Molecular epidemiology of isolates with multiple mcr plasmids from a pig farm in Great Britain: the effects of colistin withdrawal in the short and long term

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Background: The environment, including farms, might act as a reservoir for mobile colistin resistance (mcr) genes, which has led to calls for reduction of usage in livestock of colistin, an antibiotic of last resort for humans.

Objectives: To establish the molecular epidemiology of mcr Enterobacteriaceae from faeces of two cohorts of pigs, where one group had initially been treated with colistin and the other not, over a 5 month period following stoppage of colistin usage on a farm in Great Britain; faecal samples were also taken at ~20 months.

Methods: mcr-1 Enterobacteriaceae were isolated from positive faeces and was WGS performed; conjugation was performed on selected Escherichia coli and colistin MICs were determined.

Results: E. coli of diverse ST harbouring mcr-1 and multiple resistance genes were isolated over 5 months from both cohorts. Two STs, from treated cohorts, contained both mcr-1 and mcr-3 plasmids, with some isolates also harbouring multiple copies of mcr-1 on different plasmids. The mcr-1 plasmids grouped into four Inc types (X4, pO111, I2 and H12), with mcr-3 found in IncP. Multiple copies of mcr plasmids did not have a noticeable effect on colistin MIC, but they could be transferred simultaneously to a Salmonella host in vitro. Neither mcr-1 nor mcr-3 was detected in samples collected ~20 months after colistin cessation.

Conclusions: We report for the first known time on the presence in Great Britain of mcr-3 from MDR Enterobacteriaceae, which might concurrently harbour multiple copies of mcr-1 on different plasmids. However, control measures, including stoppage of colistin, can successfully mitigate long-term on-farm persistence.

Introduction

Horizontal gene transfer and plasmid/mobile genetic elements are a major driving force behind the dissemination of antimicrobial resistance (AMR), causing gene transfer between commensals and pathogenic bacteria. The discovery by Liu et al. of the plasmid-borne gene mcr-1 in Enterobacteriaceae harbouring resistance to colistin, an antibiotic of last resort, raised serious concerns. These add to fears about the rapid rise in MDR Enterobacteriaceae already restricting treatment options in humans, combined with animals and the environment potentially acting as a reservoir for important AMR genes. Since first publication there has been global reporting of mcr-1 in Enterobacteriaceae, including in Great Britain (GB). In 2015, mcr-1-positive Enterobacteriaceae were identified on a pig farm using colistin for treatment of diseased piglets and in archived human Salmonella samples. Following the detection of mcr-1 in GB, hundreds of archived pig isolates and faeces were tested for the presence of the gene, which was detected in three Escherichia coli from a further three GB pig farms, although overall prevalence was estimated to be very low. Multiple types of mcr and variants have subsequently been published or submitted to GenBank and it has been detected in E. coli from China isolated in the 1980s. The origins of mcr-1 and mcr-2 have been linked to Moraxella, from where it is thought to have mobilized, and which might be a persistent source of mcr. The deluge of reports on mcr-harbouring bacteria isolated from humans, animals and the environment worldwide is concerning. Therefore, it is important to assess the effect, if any, of cessation of on-farm colistin usage.

This work follows on from studies by Anjum et al. and Randall et al. by focusing on gaining a better understanding of the...
molecular epidemiology of mcr-positive Enterobacteriaceae, circulating plasmids and their on-farm persistence. mcr-1-positive Enterobacteriaceae isolates purified from pig faecal samples, collected from multiple visits to a farm after colistin treatment had stopped, were characterized using WGS. Faecal samples were collected from two cohorts of pigs on the same farm, one that had initially been treated with colistin following diarrhoea, and another, younger cohort that was untreated. Pig faecal samples were also collected from two cohorts of pigs on the same farm, one that had initial diagnostic submission and 15 months after the initial sampling can be found in Randall et al. (2016). A subset of positive faecal samples from each location on the farm from September 2015 to January 2016; also roughly equal numbers of isolates were chosen from each sampling point and pig cohort (Table S1). Illumina short-read assemblies contained all mcr-1 and mcr-3 plasmids in single contigs (apart from IncHI2) and were sufficiently long to determine the presence of a mcr gene; approximate sizes of the mcr plasmids were established using these contigs. Additional PacBio SMRTcell sequencing on the RSII platform was completed on three mcr-positive faecal/slurry samples taken from different pigs. The presence of mcr-1 in Enterobacteriaceae was determined by RT–PCR, as previously described.5,8

### Strain selection

#### Faecal selection

Faecal samples were collected at four timepoints from a pig farm in GB (October 2015, December 2015, January 2016 and May 2017; Table 1) following isolation of mcr-1-positive Enterobacteriaceae from a reference negative diagnostic submission in September 2015.5,6 Two slurry samples of composite faecal material were also obtained at the third sampling and processed in the same way as the faecal samples. Colistin had previously been used to control diarrhoea in weaner pigs on this farm, and amoxicillin, lincomycin/spectinomycin, zinc oxide and fleroxacin had been used in feed during disease outbreaks. Colistin use on the farm was withdrawn after the initial diagnostic submission. The samples were from two cohorts of pigs on the same farm —20 months after the initial diagnostic submission and 15 months after the initial sampling can be found in Randall et al. (2016). A subset of positive faecal samples from each location on the farm from September 2015 to January 2016; also roughly equal numbers of isolates were chosen from each sampling point and pig cohort (Table S1). Illumina short-read assemblies contained all mcr-1 and mcr-3 plasmids in single contigs (apart from IncHI2) and were sufficiently long to determine the presence of a mcr gene; approximate sizes of the mcr plasmids were established using these contigs. Additional PacBio SMRTcell sequencing on the RSII platform was completed on three mcr-positive faecal/slurry samples taken from different pigs. The presence of mcr-1 in Enterobacteriaceae was determined by RT–PCR, as previously described.5,8

#### Plasmid conjugation and mcr detection

The rate of conjugation for the mcr plasmids was established using a 1:3 or 1:10 ratio of recipient to donor, incubation at 25 or 37°C and the use of a filter-mating technique as previously described.5 Presence of mcr-1, -2 or -3 and IncA/C types in the transconjugants was determined by PCR or RT-PCR using the same running conditions as previously described and the primers are listed in the Methods section of the Supplementary data.8

#### MIC determination

The MIC of colistin for the isolates was established by broth microdilution using EUCAST-recommended control strains (Pseudomonas aeruginosa ATCC 27853 and E. coli ATCC 25922).
Results

E. coli host, AMR genes and plasmids

E. coli isolates characterized in this study by WGS were positive for mcr-1 by RT–PCR and purified from a representative selection of pig faecal samples collected over a 5 month period, from two cohorts of pigs (Table 1).

The E. coli had 15068 genes in the pangenome, and ~12.5% (1891) core genes, indicating dissemination of mcr-1 plasmids to a diverse range of isolates, which was also reflected in the core genome SNP-based phylogenetic tree (Figure 1). E. coli within the treated and untreated cohorts shared ~14.3% and ~33.5% of core genes, respectively, the latter possibly being higher due to fewer isolates being sampled (Table 2). The 53 E. coli were...
assigned to 19 unique STs, with similar diversity of STs being present in each cohort; ST10 was the most prevalent (n = 11; Figure S1).

Despite the overall diversity of STs, isolates originating from the same cohort (treated or untreated) but from different on-farm locations/sampling points generally clustered together. However, for some STs (ST10, ST23, ST1202 and ST6426) closely related isolates were found in both cohorts and slurry, as well as different locations, indicating widespread dissemination (Figure 1; Table S1). During analysis of the mcr-1-positive isolates, multiple reports of further mcr types were published. Retrospective in silico analysis of WGS data identified mcr-3 in seven of the isolates that also contained mcr-1 and in one isolate that did not contain mcr-1 but had mcr-3, mcr-2, -4, -5 and -6 were not detected. RT-PCR performed on 60 samples taken on-farm ~20 months after the initial diagnostic submission were negative for mcr-1, -2 and -3 and therefore no isolates from this time period were sequenced. These samples were not tested for mcr-4, -5 or -6 as these mcr types had not been identified from previous samplings.

Fifteen Inc types were identified and the number of plasmids present in isolates was between three and eight, with little difference in the average numbers present in each cohort/slurry at individual samplings (Table S2). However, seven isolates from the treated cohort and one from the diagnostic submission contained seven or more plasmids (Figure 2). Only four Inc types contained mcr-1: IncX4, IncI2, pO111 and HI2. Seven mcr-1 E. coli also harboured mcr-3 on an IncP-type plasmid (Figure 1). The more prevalent mcr-1 plasmids were present in isolates from both cohorts of pigs, and although the proportion of isolates with mcr-1 IncX4 and IncI2 plasmids varied in each cohort, pO111 proportions were similar (Table 2). Absence of IncHI2 and IncP in the untreated cohort and one from the diagnostic submission contained mcr-3 IncX4 in E5, an isolate that also contained mcr-3 on an IncP (Table S2).

### Characterization of mcr-1 plasmids

InX4-type mcr-1 plasmids were the most abundant (n = 22); they were detected in E. coli isolates from both cohorts (Tables 2 and 3). The IncX4-type mcr-1 plasmids could be separated into two sizes, one set ranging from 17.1 to 18.8 kb and the other set ranging from 29.8 to 33.8 kb. There was more homogeneity of gene content within mcr-1 IncX4 plasmids in either size set compared with other mcr-1 plasmids detected (Figure 3 and Figures S2 and S3). The larger IncX4 plasmids were detected in the first, second and third sampling points from both cohorts, but the smaller IncX4 plasmids, which had lost the type-IV secretion system genes present in the larger variant, were only detected in isolates from the second and third samplings from the treated cohort (Figure 3). The complete loss of genes in smaller plasmids was confirmed by mapping against the circularized larger plasmid variant present in R85 (Supplementary data). One of the IncX4-type plasmids contained a novel mcr-1 variant with a non-synonymous SNP, M62L (mcr-1.14; GenBank accession number LS398440). Conjugation experiments indicated that IncX4-type mcr-1 plasmids were transferable to a recipient Salmonella strain (S627), with no observable rate change in the smaller plasmids. In IncX4-type plasmids, mcr-1 was located next to pap2 with the dinucleotide signature from the loss of a transposon downstream. Conversely, IncI2-type mcr-1 plasmids, which also contained the conserved mcr-1–pap2 unit, contained the trinucleotide signature upstream of mcr-1.

IncX4 and IncI2 plasmids shared three other genes (dnaJ, hicB and a hypothetical protein). For the IncI2-type plasmids, the mcr-1–pap2 unit was downstream of a hypothetical protein and an insertion element belonging to the IS3 family. The 13 IncI2-type mcr-1 plasmids were originally only found in Salmonella isolated from the initial diagnostic submission and sampling 1 (Figure 2), before being found in E. coli, from the second and third samplings from both cohorts, although they were more prevalent in the untreated cohort (Table 2). The plasmid size remained stable at 60.4–63.9 kb throughout the study and IncI2 was transferred by conjugation to S627 (Table 3).

There was no mcr-1–pap2 unit found in the pO111-type mcr-1 plasmids, which had ISAp1 upstream, as in pSHP45. The pO111-type mcr-1 plasmids were the second most abundant, with 19 identified across all timepoints and cohorts. The size of these plasmids ranged from 78.6 to 95.7 kb. E4, which was isolated from the initial diagnostic samples, was smaller than pO111-type mcr-1

### Table 2. Numbers of isolates subjected to WGS, unique STs and mcr plasmid Inc types from the treated and untreated cohorts

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Number of sequenced isolates</th>
<th>E. coli STs</th>
<th>mcr-1 plasmids</th>
<th>mcr-3 plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated</td>
<td>36</td>
<td>15 (42%)</td>
<td>13 (36%)</td>
<td>5 (14%)</td>
</tr>
<tr>
<td>Untreated</td>
<td>19</td>
<td>8 (42%)</td>
<td>6 (32%)</td>
<td>5 (26%)</td>
</tr>
</tbody>
</table>

Relative proportions are shown in parentheses. 
*Includes both Salmonella isolates.*
Figure 2. Matrix of plasmid incompatibility type and their mcr or other AMR gene content in all isolates. This was determined by identification of AMR genes on the same contig as a plasmid rep gene. Isolates from the cohort treated with colistin are shown in red. S3–E5 are the diagnostic submissions, RB2–SS22215 are the first sampling, FFV8–FFV127 are the second sampling and SFV1–SFV146 are the third sampling. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
Table 3. Number of mcr plasmids identified in this study according to their incompatibility type, size, conjugation rate to S627, and core, accessory and pangenomes.

<table>
<thead>
<tr>
<th>Inc-type</th>
<th>Plasmid-mediated colistin resistance</th>
<th>Number identified</th>
<th>Size range (kb)</th>
<th>Conjugation rate (cfu/recipient cfu)</th>
<th>Genes</th>
<th>core (%)</th>
<th>core accessory total</th>
</tr>
</thead>
<tbody>
<tr>
<td>X4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>mcr-1</td>
<td>22</td>
<td>17.1–33.3</td>
<td>7.9 × 10&lt;sup&gt;–7&lt;/sup&gt;</td>
<td>23</td>
<td>20</td>
<td>43</td>
</tr>
<tr>
<td>I2</td>
<td>mcr-1</td>
<td>13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60.4–63.9</td>
<td>3.3 × 10&lt;sup&gt;–8&lt;/sup&gt;</td>
<td>70</td>
<td>14</td>
<td>84</td>
</tr>
<tr>
<td>pO111&lt;sup&gt;a&lt;/sup&gt;</td>
<td>mcr-1</td>
<td>19</td>
<td>78.6–95.7</td>
<td>–</td>
<td>74</td>
<td>49</td>
<td>123</td>
</tr>
<tr>
<td>H2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>mcr-1</td>
<td>2</td>
<td>243</td>
<td>–</td>
<td>241</td>
<td>63</td>
<td>304</td>
</tr>
<tr>
<td>P&lt;sup&gt;a&lt;/sup&gt;</td>
<td>mcr-3</td>
<td>8</td>
<td>50</td>
<td>1.07 × 10&lt;sup&gt;–7&lt;/sup&gt;</td>
<td>67</td>
<td>0</td>
<td>67</td>
</tr>
</tbody>
</table>

<sup>a</sup>Indicates plasmid types that were circularized by hybrid assembly.

<sup>b</sup>Includes both Salmonella isolates.

Figure 3. mcr-1 IncX4 plasmids from the first (a), second (b) and third (c) samplings, showing the loss of virB genes in some plasmids from later samplings. (d) The gene presence/absence dendrogram reflects the two IncX4 plasmid sets, with different-sized genomes circulating in the isolates from all three samplings. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
plasmids isolated from subsequent samplings. There was more heterogeneity in the accessory genes in this group of plasmids than observed in other mcr-1 plasmid types (Figure S2). PHASTER\textsuperscript{17} indicated that ~12% of pO111 genes were of bacterial origin and the remaining 88% were from a range of Enterobacteriaceae phages. Two isolates from sampling 1 harboured pO111-type mcr-1 plasmids and also harboured the larger IncX4 mcr-1 plasmid (Figure 1). We did not detect transfer of any of the pO111 plasmids by conjugation. Only two mcr-1 IncHI2 plasmids were identified, both in E. coli isolated from the treated cohort at sampling 3, and both had ISApI1 upstream of mcr-1. We were also unable to detect transfer of plasmids from either isolate to Salmonella S627 by conjugation. The IncHI2-type plasmids were much larger than the other mcr-1 plasmids, at ~246 kb. These were present in an ST (ST117) distinct from those of other on-farm isolates (Figure 1 and Figure S1).

Characterization of mcr-3 plasmids

We identified mcr-3 on an IncP-type plasmid in seven isolates, assigned to two STs (1011 and 3014) that also harboured mcr-1. These were only isolated from the treated cohort and were found in all samplings except the last after ~20 months (Figure 1). mcr-3 was also found on IncP in one isolate (E5) that did not contain mcr-1, from the initial diagnostic submission. IncP plasmids without mcr-3 were detected in isolates from both cohorts; these plasmids were distinct from those containing mcr-3 (Figure S4). mcr-3 was found in all seven isolates contained the same variant, mcr-3.7. The mcr-3 IncP-type plasmid, which was transferable, was ~50 kb, with no change in gene content observed over time (Table 3). One of the mcr-3-positive isolates (SFV146) originated from the slurry sample obtained in January and was closely related to isolates from the first sampling, indicating the presence of this clone and plasmid in the environment. Conjugation experiments using the recipient Salmonella S627 showed that each time the mcr-3-harboung plasmid was transferred, an mcr-1-harboung plasmid was also transferred (Supplementary data). The mcr-3 IncP-type plasmid found in these isolates was most similar to pMCR3_WCHEC-LL123 (GenBank accession number MF689760.1) from an E. coli strain originating in China, with which it showed 99% identity (Figure S5).\textsuperscript{18} Inverted repeats of TnAs2 flanked a 3.6 kb fragment that included mcr-3, as found in pWJ1.\textsuperscript{10}

MIC of colistin

Colistin MICs were determined to measure the effect, if any, on phenotypic resistance of isolates harbouring multiple copies of mcr and chromosomal mutations in genes linked to colistin resistance. The MICs of colistin for these isolates ranged from 4 to 16 mg/L, which is above the EUCAST breakpoint for resistance. The majority of isolates (n = 32) had an MIC of 8 mg/L. However, we did not detect an association between increased colistin MICs and the presence of multiple mcr plasmids and mutations in chromosomal genes.

Discussion

The aim of this study was to determine the effect, if any, of stopping colistin use on an mcr-positive farm, both in the short and long term, using WGS. Isolates were derived from faeces collected from a cohort of pigs that had previously been treated with colistin to control diarrhoea, and where various antimicrobials had been used during disease outbreaks. Isolates were also derived from farm slurry and faeces of a younger cohort that had not been treated with colistin, but overlapped in their period on the farm with the first cohort. The results confirmed that Enterobacteriaceae harbouring mcr-1 plasmids had become widely disseminated throughout the farm during this 5 month period. Presence in the younger untreated cohort may be because these pigs had moved into accommodation previously occupied by the older treated cohort, which could have had environmental contamination with mcr-1 E. coli.\textsuperscript{8} A concurrent microbiological study on the prevalence of mcr-1 E. coli in faecal samples collected from both cohorts nevertheless indicated mcr-1 prevalence was low (~0.5%).\textsuperscript{6}

Additionally, mcr-1 Enterobacteriaceae were not detected when the farm was re-sampled after 20 months, indicating that if mcr-1 was present, its prevalence was <5% with 95% confidence. The lack of mcr-1 and mcr-3 detection from a new cohort of pigs, ~20 months after the initial isolates were obtained and 15 months after the mcr-positive cohorts were slaughtered, indicates that it is possible to successfully mitigate on-farm risk of contamination and persistence by mcr-harboung Enterobacteriaceae through cessation of colistin use, alongside improved cleaning, disinfection and biosecurity. This success is especially important because colistin has been moved to the list of highest-priority critically important antimicrobials (HP-CIAs) by the WHO due to the discovery of mcr-1 and mcr-2.\textsuperscript{2}

Phylogenetic analysis of the host E. coli generally reflected the diverse nature of isolates in both cohorts; there was evidence that some STs and plasmids persisted within ‘treated’ or ‘untreated’ cohorts, while others were present in both cohorts. However, it is feasible that these isolates represent a subset of the population and are the most prevalent types, as single representative mcr-1-positive E. coli were selected for further characterization from positive faecal samples to enable us to capture the breadth of mcr-harboung E. coli. Nevertheless, others have noted the diversity of host E. coli harbouring mcr-1\textsuperscript{8,19–21} and despite the spread of mcr-1 to different hosts and multiple plasmids being present in these hosts, mcr-1 was only detected in four plasmid Inc types in our study.

In this study, colistin-resistant isolates were initially screened based on mcr-1 presence, as other mcr variants had not been reported. Results showed differences in the proportions of mcr-1-harboung IncX4 and Inc12 plasmids present in the two cohorts, while for pO111 the proportion remained similar, the reasons behind these differences was unclear. Furthermore, retrospective discovery of mcr-3, following its reporting on an IncP-type plasmid, and absence of other variants indicated the power of using WGS. This study is also the first time that mcr-3 has been reported in GB. Our study showed simultaneous presence of mcr-1 and mcr-3 in the majority of mcr-3-positive isolates, which were from the treated cohort. As with the aforementioned mcr-1 plasmid distributions, we do not know whether this difference resulted from bias in the initial screening process or is a reflection of the natural on-farm circumstances, where mcr-1/mcr-3-harboung isolates were more prevalent in the treated cohort; we also do not know why mcr-3 was not detected in isolates from the untreated cohort. mcr-3 has been reported in multiple plasmid types, indicating the
ability of the mcr-3 gene to transfer, possibly via the TnAs2-like transposon flanking this gene, which was also present in our plasmids. Studies published in 2017 from Denmark, Spain and China have found the co-occurrence of mcr-1 and mcr-3 plasmids in the same host Enterobacteriaceae; however, these studies reported only on single isolates. 

Recently Zhong et al. have speculated on the presence of multiple mcr-1 in Enterobacteriaceae isolates from humans. In this study, we demonstrate conclusively not only that Enterobacteriaceae such as E. coli can harbour multiple copies of mcr-1 on different plasmids, but also that the same isolate can harbour mcr-3 on another plasmid. In addition, the co-transfer of mcr-1 and mcr-3 plasmids concurrently from an E. coli donor to a recipient Salmonella might be of concern given the zoonotic nature of Salmonella. Therefore, the presence within Enterobacteriaceae of multiple mcr plasmids harbouring different mcr variants, which are stably maintained, especially in the short term after treatment with colistin, is likely to be a natural occurrence. Although this highlights how multiple plasmid-mediated colistin resistance could readily spread to other bacterial hosts within the microbiome, the implication of this redundancy is not yet apparent.

Even though the primary focus of this study was on colistin resistance mediated through mcr plasmids, the discovery of other AMR genes in these isolates was concerning as they may enable co-selection and persistence of mcr bacteria on this farm as a result of therapeutic treatments during disease outbreaks. Genes conferring resistance to the HP-CIAs, such as gentamicin, ampicillin and ciprofloxacin, were identified in a large proportion of isolates. Additionally, 36 of the isolates were resistant to seven or more classes of antimicrobials, including classes that had not been used for therapeutics.

In conclusion, this study indicated that although mcr E. coli had become widespread on a farm in England, measures taken to mitigate on-farm risk were successful. Differences found between mcr plasmids present in the treated and untreated cohorts mean that further work is needed and could be associated with fitness cost, especially as multiple mcr plasmids were present even 104 days after colistin treatment ceased and in the untreated cohort. As mobile genetic elements have been implicated in the spread of resistance genes, detailed understanding of how plasmids adapt and disseminate will help in the successful application of control measures.

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Supplementary data
Supplementary Methods, Tables S1 and S2 plus Figures S1 to S5 appear as Supplementary data at JAC Online.

References


