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Comparative analysis of an *mcr-4 Salmonella enterica* subsp. *enterica* monophasic variant of human and animal origin

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Objectives: In this study we compared the recently described *mcr*-4-positive *Salmonella enterica* monophasic variant, isolated in 2016 in two Italian patients affected by gastroenteritis, with the first *mcr*-4-positive *Salmonella* isolate identified in 2013 in a pig at slaughter in Italy.

Methods: WGS of the two *Salmonella* isolates of human origin was performed using a MiSeq instrument (Illumina). The phylogenetic analysis was performed by SNP analysis, comparing genomes of the *mcr*-4-positive isolates of swine and human origin with 82 *Salmonella* genomes downloaded from the EnteroBase *Salmonella* database. Complete sequences of plasmids carrying *mcr-4.2* were obtained and compared. Transformation experiments were performed to transfer the *mcr*-4 plasmids into a colistin-susceptible *Escherichia coli* recipient strain.

Results: Comparative genomics demonstrated that the *Salmonella* of swine origin did not cluster with the isolates of human origin. The *mcr-4.2* gene variant identified in the *Salmonella* of human origin was located on a ColE-like plasmid. This plasmid showed different replication and mobilization genes with respect to those previously described in the ColE plasmid carrying the *mcr-4.1* variant, identified in *Salmonella* of swine origin.

Conclusions: The divergence in genomes, plasmids and gene variants demonstrated that there was not a unique *mcr*-4-positive, monophasic *Salmonella* lineage circulating in animals and causing gastroenteritis in humans in Italy. There was no horizontal transfer of the same plasmid among *Salmonella* strains of animal and human origin, but the *mcr*-4 gene and a fragment of the plasmid identified in the animal strain were mobilized by an IS*1294* into a different ColE plasmid.

Introduction

Colistin is a decades-old drug, intensively used in veterinary medicine. In humans, the global spread of MDR Gram-negative bacteria with limited therapeutic options resulted in the return of colistin use, despite its documented toxicity.[1] Two major mechanisms of colistin resistance have been described in Gram-negative bacteria: chromosomal mutations occurring in genes involved in the synthesis and modification of LPS and the acquisition of mobile colistin resistance (*mcr*) genes encoding phosphoethanolamine transferases, resulting in a more cationic charged LPS that confers reduced susceptibility to colistin.[2] To date, five types of *mcr* genes have been described. *mcr-1* was the first to be reported and retrospective analysis on different bacterial collections demonstrated worldwide spread of the *mcr-1* gene.[3,4] The *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5* genes were more recently discovered and their distribution and prevalence are still under investigation.[5–8] The *mcr-4.1* gene was first described in an Italian strain that was a *Salmonella enterica* subsp. *enterica* monophasic variant of serovar Typhimurium with antigenic formula 4,[5],12,i:- isolated from a pig slaughtered in Italy in 2013 (*Salmonella* strain R3445).[7] Recently, *mcr-4.2*-positive *Salmonella* serovar 4,[5],12,i:- strains were reported from two apparently unrelated human gastroenteritis cases occurring in Italy in 2016.[9] In this study the analysis of the plasmid carrying the *mcr-4.2* gene and the genomic comparison of *Salmonella* strains of human and animal origin was performed, with the aim to better describe characteristics, similarities and differences among the *mcr*-4-positive strains from Italy.

Materials and methods

Bacterial strains

Two *S. enterica* with antigenic formula 4,[5],12:i:- were isolated from faecal samples of two Italian patients with gastroenteritis, collected in October 2016 (strain AB-160) and November 2016 (strain AB-243), at S. Agostino-Estense Hospital.[9] The R3445 *S. enterica* monophasic variant 4,[5],12:i:-

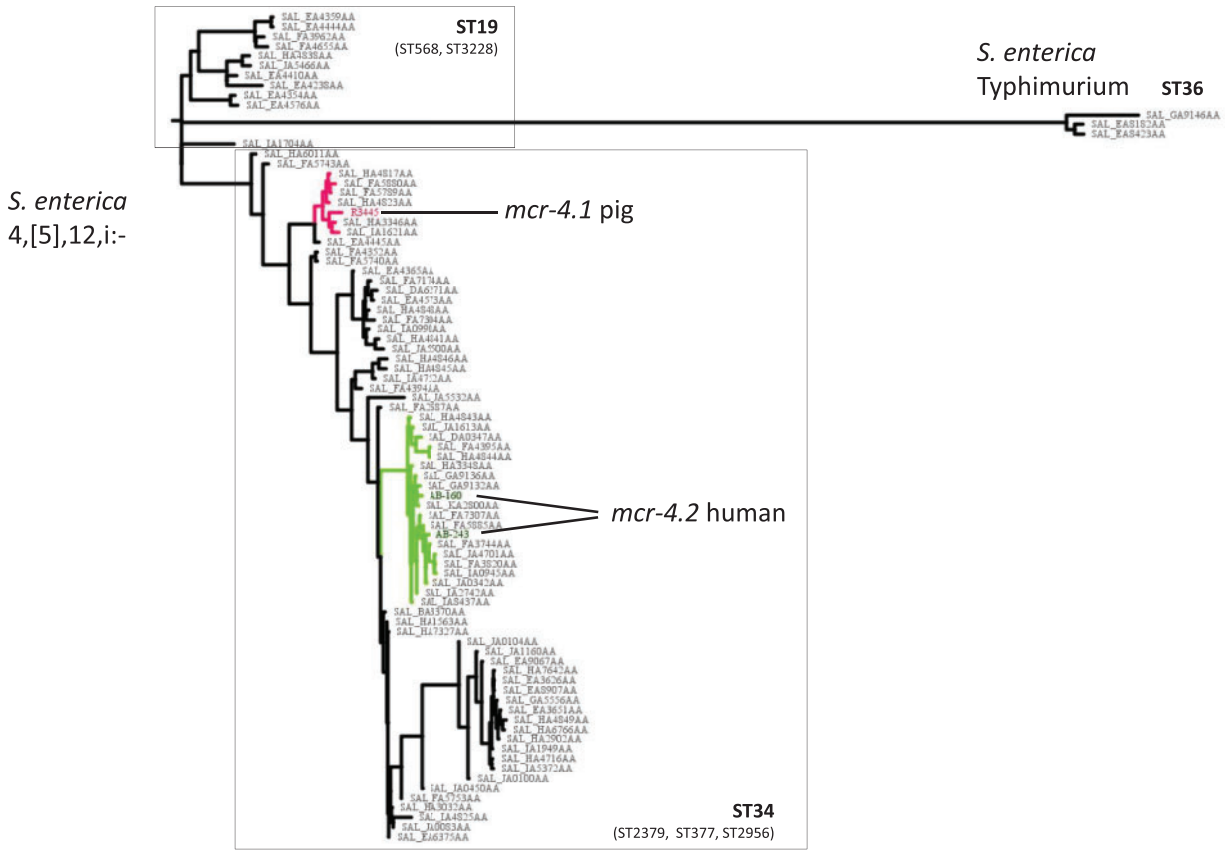

Figure 1. Phylogenetic tree of human and animal *mcr*-4-positive *Salmonella* genomes. Phylogenetic maximum-likelihood tree generated using FigTree v1.4.3 software of the SNP analysis performed using the kSNP3 tool. The clades of the *Salmonella* of pig (R3445) and human (AB-160 and AB-243) origin are highlighted in bold. The labels of the branches correspond to the Uberstrain codes as they appear in the EnteroBase *Salmonella* database. Three genomes (SAL_GA9146AA, SAL_EA8182AA and SAL_EA8423AA) were from *S. enterica* serovar Typhimurium; all the others were *S. enterica* monophasic variants. The branches generated by genomes assigned to ST34 or to ST19 and their related STs (differing from ST34 and ST19 by one single allele of the MLST profile, respectively) are boxed. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

was identified in a pig at slaughter in Italy in 2013.[7] Colistin MICs for *Salmonella* strains and their respective *mcr*-4-positive transformants were determined using a broth microdilution (BMD)-based commercial system (SensiTest Colistin, Liofilchem, Italy) and then confirmed using the BMD method according to CLSI and EUCAST (ISO standard method 20776-1).[10–12] All the isolates were tested in triplicate. Three reference strains (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *E. coli* NCTC 13846) were used as controls.

Transfer of pMCR4 plasmids

Plasmid DNA was purified from strains AB-160 and AB-243 with the Plasmid Mini Kit (QIAGEN) and used to transform *E. coli* DH5α chemically competent cells (Invitrogen). Transformants were selected on LB agar plates (Sigma) containing colistin (2 mg/L) and the colonies were tested by PCRs using *mcr*-4-specific primers.[7] Transformants of pMCR-4.1_R3445 in the *E. coli* DH5α recipient were previously obtained and used for comparative analysis.[7] MICs conferred by the different plasmids to the isogenic *E. coli* DH5α recipient were determined as above.

WGS

WGS was performed on DNA extracted from the *Salmonella* strains using the Macherey Nagel Kit. Genomic DNA paired-end libraries were generated using the Nextera XT DNA Sample Preparation Kit (Illumina) and sequenced using a MiSeq instrument with the 2 × 300 PE protocol (Illumina). *De novo* assembly of Illumina reads was performed using the Galaxy version 20150522 of the SPAdes pipeline through the ARIES public Galaxy server (https://w3.iss.it/site/aries/). Draft genome sequences were annotated using the RAST server (http://rast.nmpdr.org/). Antimicrobial resistance, replicon genes and MLST STs were detected using the ResFinder, PlasmidFinder and MLST online tools at the Center for Genomic Epidemiology (CGE) of the Danish Technical University (https://cge.cbs.dtu.dk/services/), respectively. IS elements were detected using ISFinder (https://www-is.biotoul.fr/).

Seventy-nine genomes were selected and downloaded from the EnteroBase *Salmonella* database (http://enterobase.warwick.ac.uk/), using '4,[5],12,i:-' as the keyword. For comparison, three *Salmonella* Typhimurium genomes were also downloaded from the EnteroBase *Salmonella* database and included in an SNP analysis, performed using the kSNP3 tool through the ARIES public Galaxy server. A phylogenetic maximum-likelihood tree was generated using FigTree v1.4.3 software.

The *mcr*-4 and replicon genes were identified on the same contig in assembled genomes, determining *in silico* the *mcr*-4 gene location on plasmids. The complete, circularly closed plasmid was obtained by PCR-based gap closure and sequencing of the amplicon. Purified plasmid DNA was extracted (PureLink HiPure Plasmid Filter Midiprep Kit, Invitrogen) from transformants and the expected plasmid size was verified by restriction with SalI. SalI was chosen to linearize the plasmid since one single

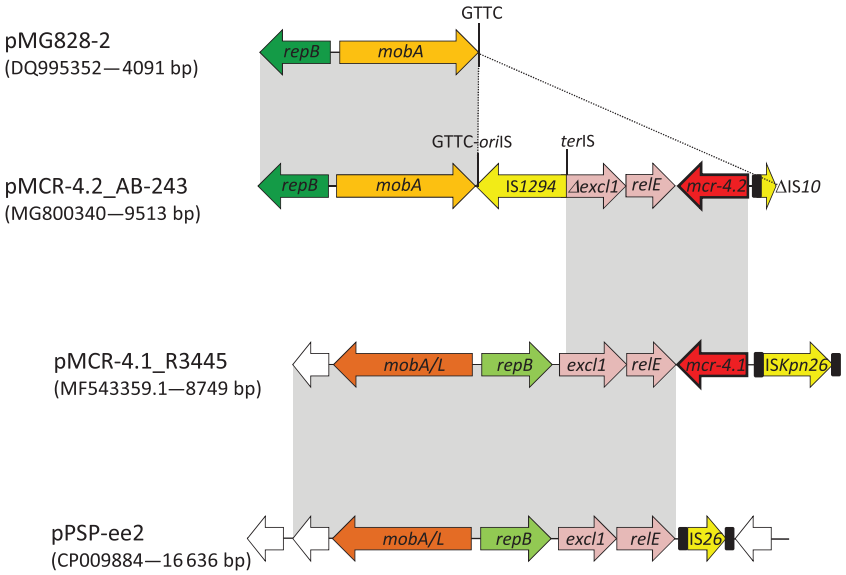

Figure 2. Comparative linear maps of pMCR-4.2_AB-243 and pMCR-4.1_R3445 plasmids and their close relatives. pMCR-4.2_AB-243 and pMCR-4.1_R3445 plasmid maps were drawn in comparison with the pMG828-2 and pPSP-ee2 reference plasmid maps. The grey shading indicates common regions among plasmids (at nucleotide identity >98%). Predicted coding sequences are indicated by arrows orientated in the direction of transcription of each respective gene; the sizes of the arrows are not to scale. The IS*1294* tetranucleotide (GTTC) sequence-specific binding site is indicated. The *ori*IS and *ter*IS sequences flanking IS*1294* are also indicated. IRs flanking the other IS elements are indicated by black boxes. The sequence of the pMCR-4.2_AB-243 plasmid is available at NCBI GenBank under accession number MG800340. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

restriction site was expected from the *in silico* analysis of the plasmid (data not shown).

Results and discussion

Phylogenetic analysis of the Salmonella isolates

MLST STs deduced from WGS revealed that the *Salmonella* strains of animal (R3445) and human (AB-160 and AB-243) origin all belonged to ST34. The three strains also showed the same antigenic formula, being monophasic variants of serovar Typhimurium 4,[5],12,i:-.

Comparative genome analysis of the three *mcr-4*-positive *Salmonella* isolates of Italian origin was performed against a selection of 82 genomes of *Salmonella*, 3 serovar Typhimurium and 79 monophasic variant 4,[5],12,i:- of human, animal and environmental origin, isolated in Europe, Asia and North America and showing ST36, ST34, ST19, ST2379, ST3377, ST2956, ST3228 and ST568, downloaded from the EnteroBase Salmonella database. The phylogenetic tree showed that AB-160 and AB-243 strains of human origin were on closely related clades, which were distant from that of the R3445 genome from the pig (Figure 1).

mcr-4 plasmids

In the AB-243 genome, the ResFinder tool detected the *mcr-4.2* gene variant on a single contig of 9568 bp with overlapping extremities, which was also identified by the Col8282 PlasmidFinder probe. The presumptive plasmid was circularized by PCR-based gap closure, obtaining the complete sequence of a ColE-like plasmid of 9513 bp, here named pMCR-4.2_AB-243 (accession number MG800340). In the AB-160 genome a plasmid identical to pMCR-4.2_AB-243 was identified. pMCR-4.2_AB-243 carried *repB* and *mobA* genes (nt positions 5462 to 9513) highly similar to those of plasmid pMG828-2 (accession number DQ995352). In pMCR-4.2_AB-243, a truncated IS*10* (nt positions 4975 to 5465) and an entire IS*1294* (nt positions 34 to 1721) were identified, which were not present in pMCR-4.1_R3445 (accession number MF543359.1), the ColE plasmid previously described in the *Salmonella* of swine origin.[7] However, upstream of the IS*1294* element, a common region (nt positions 1719 to 4974) was shared between pMCR-4.2_AB-243 and pMCR-4.1_R3445, including the *mcr-4* gene, the *excl1* and *relE* (belonging to the RelE/ParE toxin–antitoxin family) genes (Figure 2). The comparative analysis of pMG828-2 and pMCR-4.2_AB-243 suggested that *excl1*, *relE*, *mcr-4.2* genes and the IS*10* fragment were mobilized by IS*1294* and integrated into a ColE plasmid highly similar to pMG828-2, in a tetranucleotide GTTC site, known to be one of the IS*1294* preferred target sites (Figure 2).[13]

The pMCR-4.2_AB-243 backbone differed from that of pMCR-4.1_R3445. The latter was positive for the ColE10 PlasmidFinder probe and carried the *mcr-4.1* gene variant flanked by an IS*Kpn26* element (Figure 2). The *mobA/L*, *repB* and *excl1* genes of pMCR-4.1_R3445 showed 99% nucleotide identity to the pPSP-ee2 plasmid of the *Pantoea* sp. PSNIH1 isolate.[7] Nucleotide identity between pMCR-4.1_R3445 and pPSP-ee2 plasmids ended at 214 bp from the *mcr-4.1* stop codon because of an IS*26* integration occurring in pPSP-ee2 at this site (Figure 2).

Colistin MIC conferred by the pMCR-4.1 and pMCR-4.2 plasmids

The two *mcr-4*-positive plasmids conferred the same colistin MIC of 4 mg/L when transferred by transformation into the isogenic

E. coli DH5α strain. The colistin MIC for the recipient *E. coli* DH5α was 0.5 mg/L, while the colistin MIC for the *Salmonella* from human or animal sources was 8 mg/L. The mutation of the *mcr-4.1* into the *mcr-4.2* variant and its transfer into a novel ColE-type gave no apparently measurable MIC difference.

Conclusions

Since the isolation of R3445 in 2013, other additional variants of the *mcr-4.1* gene have been described.[9,14] The *mcr-4.2* variant was initially described in *Salmonella* from human gastroenteritis, but is not exclusive to human isolates since it was also found in a collection of *E. coli* and *Salmonella* of animal origin.[14] However, among the *Salmonella* isolates from Italy, the *mcr-4* gene variants were different in strains of human and animal origin, being *mcr-4.2* and *mcr-4.1*, respectively.[7] Moreover, genomic analysis demonstrated that the monophasic *Salmonella* of human origin did not belong to the same cluster as that of animal origin. Currently, all *mcr-4* gene variants seem to be preferentially carried by ColE-like plasmids in both *E. coli* and *Salmonella* species, even though the ColE plasmids found in human isolates were different from that previously reported in the *Salmonella* of animal origin.[7]

As demonstrated by the wide distribution of *mcr-1* worldwide, it could be hypothesized that *mcr-4* has also been spreading undetected among Enterobacteriaceae, with *Salmonella* representing a hidden reservoir.[2] The source of the *mcr-4*-positive *Salmonella* infecting humans in 2016 in Italy is still unknown. Further epidemiological analyses at the local and national level may be useful to understand the extent of the circulation of this gene in the different sources.

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Transparency declarations

None to declare.

References

1 Landman D, Georgescu C, Martin DA *et al*. Polymyxins revisited. *Clin Microbiol Rev* 2008; **21**: 449–65.

2 Poirel L, Jayol A, Nordmann P. Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clin Microbiol Rev* 2017; **30**: 557–96.

3 Liu YY, Wang Y, Walsh TR *et al*. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 2016; **16**: 161–8.

4 Schwarz S, Johnson AP. Transferable resistance to colistin: a new but old threat. *J Antimicrob Chemother* 2016; **71**: 2066–70.

5 Xavier BB, Lammens C, Ruhal R *et al*. Identification of a novel plasmid-mediated colistin-resistance gene, *mcr-2*, in *Escherichia coli*, Belgium, June 2016. *Euro Surveill* 2016; **21**: pii=30280.

6 Yin W, Li H, Shen Y *et al*. Novel plasmid-mediated colistin resistance gene *mcr-3* in *Escherichia coli*. *mBio* 2017; **8**: e00543–17.

7 Carattoli A, Villa L, Feudi C *et al*. Novel plasmid-mediated colistin resistance mcr-4 gene in *Salmonella* and *Escherichia coli*, Italy 2013, Spain and Belgium, 2015 to 2016. *Euro Surveill* 2017; **22**: pii=30589.

8 Borowiak M, Fischer J, Hammerl JA *et al*. Identification of a novel transposon-associated phosphoethanolamine transferase gene, *mcr-5*, conferring colistin resistance in *d*-tartrate fermenting *Salmonella enterica* subsp. *enterica* serovar Paratyphi B. *J Antimicrob Chemother* 2017; **72**: 3317–24.

9 Carretto E, Brovarone F, Nardini P *et al*. Detection of *mcr-4* positive *Salmonella enterica* serovar Typhimurium in clinical isolates of human origin, Italy, October to November 2016. *Euro Surveill* 2018; **23**: pii=17-00821.

10 Carretto E, Brovarone F, Russello G *et al*. Clinical validation of SensiTest Colistin, a broth microdilution-based method to evaluate colistin MICs. *J Clin Microbiol* 2018; **56**: e0152317.

11 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing: Twenty-Seventh Informational Supplement M100-S27*. CLSI, Wayne, PA, USA, 2017.

12 International Organization for Standardization (ISO). *ISO 20776-1:2006 Clinical Laboratory Testing and In Vitro Diagnostic Test Systems—Susceptibility Testing of Infectious Agents and Evaluation of Performance of Antimicrobial Susceptibility Test Devices—Part 1: Reference Method for Testing the In Vitro Activity of Antimicrobial Agents Against Rapidly Growing Aerobic Bacteria Involved In Infectious Diseases*. Geneva, Switzerland: ISO, 2006. https://www.iso.org/standard/41630.html.

13 Tavakoli N, Comanducci A, Dodd HM *et al*. IS1294, a DNA element that transposes by RC transposition. *Plasmid* 2000; **44**: 66–84.

14 Rebelo AR, Bortolaia V, Kjeldgaard JS *et al*. Multiplex PCR for detection of plasmid-mediated colistin resistance determinants, *mcr-1, mcr-2, mcr-3, mcr-4* and *mcr-5* for surveillance purposes. *Euro Surveill* 2018; **23**: pii=17-00672.