Genetic analysis of plasmid-encoded mcr-1 resistance in Enterobacteriaceae derived from poultry meat in the Netherlands

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Background: Colistin is classified as the highest priority and critically important antimicrobial for human medicine by WHO as it is the last resort agent for treatment of carbapenem-resistant Enterobacteriaceae in humans. Additional research is necessary to elucidate the genetic structure of mcr-1 resistance genes, commonly found on plasmids, using WGS.

Objectives: To map and compare the genetic characteristics of 35 mcr-1-mediated colistin-resistant Enterobacteriaceae isolated from chicken meat to highlight the genetic variation of the mcr-1-containing plasmids.

Methods: Sequencing was performed using Illumina HiSeq2500, Novaseq6000 and ONT’s GridION. GridION data was locally basecalled and demultiplexed using ONT’s Albacore 2.3.4 followed by Porechop 2.3. Quality filtering was performed using Filtlong 2.0. Hybrid Assembly was performed using Unicycler 4.7. Plasmids were compared with reference sequences in plasmid-RefSeq and pATLAS.

Results: A total of 35 mcr-1 positive Enterobacteriaceae were investigated, which resulted in 34 qualitatively robust hybrid assemblies of 2 Klebsiella pneumoniae and 32 Escherichia coli. mcr-1.1 was present in 33/34 isolates. One isolate contained an mcr-1.1-like resistance gene, due to a deletion of one codon. Two mcr-1.1 genes were located on the chromosome, while the majority of the mcr-1 genes were found on IncX4 type plasmids (n = 19). Almost all plasmids identified in this study were highly similar to plasmids found in human-derived strains.

Conclusions: The mcr-1.1-containing plasmids from retail chicken show high sequence similarity to human mcr-1.1 plasmids, suggesting that this may be a contributor to the presence of colistin resistance in humans.

Introduction
In 2015, a plasmid-mediated colistin resistance gene was reported in China.1 From that moment on, many more mobile colistin resistance (mcr) genes and variants have been detected all over the globe.2 This discovery represents a mechanism for an easy transferable resistance mechanism to colistin, which is seen as a last-resort antibiotic to treat carbapenem-resistant Enterobacteriaceae.3 In Europe, colistin is used to treat infections caused by Enterobacteriaceae in sheep, cows, pigs, goats and chicken.4 Therefore, the detection of mcr-1-harbouring Enterobacteriaceae isolates in chicken meat was self-evident.5,6

In order to understand the molecular epidemiology and resistance mechanism of mcr genes, WGS approaches should be used. Characteristically, high-throughput sequencing platforms (e.g. Illumina) are used in order to sequence the full bacterial genome.7 However, short reads from these high-throughput sequencers can make it challenging to reconstruct plasmids and therefore they are inaccurate for studying antibiotic resistance epidemiology.8

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Single-molecule sequencing platforms such as the Oxford Nanopore Technologies (ONT) MinION, GridION and PromethION are able to sequence long fragments of DNA. Subsequently, with the use of a hybrid assembly, increased information content can be generated since the genome completeness is increased and the location of resistance genes in the genome can be determined. 9

In this study, short- and long-read sequencing platforms were used in order to study the mcr-1-containing Enterobacteriaceae isolated from retail chicken meat.5,6 We used a hybrid-assembly approach to extract the plasmid sequences that contain mcr-1 and studied the plasmid relationship compared with publicly available mcr-1 plasmid sequences.

Methods
Sample collection
In total, 35 confirmed mcr-1-holding Enterobacteriaceae were subjected to Illumina short read and ONT sequencing. The isolates derived from previous studies,5,6 with the exception of EC-MCR34. All samples derived from three prevalence surveys in Dutch retail chicken meat performed in 2009, 2014 and 2015, which were initially performed to study the presence of ESBL-producing Enterobacteriaceae. 5,6 The isolates in this study were genotypically mcr-1 PCR positive and phenotypically colistin resistant. 5

Illumina sequencing
The 35 samples were sequenced using paired-end Illumina HiSeq2500.

The library prep for 35 samples was performed using the Nextera XT DNA library prep kit and the Nextera XT Index Kit v2 (Illumina, Eindhoven, The Netherlands), according to the manufacturer's instructions. Libraries were subsequently purified using Agencourt AMPure XP beads (Beckman Coulter, Woerden, The Netherlands) and quantified using the Quant-it dsDNA HS-kit (Thermo Fisher, Bleiswijk, The Netherlands) and using a Fragment Analyzer (Agilent, The Netherlands.) Samples were then loaded on a HiSeq2500 system and run for 251 cycles (PE125) using HiSeq Rapid SBS Kit v2 chemistry.

Due to low quality, EC-MCR10 and EC-MCR21 were re-sequenced using the Illumina NovaSeq 6000 The library prep for these two samples was performed using the Nextera XT DNA library prep kit and the IDT for Illumina Nextera DNA Unique Dual Indexes (Indexa) according to the manufacturer's instructions. Libraries were subsequently purified using Agencourt AMPure XP beads (Beckman Coulter) and quantified using the Quant-it dsDNA HS-kit (Thermo Fisher) and using a Fragment Analyzer (Agilent). Samples were then loaded on an S1 flow cell on the NovaSeq6000 system and run for 301 cycles (PE150) using HiSeq Rapid SBS Kit v2 chemistry.

Fastq read sequence files were generated using bc12fastq2 version 2.1.8. Initial quality assessment was based on data passing the Illumina Chastity filtering. Subsequently, reads containing PhiX control signal were removed using an in-house filtering protocol. In addition, reads containing (partial) adapters were clipped (up to a minimum read length of 50 bp). The second quality assessment was based on the remaining reads using the FASTQC quality control tool version 0.11.5.

ONT sequencing
All 35 samples were sequenced using the ONT GridION (Oxford Nanopore Technologies, Oxford, UK). Libraries were prepared using shearing by needle shearing (KP-MCR01–02 and EC-MCR03–31) or using the Covaris G-tube (EC-MCR32–35). The library was prepared using the ONT 1D ligation sequencing kit (SQK-LSK109) with the native barcoding kit (EXP-NBD103). Samples KP-MCR01–02 and EC-MCR03–29 were loaded on FLO-MIN107 R9.5.1 flow cells and the remaining on a FLO-MIN106 R9.4.1 flow cell.

Sequence data availability
All data is available from the National Center for Biotechnology Information (NCBI) under BioProject number PRJEB44175. Raw short-read Illumina and long-read ONT sequencing data and metadata for all 35 isolates used in this study are available from the NCBI Sequence Read Archive database under accession numbers ERR5727763 to ERR5727797 (short read) and ERR5726838 to ERR5726872 (long read).

Assembly
GridION data were locally basecalled and demultiplexed using ONT's Albacore 2.3.4 followed by Porechop 2.3 to demultiplex the unclassified reads. Quality filtering was performed using Filtlong 2.0 using the following settings: (i) maximum size of 500 Mbp; (ii) keep 90% percentage of the best reads of the data; and (iii) minimum size of 1000 bp. The long-read quality was evaluated using FastQC and NanoPlot v1.13.0 and the short-read quality using FastQC. Hybrid assembly was performed using Unicycler 4.7 using default settings and a minimum length of 1000bp and subsequently assessed using QUAST 5.0.7 Genetic characterization of the hybrid assemblies was performed using the online service of goseqit.com.

The coverage of the ONT sequence reads was calculated by mapping the long reads back to the assembly using minimap2 (v2.13) and SAMtools (v1.9) using the in-house scripts. Sequence annotation was done using Bakta (v1.1).16

Plasmid analysis
The mcr-1 plasmid sequences were manually identified and extracted from the assembly graphs (.gfa files) using Bondage.14 The mcr-1 gene sequence (AKF16168.1) was used to locate the mcr-1-containing plasmids. mcr-1 gene-containing plasmids from RefSeq plasmid database and pATLAS (accessed April 8, 2020) were retrieved.15 Any duplicates entries were removed prior subsequent analyses. In total 69 publicly available plasmids and mcr-1-containing plasmids from this study were used. Plasmid sequences were clustered using PlasmidSimilarity (v0.3.0, https://github.com/Caserjian/PlasmidSimilarity). In short, dissimilarity among plasmids was calculated using the Jaccard index, using the complete k-mer composition (all subsequences in a sequence of length k) of each plasmid sequence, using k length of 31 bp. Antimicrobial resistance (AMR) genes, virulence genes and plasmid origin of replications were identified with Abricate (v1.1.0, default settings) using the NCBI, virulence factor database and PlasmidFinder database respectively (retrieved on 10 September 2019).16,17

Results and discussion
A total of 35 mcr-1-positive Enterobacteriaceae were investigated, which resulted in 34 qualitatively robust hybrid assemblies of 2 Klebsiella pneumoniae and 32 Escherichia coli isolates (Table S1, available as Supplementary data at JAC-AMR Online). The hybrid assembly substantially improved the reconstruction of the microbial genome (data not shown). The mcr-1.1 gene was present in 33/34 isolates (Table 1). The most common STs for E. coli were ST624 (n = 7), ST10 (n = 5) and ST997 (n = 4). The two K. pneumoniae isolates belonged to ST107 and ST1944. One isolate contained an mcr-1.1-like resistance gene, due to a mutation in the start codon, but still remained resistant to colistin.5 The second codon in mcr-1.1 is ATG and will likely replace the first codon as start codon, leading to a truncated but functional gene. Two mcr-1.1 genes were located on the chromosome, while the majority of the mcr-1 genes were found on IncX4 type plasmids (n = 19, Table 1), which is a common plasmid type harbouring mcr-1 found in Europe.18,19
Table 1. Overview of mcr-1-positive isolates with corresponding Inc type, size and other genetic characteristics

| Sample    | Species     | ST   | Mcr type | Inc type on mcr1.1 plasmid | Other AMR genes                            | Transposase gene located near mcr1.1 | Contig no. | Contig size | Study reference |
|-----------|-------------|------|----------|---------------------------|--------------------------------------------|---------------------------------------|------------|-------------|-----------------|
| KP-MCR01  | K. pneumoniae | ST107 | 1.1      | IncX4                     | —                                          | —                                     | 6          | 33303       |                 |
| KP-MCR02  | K. pneumoniae | ST1944 | 1.1      | IncHI2, IncHI2A           | ahp(3')-Ia, sul3, aadA1, dfrA12           | IS30-like element ISApl1 family transposase | 2          | 211949      |                 |
| EC-MCR03  | E. coli     | ST10  | 1.1      | IncX4                     | —                                          | —                                     | 7          | 33303       |                 |
| EC-MCR04  | E. coli     | ST8262 | 1.1      | IncX4                     | —                                          | —                                     | 5          | 33303       |                 |
| EC-MCR05  | E. coli     | ST8262 | 1.1      | IncX4                     | —                                          | —                                     | 6          | 33303       |                 |
| EC-MCR06  | E. coli     | ST1564 | 1.1      | IncX4                     | —                                          | —                                     | 6          | 33303       |                 |
| EC-MCR07  | E. coli     | ST752 | 1.1      | IncB/O/K/Z                | sul2                                      | IS30-like element ISApl1 family transposase | 5          | 93122       |                 |
| EC-MCR08  | E. coli     | ST10  | 1.1      | IncX4                     | —                                          | —                                     | 5          | 23832       |                 |
| EC-MCR09  | E. coli     | ST162 | 1.1      | IncX4                     | —                                          | —                                     | 5          | 35016       |                 |
| EC-MCR11  | E. coli     | ST1842 | 1.1      | IncX4                     | —                                          | —                                     | 3          | 33303       |                 |
| EC-MCR12  | E. coli     | ST10  | 1.1      | IncX4                     | —                                          | —                                     | 6          | 33303       |                 |
| EC-MCR13  | E. coli     | ST641 | 1.1      | IncX4                     | —                                          | —                                     | 7          | 33303       |                 |
| EC-MCR14  | E. coli     | ST155 | 1.1      | IncHI2, IncHI2A           | aadA2, cmlA1, aadA1, sul3                 | IS30-like element ISApl1 family transposase | 2          | 243755      |                 |
| EC-MCR15  | E. coli     | ST10  | 1.1      | IncX4                     | —                                          | —                                     | 4          | 34755       |                 |
| EC-MCR16  | E. coli     | ST997 | 1.1      | IncHI2, IncHI2A           | tet(A), sul1, aadA1, dfrA10, ahp(6)-Ia, ahp(3')-Ia, aadA1, sul3, aadA1, dfrA10 | IS30-like element ISApl1 family transposase | 2          | 2114156     |                 |
| EC-MCR17  | E. coli     | ST57  | 1.1      | IncHI2, IncHI2A           | —                                          | —                                     | 2          | 211552      |                 |
| EC-MCR18  | E. coli     | ST997 | 1.1      | IncX4                     | —                                          | —                                     | 5          | 33303       |                 |
| EC-MCR19  | E. coli     | ST997 | 1.1      | IncX4                     | —                                          | —                                     | 5          | 33303       |                 |
| EC-MCR20  | E. coli     | ST624 | 1.1      | IncX4                     | —                                          | —                                     | 5          | 33303       |                 |
| EC-MCR21  | E. coli     | ST624 | 1.1      | IncX4                     | —                                          | —                                     | 6          | 33303       |                 |
| EC-MCR22  | E. coli     | ST10  | 1.1      | IncHI2, IncHI2A           | btaTEM-1, tet(A), sul1, aadA1, dfrA1, Inu(F), ahp(3')-Ia | IS30-like element ISApl1 family transposase | 2          | 234218      |                 |
| EC-MCR23  | E. coli     | ST93  | 1.1      | none                      | —                                          | —                                     | 1 chromosomal |                 |                 |
| EC-MCR24  | E. coli     | ST48  | 1.1      | IncX4                     | —                                          | —                                     | 6          | 34639       |                 |
| EC-MCR25  | E. coli     | ST624 | 1.1      | IncX4                     | —                                          | —                                     | 3          | 33303       |                 |
| EC-MCR26  | E. coli     | ST997 | 1.1      | IncHI2, IncHI2A, IncQ1    | tet(A), sul1, aadA1, dfrA10, ahp(6)-Ia, ahp(3')-Ia, btaTEM-150, aadA2, cmlA1, sul3, aadA1, dfrA1, Inu(F), ahp(3')-Ia | IS30-like element ISApl1 family transposase | 2          | 267214      |                 |
| EC-MCR27  | E. coli     | ST1011 | 1.1    | IncX4                     | —                                          | —                                     | 8          | 33303       |                 |
| EC-MCR28  | E. coli     | ST354 | 1.1      | IncHI2, IncHI2A, IncQ1, Col(MG828) | tet(A), sul1, aadA1, dfrA10, ahp(6)-Ia, ahp(3')-Ia, btaTEM-150 | IS30-like element ISApl1 family transposase | 3          | 252468      |                 |
| EC-MCR29  | E. coli     | ST624 | 1.1      | IncHI2, IncHI2A           | cmlA1, aadA10, sul3, ahp(3')-Ia, btaTEM-150, tet(A), aadA2, aac(3)-Vla | IS30-like element ISApl1 family transposase | 2          | 261285      |                 |
| EC-MCR30  | E. coli     | ST624 | 1.1      | IncHI2, IncHI2A           | —                                          | —                                     | 2          | 261102      |                 |

Continued
### Table 1. Continued

| Sample         | Species | ST  | Mcr type | Inc type on mcr.1 plasmid | Other AMR genes                         | Transposase gene located near mcr.1 | Contig no. | Contig size | Study reference |
|----------------|---------|-----|----------|---------------------------|-----------------------------------------|-------------------------------------|------------|-------------|-----------------|
| EC-MCR31       | E. coli | ST624| 1.1      | IncHI2, IncHI2A           | cmvA1, adA1, sul3, aph(3')-Ia, blt(A), adA2, oac(3')-Vla | IS30-like element ISApl1 family transposase | 2          | 260457      |                 |
| EC-MCR32       | E. coli | ST624| 1.1      | IncHI2, IncHI2A           | cmvA1, adA1, sul3, aph(3')-Ia, blt(A), adA2, oac(3')-Vla | IS30-like element ISApl1 family transposase | 2          | 261285      |                 |
| EC-MCR33       | E. coli | ST1564| 1.1     | IncX4                     | none                                    | IS30-like element ISApl1 family transposase | 4          | 33303       | chromosomal     |
| EC-MCR34       | E. coli | ST117| 1.1      | none                      | —                                       | IS30-like element ISApl1 family transposase | 1          | 254841      |                 |
| EC-MCR35       | E. coli | ST2079| 1.1     | IncHI2, IncHI2A           | tet(A), sul1, adA1, dfrA1, aph(6)-Id, aph(3'')-Ib, sul3, cmvA1, adA2, catA1 | IS30-like element ISApl1 family transposase | 2          | 248481      |                 |

*Identity or alignment length is not 100%.
Substitution in second base pair of first starting codon.
Resistance gene detected twice.
Figure 1. Heatmap and dendrogram showing all plasmids analysed in this study. The dendrogram represents the similarity among plasmid sequences based on the Jaccard dissimilarity of 31-mers of each plasmid. Coloured cells in the heatmap indicate either the presence of this gene or the origin of replication of this plasmid.
the possibility of retail meat to be a significant contributor to
the dissemination of mobile colistin resistance in the Netherlands.

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Transparency declarations
None to declare.

Supplementary data
Table S1 is available as Supplementary data at JAC-AMR Online.

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