 35 kb; 4 transferable) and IncHI2 (n = 6), either of ST4 subtype (n = 3; 200–300 kb; all transferable) or non-typeable (n = 3; 120–125 kb; all non-transferable) and mostly associated with the IS*Apl1* transposable element (Table 2). IncHI2/ST4 and IncX4 plasmids have been widely implicated in the spread of *mcr-1* gene in diverse *Salmonella* serotypes and other *Enterobacteriaceae* in European and non-European countries, both from human and animal sources [2,12–14]. Transferability of the *mcr-1* gene was achieved from *S.* Rissen (n = 1) and *S.* 1,4,[5],12:i:- (n = 6) isolates and was associated with a 32–64-fold increase in the colistin MIC and, in some isolates, with acquisition of

**TABLE 2**

Characterisation of *Salmonella* isolates recovered from clinical and food samples and carrying the *mcr-1* gene, Portugal, 2011–2015 (n = 11)

Serotype <sup>a</sup> (number of isolates)	Source-origin (number of isolates)	Clone designation; ST(eBG); PFGE-type <sup>b</sup> (number of isolates, source)	Year/ Regions	Antibiotic resistance phenotype/genotype <sup>c</sup> (number of isolates)	Metal tolerance genes (number of isolates) <sup>d</sup>	Plasmid-mediated colistin resistance <i>mcr-1</i>		
						Colistin MIC-mg/L, donor (transconjugant/ number of isolates) <sup>e</sup>	Plasmid type carrying <i>mcr-1</i> gene (pMLST, Kb) <sup>f</sup> (number of isolates)	ISA <sub>1</sub> upstream of <i>mcr-1</i> (number of isolates)
1,4,[5],12:i:- (n=9)	Clinical faeces/blood (n=4)	European clone; ST34(eBG1); C (n=1, 1 hospital), E (n=3, 2 hospitals)	2011–12 North	AMP, (GEN), STR, SUL, TET/ <i>bla</i> <sub>TEM</sub> , [ <i>aac</i> (3)-IV], <i>strA</i> - <i>strB</i> , <i>sul2</i> , <i>tet</i> (B) (n=4)	<i>pcdD</i> + <i>silA</i> + <i>merA</i> + ( <i>terF</i> ) (n=4)	4–8 (4–8/n=1)	X <sub>4</sub> (35) (n=1) HI2 (NT, 120–125) (n=3)	No (n=1) Yes (n=3)
	Pork carcass (n=4)	European clone; ST34(eBG1); A (n=1, 1 slaughterhouse), B (n=2, 2 slaughterhouses), F (n=1, 1 slaughterhouse)	2014–15 North, Centre	AMP, (CLO), (CIP, PEF), STR, SUL, TET, (TMP)/ <i>bla</i> <sub>TEM</sub> , ( <i>floR</i> )/( <i>catA</i> - <i>cmiA</i> ), ( <i>aadA1/aadA2</i> ) – <i>strA</i> - <i>strB</i> , <i>sul1-sul3/sul2</i> , <i>tet</i> (A)/ <i>tet</i> (B), ( <i>df</i> rA1/ <i>df</i> rA12) (n=4)	<i>pcdD</i> + <i>silA</i> + ( <i>merA</i> ) + ( <i>terF</i> ) (n=4)	4–8 (4–8/n=4)	X <sub>4</sub> (35) (n=2) HI2 (ST4, 230–300) (n=2)	No (n=2) Yes (n=2)
Rissen (n=2)	Pork meat (n=1)	European clone; STNew/ Single locus variant of ST34; B (n=1, 1 meat production unit)	2015 South	AMP, STR, SUL, TET/ <i>bla</i> <sub>TEM</sub> , <i>strA-strB</i> , <i>sul2</i> , <i>tetB</i> (n=1)	<i>pcdD</i> + <i>silA</i> + <i>merA</i> + <i>terF</i> (n=1)	4 (4/n=1)	HI2 (ST4, 200) (n=1)	No (n=1)
	Pork carcass (n=2)	ST469(eBG66); N (n=2, 2 slaughterhouses)	2014–15 North	AMP, CLO, STR, SUL, (TET), TMP/ <i>bla</i> <sub>TEM</sub> , <i>cmiA</i> , <i>aadA1</i> , <i>sul1-sul3</i> , [ <i>tet</i> (A)], <i>df</i> rA1 (n=2)	<i>pcdD</i> + <i>silA</i> + <i>merA</i> (n=2)	4 (4/n=1)	X <sub>4</sub> (35) (n=2)	No (n=2)

AMP: ampicillin; CIP: ciprofloxacin; CLO: chloramphenicol; GEN: gentamicin; MIC: minimum inhibitory concentration; PEF: pefloxacin; pMLST: plasmid multilocus sequence type; STR: streptomycin; SUL: sulfamethoxazole; TET: tetracycline; TMP: trimethoprim.

<sup>a</sup> The serotypes of *Salmonella* isolates were determined by classical serotyping, performed at the National Centre of Salmonella (NSA, Lisbon, Portugal) and/or PCR assay for determination of S. 4:[5],12:i:- [5].

<sup>b</sup> PFGE types are designated by capital letters and include previously described types [5,6] and types described for the first time in this study. The human clinical isolates (n=4 from four patients) were recovered from three hospitals, and pork products (n=7) were recovered from six slaughterhouses and one meat production unit.

<sup>c</sup> Antimicrobial susceptibility was evaluated by disc diffusion assay. Variable antibiotic resistance phenotypes and genotypes are presented between brackets; Antibiotic resistance patterns and genes transferred by conjugation are underlined; In two S. 1,4,[5],12:i:- isolates, transfer of genes *strA-strB* and/or *bla*<sub>TEM</sub> was observed; Some genes were included on class 1 integrons (1,700bp (*df*rA1-*aadA1*) or 2,000bp (*df*rA12-*orfF-aadA2*)); Integrons were located on the chromosome in S. Rissen (n=2) and on the IncHI2/ST4 plasmid in S. 1,4,[5],12:i:- isolates (n=2).

<sup>d</sup> Screening for genes encoding tolerance to metals were done by PCR [20]. Metal tolerance genes that were not observed in all the isolates are presented between brackets; Metal tolerance genes transferred by conjugation are underlined. All *pcdD* + *silA* genes were chromosomally located.

<sup>e</sup> Recipient strain used in conjugation assays: *Escherichia coli* HB101 (azide sodium, resistant to streptomycin and kanamycin); colistin MIC = 0.125 mg/L).

<sup>f</sup> Plasmid types carrying the *mcr-1* gene transferred by conjugation are underlined.

resistance to other antibiotics and metals tolerance genes (Table 2). The fact that successful MDR *S. 1,4,[5],12:i:-* and *S. Rissen* clones have the ability to acquire plasmids carrying the *mcr-1* gene is of concern because colistin resistance may contribute to their further expansion, particularly in the pig reservoir. In addition, those strains could act as reservoir of *mcr-1*-carrying plasmids with a broad host range enhancing colistin resistance transmission for other clinically relevant bacteria sharing the same ecological niche.

## Conclusions

This study has evidenced the acquisition of *mcr-1*-carrying plasmids by two clinically relevant MDR and copper-tolerant clones of *S. 1,4,[5],12:i:-* and *S. Rissen*, strongly associated with pork food products and which were dominant in the collection studied. The detection of *S. 1,4,[5],12:i:-* from human infections, already in 2011, is also of note, suggesting long-term dissemination of this resistance gene in humans in Portugal. Finally, the detection of *mcr-1* in copper-tolerant clones raises questions about the efficacy of recently suggested metal-based interventions (e.g. copper) to reduce the use of colistin and contain *mcr-1* dissemination [3].

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## Conflict of interest

None declared.

## Authors' contributions

JC, LP and PA designed the study and analysed epidemiological, microbiological and molecular data, JC and LC performed the phenotypic and molecular assays, JC and PA wrote the first draft of the manuscript, PA and LP participated in the coordination and concept of the manuscript and revised the final version.

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