Rapid communication

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 (IncX4/IncHI2) in pig-associated multidrug-resistant, copper-tolerant S.1,4,[5],12:i:-/ST34 and S. Rissen/ST469 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’-CGGTCAGTCCGTTTGTTC-3’) and Mrc1-Rv2 (5’-CCAGCGTATCCAGCACATTT-3’) [1] and Mrc1-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-CeuI/S1-PFGE nuclease) [5,9] and detection of the insertion sequence element ISApl1 was performed in Salmonella strains and transconjugants. The presence and location of ISApl1 was determined using primers ISApl1-Fw (5’-GTCGCTTTGGACATTGGGAA-3’) and ISApl1-Rv (5’-GATTGATGTCTTGGTGCTTCGG-3’) designed as part of this study, and CLR5-R (5’-CTTGCGGTCTGCTAGGG-3’) [1]. Clonal relatedness of Salmonella strains was assessed by XbaI 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
we found them associated with particular successful
[5,6,20-22] including in Portugal [23]. In both cases,
production and caused human infections in Europe
2), which have been strongly associated with pig
in cases of the serotypes
Salmonella
during the study period (2002 to 2015),
4–8 mg/L for resistance to colistin (Table 2).
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2). Colistin has been widely used in veterinary medi-
1). Colistin has been widely used in veterinary medi-
cine, particularly in food-producing animals, primar-
icles harbouring the
mcr-1
gene were only recovered
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 pic-
ture 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 bac-
teria 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 epidemic-
ally 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-carry-
ing 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 suc-
cessful clones presented higher tolerance to copper, as
previously demonstrated [6,20], can contribute to their
selection and wider expansion with potential repercus-
sions 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 transfer-
able) or non-typeable (n = 3; 120–125 kb; all non-
transferable) and mostly associated with the IS
Apil
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

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

* The serotypes of
Salmonella
isolates are presented only for those among which
mcr-1-positive ones were detected.
* Other studied food products comprised: poultry, beef, cow, quail, clam and cooked meals.
Table 2

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

<table>
<thead>
<tr>
<th>Serotype (number of isolates)</th>
<th>Source-origin (number of isolates)</th>
<th>Clone designation; ST(eBG); PFGE-type (number of isolates, source)</th>
<th>Year/ Regions</th>
<th>Antibiotic resistance phenotype/genotype (number of isolates)</th>
<th>Metal tolerance genes (number of isolates)</th>
<th>Plasmid-mediated colistin resistance <em>mcr-1</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1,4,[5],12:i:- (n = 9)</td>
<td>Clinical faeces/blood (n = 4)</td>
<td>European clone; ST14(eBG1); C (n = 1, 1 hospital), E (n = 3, 2 hospitals)</td>
<td>2011–12 North</td>
<td>AMP, (GEN), STR, SUL, TEM, bla&lt;sub&gt;TEM&lt;/sub&gt; (J&lt;sup&gt;loc&lt;/sup&gt;—IV), strA-strB, sul2, tet(B) (n = 4)</td>
<td>pcoD + silA + merA + (terF) (n = 4)</td>
<td>X&lt;sub&gt;4&lt;/sub&gt; (35) (n = 4) H&lt;sub&gt;2&lt;/sub&gt; (NT, 120–125)</td>
</tr>
<tr>
<td></td>
<td>Pork carcass (n = 4)</td>
<td>European clone; ST14(eBG1); A (n = 1, 1 slaughterhouse), B (n = 2, 2 slaughterhouses), F (n = 1, 1 slaughterhouse)</td>
<td>2014–15 North, Centre</td>
<td>AMP, (CLO), (CIP), PEF, STR, SUL, TEM, (TMP), bla&lt;sub&gt;TEM&lt;/sub&gt;, (J&lt;sup&gt;loc&lt;/sup&gt;—IV), (catA—cmiA), (aadA1/aadA2) – strA-strB, sul2-sul3/sul2, tet(A)/tet(B), (dfrA1/dfrA12) (n = 4)</td>
<td>pcoD + silA + (merA) + (terF) (n = 4)</td>
<td>X&lt;sub&gt;4&lt;/sub&gt; (35) (n = 2) H&lt;sub&gt;2&lt;/sub&gt; (ST&lt;sub&gt;4&lt;/sub&gt;, 230–300)</td>
</tr>
<tr>
<td></td>
<td>Pork meat (n = 1)</td>
<td>European clone; STNew/ Single locus variant of ST134; B (n = 1, 1 meat production unit)</td>
<td>2015 South</td>
<td>AMP, STR, SUL, TEM, bla&lt;sub&gt;TEM&lt;/sub&gt;, strA-strB, sul2, tet(B) (n = 1)</td>
<td>pcoD + silA + merA + terF (n = 4)</td>
<td>4 (n = 1)</td>
</tr>
<tr>
<td></td>
<td>Pork carcass (n = 1)</td>
<td>ST469(eBG66); N (n = 2, 2 slaughterhouses)</td>
<td>2014–15 North</td>
<td>AMP, CLO, STR, SUL, TEM, TMP, bla&lt;sub&gt;TEM&lt;/sub&gt;, cmlA, aadA1, sul1-sul3, tet(A), dfrA1 (n = 2)</td>
<td>pcoD + silA + merA (n = 2)</td>
<td>X&lt;sub&gt;4&lt;/sub&gt; (35) (n = 2)</td>
</tr>
</tbody>
</table>

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

* The serotypes of *Salmonella* isolates were determined by classical serotyping, performed at the National Centre of Salmonella (INSA, Lisbon, Portugal) and/or PCR assay for determination of *S. 1,4,[5],12:i:-*.

* 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.

* 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 (dfrA1-aadA1) or 2,000bp (dfrA12-orfF-aadA2)); Integrons were located on the chromosome in *S. 1,4,[5],12:i:-* isolates (n = 2) and on the IncHI2/ST4 plasmid in *S. Rissen* (n = 2).

* 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 *pcoD + silA* genes were chromosomally located.

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

* 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:: 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:: 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:: 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.

References


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