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The characterization of mobile colistin resistance (mcr) genes among 33 000 Salmonella enterica genomes from routine public health surveillance in England

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Abstract
To establish the prevalence of mobile colistin resistance (mcr) genes amongst Salmonella enterica isolates obtained through public health surveillance in England (April 2014 to September 2017), 33 205 S. enterica genome sequences obtained from human, food, animal and environmental isolates were screened for the presence of mcr variants 1 to 8. The mcr-positive genomes were assembled, annotated and characterized according to plasmid type. Nanopore sequencing was performed on six selected isolates with putative novel plasmids, and phylogenetic analysis was used to provide an evolutionary context for the most commonly isolated clones. Fifty-two mcr-positive isolates were identified, of which 32 were positive for mcr-1, 19 for mcr-3 and 1 for mcr-5. The combination of Illumina and Nanopore sequencing identified three novel mcr-3 plasmids and one novel mcr-5 plasmid, as well as the presence of chromosomally integrated mcr-1 and mcr-3. Monophasic S. enterica serovar Typhimurium accounted for 27/52 (52 %) of the mcr-positive isolates, with the majority clustering in clades associated with travel to Southeast Asia. Isolates in these clades were associated with a specific plasmid range and an additional extended-spectrum beta-lactamase genotype. Routine whole-genome sequencing for public health surveillance provides an effective screen for novel and emerging antimicrobial determinants, including mcr. Complementary long-read technologies elucidated the genomic context of resistance determinants, offering insights into plasmid dissemination and linkage to other resistance genes.

DATA SUMMARY
All fastq files and assemblies were submitted to the National Centre for Biotechnology Information (NCBI). All data can be found under BioProject: PRJNA248792, PRJNA248064 https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA315192. Strain-specific details can be found in Table S1.

INTRODUCTION
Colistin has been utilized as an antimicrobial last resort against Gram-negative bacteria exhibiting pan-resistance to multiple antimicrobials [1]. Colistin resistance has historically been attributed to intrinsic resistance or chromosomal mutations that are not transmissible within the population; however, the acquisition of mobile colistin resistance (mcr) genes borne on plasmids has recently been described [2, 3]. Since 2015, eight variants of plasmid-mediated mcr genes have been detected in Gram-negative bacteria, including Salmonella enterica, Escherichia coli, Moraxella spp. and Klebsiella pneumoniae isolated from clinical human cases as well as pigs, calves, poultry, food and environmental sources [3–9].

The variety of mcr genes reported is mirrored in the variability of their genetic context. For mcr-1, at least 12 different
plasmid replicons have been described, namely: IncX3, IncX4, an IncX3-4 hybrid, IncH1, IncH11, IncH12, IncP, IncI2, IncF, IncFII, an IncI2-IncFIB hybrid and IncY [3, 10–12]. mcr-2 has been associated with an IncX4 plasmid [8], mcr-3 has been found on an IncH12, IncP and an IncR plasmid type [9, 11, 13], and both mcr-4 and mcr-5 have been described on a ColE-like plasmid [5, 6]. mcr-6 has been integrated into the chromosome and mcr-7 and mcr-8 have been reported on an IncI2 and IncFII plasmid, respectively [4, 7, 14]. Recently, mcr-9 was described in a *Salmonella* Typhimurium isolated in 2010 from a human patient in the USA [15].

Whole-genome sequencing (WGS) of bacterial isolates facilitates the detection of known resistance genes and resistance-associated mutations. The adoption of WGS for routine pathogen surveillance has consequently improved antimicrobial resistance (AMR) surveillance by enabling earlier detection of resistant strains [16] and by providing a framework for passive AMR surveillance. In 2016, a retrospective study to understand the prevalence of *mcr-1* in England identified 15 *mcr-1*-positive isolates within a large collection of genomes obtained from the surveillance of Gram-negative pathogens by Public Health England [17].

The aim of this study was to further investigate the prevalence of *mcr-1* to -8 in *S. enterica* isolates submitted to Public Health England (PHE) through routine surveillance, to characterize the genomic context of *mcr* and to provide an insight into the dissemination of *mcr*-mediated colistin resistance in England from 2012 to September 2017.

**METHODS**

**WGS isolates**

Since April 2014, WGS analysis has been performed routinely for the identification and typing of presumptive *S. enterica* isolates at PHE. Data obtained from WGS have been deposited and archived in the National Center for Biotechnology Information (NCBI) Sequence Read Archive – BioProject: PRJNA248064. From April 2014 to September 2017, 31,292 *Salmonella* isolates were referred; 27,611 were isolated from human faeces, 14,690 were isolated from human blood isolates, 335 were isolated from human urine, 171 were isolated from other human sites, 769 were isolated from non-human sources (environmental, animal and unspecified samples) and 937 were isolated from food. Additionally, the database also consisted of 1913 genome sequences obtained from clinical isolates from 2012 to 2013, making up 5% of the collection. For clinical isolates, information concerning the age, sex and recent foreign travel of patients was obtained from the isolate referral form.

**Sample preparation**

Genomic DNA purification was performed in accordance with the manufacturer’s procedures using the Qiasymphony DSP DNA Midi kit (Qiagen, Hilden, Germany), while DNA extraction was performed using a Qiasymphony SP (Qiagen, Hilden, Germany) with DNA eluted with nuclease-free water.

**Assembly and annotation**

Genomes were assembled using SPades v3.8.0 [23] with the options ‘--only-assembler’ and ‘--k 21, 33, 55, 77’. Quast v4.5 [24] was used with default settings to check the contiguity of assembled reads before visualizing the assembly graph with Bandage v0.8.1 [25]. Prokka v1.12 [26] was used with the

**Impact Statement**

In this study we present the results of screening for *mcr* variants against Public Health England’s considerable set of *Salmonella* genomes collected via routine national surveillance of gastrointestinal disease over a 4-year period, thereby elucidating the presence of *mcr*-containing *Salmonella* entering the food chain. Employing combined Illumina and Nanopore sequencing, we identified three novel *mcr-3* plasmids and one novel *mcr-5* plasmid, as well as the presence of chromosomally integrated *mcr-1* and *mcr-3*. This study highlights how routine whole-genome sequencing for public health surveillance provides an effective screen for novel and emerging antimicrobial determinants.
options ‘--kingdom Bacteria’ to annotate putative contigs of significance identified in Bandage, before being deposited into Artemis v16.0.0 [27] to be viewed interactively for any notable coding sequences flanking the mcr gene.

Screening for plasmids
PlasmidFinder v1.3 [28] was used to identify known replicons that could be present within the queried genome. BLASTN was used to align the mcr-containing contig to a representative plasmid of that replicon type. Once a plausible comparator plasmid was identified, Mauve v2.4.0 [29] was used to position the contigs of the query sequence to their aligned location on the reference plasmid. Genomes associated with the same putative plasmid type were then grouped and viewed using BLAST Ring Image Generator (BRIG) v0.95 [30] and the genetic context of the mcr gene was analysed using Easyfig [31].

Phylogenetic analysis of mcr-positive genomes
To explore the evolutionary relationships between isolates harbouring mcr genes, phylogenetic analysis was performed on the most common mcr-positive isolated Salmonella serotype, Salmonella Typhimurium. Illumina fastq files have been uploaded to NCBI BioProject – PRJNA248792.

RESULTS
Prevalence
Of the 33 205 Salmonella isolates screened for mcr variants, 52 genomes were positive for an mcr determinant. mcr-1 was detected in 32 isolates, mcr-3 was detected in 19 isolates and mcr-5 was detected in a single isolate. Of the mcr-1-positive isolates, 19/32 were identified as S. enterica serovar Typhimurium, of which 13 were eBG1 (ST34 n=12, ST19 n=1) and 6 were eBG138 (all ST36). All S. Typhimurium-positive genomes were isolated from stool samples. The remaining mcr-1-positive isolates were identified as S. enterica serovars Stanley (n=3), Rissen (n=2) and Java (n=2), as well as single isolates of S. enterica serovars 4,[5],12:b:-. Thompson, Newport, Enteritidis, Agona and Virchow. One of the S. enterica serovar Java isolates was obtained from a poultry meat sample. The age distribution of mcr-1-positive cases was between 1 and 88 years old. Ten patients reported travel to Southeast Asia before the onset of symptoms, with Thailand (n=6) and Malaysia (n=2) being the countries visited most frequently, with single reports of travel to India, Borneo, Egypt, Spain and Poland. The highest number of mcr-1-positive isolates was received in 2017 (n=11), followed by 2016 (n=8), 2015 (n=8), 2014 (n=4) and 2012 (n=1).

Of the 19 mcr-3-positive isolates, 16 were S. Typhimurium, of which 13 were eBG1 (all ST34) and 3 were eBG138 (all ST36). Fifteen were from human faecal samples and one was from a food product. mcr-3 was identified in a Salmonella cholerae-suis ST139 isolate from human blood. Single isolates of Salmonella bovis-morbificans and Salmonella Stanley were also positive for mcr-3. The age distribution of patients with mcr-3-positive samples was 3 to 68 years old, with eight patients having travelled to Thailand and single reports of travel to Vietnam, Malaysia and Cambodia. The greatest number of mcr-3-positive isolates was received in 2015 (n=9), followed by 2014 (n=6), 2016 (n=4) and 2017 (n=1).

The mcr-5 gene was detected in an S. Typhimurium ST34 food isolate received in 2014. No isolates were positive for variants of mcr-2, mcr-4, mcr-6, mcr-7 or mcr-8.

Plasmid types and mcr context mcr-1
The mcr-1-positive isolates contained one of four plasmid types that have been previously described, namely: pESTmcr (KU743383), pHNSHP45 (KP347127), pHNSHP45-2 (KU341831) and pH226B (KX129784). Thirteen shared a similar backbone to pESTmcr, an IncX4 plasmid (88–100 % identity) (Fig. 1a); nine to pHNSHP45, an IncI2 plasmid (80–95 % identity) (Fig. 1b); seven to pHNSHP45-2, an IncHI2 plasmid (70–90 % identity) (Fig. 1c); while one isolate had a similar backbone to pH226B, an IncHI1 plasmid (80 % identity) (Fig. 1d). Neither S. Rissen isolate (S37 and
Fig. 1. Brig plots showing mcr-1-positive plasmids aligned to (a) pESTMCR, (b) pHNHP45, c) pHNHP45-2 and (d) pH226B. (e) The genetic context upstream and downstream of mcr-1.
S38; obtained from second-degree relatives) aligned to any reference plasmid. Nanopore sequencing detected two copies of the *mcr-1* gene on the chromosome of isolate S37.

Among the 32 *mcr-1*-positive isolates, 3 distinct genetic environments were observed immediately upstream and downstream of the *mcr-1* gene (Fig. 1e). The ISA*plI* element, thought to be involved in *mcr-1* mobilization, was found upstream of the *mcr-1* gene in two isolates with the IncI2 plasmid type, two isolates with an IncH12 plasmid type and one isolate with the IncH1 plasmid type, but was not present in all isolates with the IncX4 backbone. Both copies of chromosomally located *mcr-1* were located within a composite ISA*plI* transposon designated as Tn6330 [38]. All 32 isolates, including both chromosomally located *mcr-1* copies, had the *pap2* gene located downstream of *mcr-1*, with no flanking transposons near the *mcr-1* region when the ISA*plI* element was absent. These three genetic environments have previously been observed in other studies [3, 3, 36, 37, 38, 39, 40, 41].

**mcr-3**

Seven isolates were found to harbour the *mcr-3* gene on an IncHI2 plasmid with a similar backbone (95–95% identity) to pWJ1 (KY924928) (Fig. 2a). The remaining 12 *mcr-3*-positive isolates did not align to a reference plasmid and a range of replicon types were detected by PlasmidFinder. To characterize the putative novel plasmids, a hybrid long-read/short-read sequencing strategy was deployed and three novel environments were observed immediately upstream and downstream of the *mcr-1* gene (Fig. 2c). Finally, isolate S62 (the *S. bovis* ST34 isolate) was found to harbour *mcr-3* (Fig. 2c). Among the 32 isolates containing the *mcr-3* gene along with both IncFII and IncFIB replicons, the IncFII/IncFIB plasmid encoded *sul2*, *sfd*, *floR*, *catA*, and downstream of the *ermB* region when *ermB* was found on a small novel 10 kb plasmid with an uncataloged plasmid replication protein (Fig. 3). This plasmid harboured the same *mcr-5* Tn3 family transposon, Tn6452, described in pSE13-SA01718, pEC2380 and pEC1066 [5, 42].

**mcr-5**

The *mcr-5*-positive isolate identified in this study did not align to the previous *mcr-5* ColE plasmids pSE13-SA01718 (KY807921), pEC2380 (MG587004), pEC1066 (MG587003), pECO674 (MF684783) and pI0642 (MG800820), and instead was found on a small novel 10 kb plasmid with an uncataloged plasmid replication protein (Fig. 3). This plasmid harboured the same *mcr-5* Tn3 family transposon, Tn6452, described in pSE13-SA01718, pEC2380 and pEC1066 [5, 42].

**Co-resistance to other antimicrobials**

Of the *mcr*-positive *Salmonella* isolates, all but four were defined as multidrug-resistant (MDR), with genotypically inferred resistance to at least three classes of antibiotics. Forty-seven isolates possessed β-lactamases, of which 10 encoded for extended-spectrum beta-lactamase (ESBL) production. Forty isolates were positive for *bla*<sub>TEM-1</sub> and six isolates had an alternative TEM variant. Three isolates were positive for *bla*<sub>CTX-M-2</sub>, whilst none of the isolates possessed carbapenemase genes. The ESBL *bla*<sub>CTX-M-55</sub> was found in nine isolates and one isolate contained *bla*<sub>CTX-M-14</sub>. The ESBL genotype was restricted to *mcr-3* *Salmonella* samples. Forty-seven isolates harboured genes conferring resistance to a range of aminoglycosides. *dfrA*-mediated trimethoprim resistance determinants were detected in 24 isolates, with *dfrA12* being the most common gene (*n* = 16). Forty-seven isolates contained tetracycline resistance determinants [tet(A)], [tet(M) or tet(X)], while 34 isolates contained *catA*, *floR or cmlA* conferring resistance to chloramphenicol and/or florfenicol. Predicted reduced susceptibility to fluoroquinolones was largely mediated by *qnr* genes (*n* = 24), with only five isolates displaying *gyrA* mutations in the quinolone resistance-determining region. Nine isolates displayed macrolide resistance determinants, with six isolates containing *mph*(A) in isolation or in conjunction with an *erm* gene. All genotypic profiles are described in Table S1 (available in the online version of this article).

Co-location of resistance determinants within the *mcr*-harbouring plasmids was also explored. *mcr-1*-positive isolates with an IncH12-like backbone had additional resistance genes located on the same plasmid conferring resistance to aminoglycosides, tetracyclines and phenicols (Fig. 1c). All *mcr-3*-positive isolates with the IncH12 plasmid, pWJ1, also co-harboured resistance genes, including four that acquired *floR*, five that acquired *tet(A)* and *aph [6]-Id*, two with *mph*(A) and one with *qnrS1* and *bla*<sub>CTX-M-55</sub>. Of the novel *mcr-3* plasmids, the IncA/C2 plasmid co-harboured *bla*<sub>CTX-M-55</sub> in 5/8 isolates and the *erm* gene in a single isolate, while all contained the resistance genes *floR*, *tet(A)*, *aph [6]-Id* and *sul2*. The IncFII/IncFIB plasmid encoded *bla*<sub>CTX-M-55</sub> and *qnrS1* in conjunction with *mcr-3*, and the IncH12A plasmid encoded *bla*<sub>TEM-1</sub> and a tetracycline resistance operon. The small *mcr-5* plasmid did not harbour any other resistance genes.
Fig. 2. Brig plots showing mcr-3-positive plasmids aligned to (a) pWJ1, (b) the novel IncA/C2 plasmid, (c) the novel IncFII/IncFIB plasmid and (d) the novel IncHI2A/IncY plasmid. (e) The genetic context upstream and downstream of mcr-3.
Phylogenetic analysis

The majority (36/52) of the mcr-positive samples were S. Typhimurium, with 27 of these isolates belonging to clonal complex eBG1. Placing the mcr-positive isolates into a phylogeny of 288 diverse S. Typhimurium genomes (100 SNP representatives) revealed that 26/27 clustered within the monophasic variant 4,[5],12:i- ST34 clade (Fig. 4). One mcr-positive isolate (MLST 19) clustered adjacent to the ST34 monophasic clade, as defined by BAPS. Comparing the ST34 mcr-positive isolates against 1284 deduplicated (5 SNP representatives) ST34 genomes from routine surveillance of human clinical cases in England revealed that neither the mcr-1-positive isolates nor the mcr-3-positive isolates form a monophyletic group (Fig. 5). Overlaying patient travel information onto the phylogeny revealed several broad clusters where reported travel to Southeast Asia predominated. Of the mcr-positive ST34 EBG1 isolates, 16/26 cluster in clades where most background isolates are from patients reporting travel to Asia, including 7/16 cases that did not report any travel information. The remaining 10 mcr-positive EBG1 isolates are found in a diverse set of clusters that tend to be more associated with travel to Europe.

Several mcr plasmids were restricted to Asian-associated clades. The mcr-3 IncH12 plasmid PJW1 was exclusively found in multiple Asian-associated clades of EBG1 but was also found in EBG36 associated with cases who had reported travel to Asia. The novel mcr-3 IncA/C2 plasmid was also restricted to the Asian-associated clades and furthermore associated with bla<sub>CTX-M-55</sub>. In contrast, the IncX4 mcr-1 plasmid pESTMCR was more commonly associated with EBG1 isolates that cluster with patients reporting travel within Europe, as was the IncH12 plasmid pHNHSHP4 5–2.

**DISCUSSION**

All mcr-positive S. enterica serovars isolated in this study are established zoonotic pathogens with serovars Typhimurium, Stanley, Rissen and cholerae-suis commonly isolated in pigs [43, 44]. This is consistent with the premise that the rise in colistin resistance in *S. enterica* has been driven by the selective pressure from colistin usage in agricultural animals rather than therapeutic use in humans [45]. Twenty-one patients that harboured mcr-positive strains reported travel to Southeast Asian countries, generally Thailand, where traditionally there are no veterinary antibiotic restrictions and antibiotics are readily available [46]. Furthermore, co-location of tet(A), tet(B), tet(M), catA, floR or cmlA1 on mcr-harbouuring plasmids as a result of tetracycline and/or florfenicol use in food-producing animals could have also contributed to the selection of colistin resistance in *Salmonella* strains. Since the emergence of mcr genes, the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) have implemented guidelines for the restricted use of colistin in veterinary medicine in Europe and Thailand. However, plasmids harbouring mcr genes appear to be well established in bacterial species belonging to *Enterobacteriaceae* and it is likely that the number of mcr-positive isolates and mcr-harbouuring plasmids will continue to increase.

The predominant plasmid types harbouring mcr-1 in this routine surveillance study were IncX4 (n=13), IncI2 (n=9) and IncH12 (n=7), which were the common plasmid types identified in other studies involving human and food *S. enterica* and *E. coli* isolates from Asia, Europe, the Americas and Africa [12, 38, 47, 48]. For mcr-3-positive samples, 8/19 had a novel IncA/C2 replicon type with the second most common (7/19) being an IncH12 plasmid backbone previously described in Danish travellers returning from Asia [49, 50]. Two other novel mcr-3-containing plasmids were described in our data set, with one isolate harbouring an IncFII/FIB plasmid and another an IncH2A/Y plasmid.

The mcr-1-pap2 and mcr-3-dgkA gene cassette was shared by all mcr-1 and mcr-3-positive isolates, respectively. Snesrud *et al.* demonstrated that mobilization of the mcr-1 cassette involves a composite transposon of ISApl1 [51]. The loss of a
Fig. 4. Maximum-likelihood SNP phylogeny of the 28 mcr-positive Salmonella Typhimurium eBG1 isolates against a background of 288 100-SNP representatives encompassing the diversity of the PHE Salmonella Typhimurium collection. Taxa are labelled and coloured by MLST and predominant phage types are labelled.

single copy of ISAppl, as seen in isolates with pHNSHP45-2, pHNSHP45 and pH226B backbones, or both copies of ISAppl, as seen in isolates with an pESTMCR backbone, is a result of its ‘copy out–paste in’ mechanism, which prevents the transposition of the mcr-1 cassette and transfixes it in the plasmid [51]. However, mcr-1 cassettes with a single-ended ISapl could still transpose if they maintained the inverted right repeat sequence of ISapl immediately after the pap2 gene [51]. Meanwhile, conjugation experiments have determined two circular forms that could have facilitated the dissemination of the mcr-3 gene. One circular form, mcr-3-dgkA-ISKpn40, was a 3535 bp derivative of the ISKpn40-mcr-3-dgkA-ISKpn40 fragment [41]. The second circular intermediate was ΔIS26-TnAs2-ΔISKpn40-mcr-3-dgkA-ISKpn40, 5990 bp in size, mediating mcr-3 transposition via homologous recombination between IS26 and IS15DI components [13].

These genetic combinations were also observed in the chromosomal integration of the mcr-1 and the mcr-3 gene cassettes, which adds a new perspective to their transmissible nature. mcr genes can therefore co-occur with other chromosome- and plasmid-located resistance genes and transpose themselves from plasmid-to-plasmid, from plasmid-to-chromosome, and vice versa. This transmissibility is of great concern in the clinical setting with respect to the treatment of invasive pathogens such as S. cholerae-suis. In silico analysis determined that mcr-3-positive isolates had more resistance genes co-located on the same mcr plasmid compared to mcr-1-positive isolates, and that the IncHI2 plasmid type harboured more resistance genes compared to other plasmid types. A study by Ruichao Li et al. hypothesized that IncHI2 plasmid types could be the most efficient vessels for the dissemination of co-located mcr genes due to their diverse MDR region, which incorporates several transposons, IS and resistance genes, supplying multiple insertion sites for mcr-1 [38].

Phylogenetic analysis of the most commonly detected mcr-positive serovar Typhimurium confirms previous reports of mcr-positive S. enterica circulating in Southeast Asia. The tree topology suggests multiple acquisitions of a restricted range of both mcr-1- and mcr-3-encoding plasmids within Asia and that those plasmids are more likely to encode additional antimicrobial resistance determinants, including ESBLs. These plasmids were also associated with other serovars of Salmonella associated with patients who reported travel to Asia. These data suggest that within Europe there appear...
to be alternative \textit{mcr}-harbouring plasmids circulating that in general encode fewer additional antimicrobial resistance determinants.

This study demonstrates that routine WGS for public health surveillance can provide an effective screen for antimicrobial resistance determinants, including \textit{mcr} genes. In addition, complementary long-read technologies can elucidate the genomic context of these determinants, offering insights into plasmid dissemination and linkage to other resistance genes. Finally, the phylogenetic context offers a perspective on the likely geographical origin of infection and plasmid transmission dynamics.

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\textbf{Author contributions}

T. J. D., N. W., M. D., G. G. and C. J. conceptualized the project. D. G. and M. D. performed the DNA extraction. D. G. and C. M. performed the ONT sequencing. C. M., D. G., H. H. and A. P. performed the bioinformatics analysis. T. D. J. and C. M. wrote the manuscript. M. A. C., K. L. H. and D. M. critically reviewed the manuscript.

\textbf{Conflicts of interest}

The authors declare that there are no conflicts of interest.
Data bibliography
NCBI BioProject: PRJNA248792, PRJNA248064.

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