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

Casper Jamin[1], Bazante K. Sanders[2,3], Miaomiao Zhou[2], Adalberto Costessi[4], Danny Duijsings[4]†, Jan A. J. W. Kluytmans[5,6], Lieke B. van Alphen[1] and Eefje J. A. Schrauwen[2,3]*

[1]Department of Medical Microbiology, Care and Public Health Research Institute (CAPHRI), Maastricht University Medical Center, Maastricht, The Netherlands; [2]Academy for Technology of Health and Environment, Avans University of Applied Science, Breda, The Netherlands; [3]Research Group Analysis Techniques in Life Sciences, Avans University of Applied Sciences, Breda, The Netherlands; [4]BaseClear B.V., Leiden, The Netherlands; [5]Department of Infection Control, Amphia Hospital, Breda, The Netherlands; [6]Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands

*Corresponding author. E-mail: eja.schrauwen@avans.nl
†Present address: Viroclinics Biosciences B.V., Rotterdam, 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]

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.[10,11] The isolates in this study were genotypical *mcr-1* PCR positive and phenotypical 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, Amstelveen, 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 (Illumina), 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).

Fastq read sequence files were generated using bcl2fastq2 version 2.18. 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 1000 bp and subsequently assessed using QUAST 5.0.[12] 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).[13]

Plasmid analysis

The *mcr-1* plasmid sequences were manually identified and extracted from the assembly graphs (.gfa files) using Bandage.[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/Casperjamin/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 (v1.0.1, 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	33 303	5
KP-MCR02	*K. pneumoniae*	ST1944	1.1	IncHI2, IncHI2A[a]	*aph(3')-Ia*[a], *sul3*, *aadA1*[a], *dfrA12*	IS*30*-like element ISApl1 family transposase	2	211 949	5
EC-MCR03	*E. coli*	ST10	1.1	IncX4	—	—	7	33 303	5
EC-MCR04	*E. coli*	ST8262	1.1[b]	IncX4	—	—	5	33 310	5
EC-MCR05	*E. coli*	ST8262	1.1	IncX4	—	—	6	33 310	5
EC-MCR06	*E. coli*	ST1564	1.1	IncX4	—	—	6	33 303	5
EC-MCR07	*E. coli*	ST752	1.1	IncB/O/K/Z	*sul2*	IS*30*-like element ISApl1 family transposase	5	93 122	5
EC-MCR08	*E. coli*	ST10	1.1	IncX4	—	—	5	23 832	5
EC-MCR09	*E. coli*	ST162	1.1	IncX4	—	—	5	35 016	5
EC-MCR11	*E. coli*	ST1842	1.1	IncX4	—	—	3	33 310	5
EC-MCR12	*E. coli*	ST10	1.1	IncX4	—	—	6	33 303	5
EC-MCR13	*E. coli*	ST641	1.1	IncX4	—	—	7	33 303	5
EC-MCR14	*E. coli*	ST155	1.1	IncHI2, IncHI2A[a]	*aadA2*, *cmlA1*[a], *aadA1*[a], *sul3*	IS*30*-like element ISApl1 family transposase	2	243 755	5
EC-MCR15	*E. coli*	ST10	1.1	IncX4	—	—	4	34 755	5
EC-MCR16	*E. coli*	ST997	1.1	IncHI2, IncHI2A[a]	*tet*(A), *sul1*, *aadA1*[a], *dfrA1*[a], *aph(6)-Id*, *aph(3'')-Ib*[a]	IS*30*-like element ISApl1 family transposase	2	214 156	5
EC-MCR17	*E. coli*	ST57	1.1	IncHI2, IncHI2A[a]	*aadA1*[a], *sul3*, *aph(3')-Ia*[a]	IS*30*-like element ISApl1 family transposase	2	211 552	5
EC-MCR18	*E. coli*	ST997	1.1	IncX4	—	—	5	33 310	5
EC-MCR19	*E. coli*	ST997	1.1	IncX4	—	—	5	33 310	5
EC-MCR20	*E. coli*	ST624	1.1	IncX4	—	—	5	33 310	5
EC-MCR21	*E. coli*	ST624	1.1	IncX4	—	—	6	33 310	5
EC-MCR22	*E. coli*	ST10	1.1	IncHI2, IncHI2A[a]	bla_{TEM-1}, *tet*(A), *sul1*, *aadA1*[a], *dfrA1*, *lnu*(F), *aph(3')-Ia*	IS*30*-like element ISApl1 family transposase	2	234 218	5
EC-MCR23	*E. coli*	ST93	1.1	none	—	IS*30*-like element ISApl1 family transposase	1	chromosomal	5
EC-MCR24	*E. coli*	ST48	1.1	IncX4	—	—	6	34 639	5
EC-MCR25	*E. coli*	ST624	1.1	IncX4	—	—	3	33 310	5
EC-MCR26	*E. coli*	ST997	1.1	IncHI2, IncHI2A[a], IncQI	*tet*(A), *sul1*, *aadA1*[a], *dfrA1*[a], *aph(6)-Id*[c], *aph(3'')-Ib*[a,c], *sul2*[a], *aph(3')-Ia*, *aac(3)-IIe*, $bla_{TEM-150}$[a]	IS*30*-like element ISApl1 family transposase	2	267 214	5
EC-MCR27	*E. coli*	ST1011	1.1	IncX4	—	—	8	33 310	5
EC-MCR28	*E. coli*	ST354	1.1	IncHI2, IncHI2A[a], IncQI, Col(MG828)	*tet*(A)[a], *sul1*, *aadA1*[a,c], *dfrA1*[a], *aph(6)-Id*[c], *aph(3'')-Ib*[a,c], *sul2*[a], *aadA2*, *cmlA1*[a], *sul3*, *aac(3)-IIe*, $bla_{TEM-150}$[a]	IS*30*-like element ISApl1 family transposase	3	252 468	5
EC-MCR29	*E. coli*	ST624	1.1	IncHI2, IncHI2A[a]	*cmlA1*, *aadA1*[a,c], *sul3*, *aph(3')-Ia*, bla_{TEM-1}[a], *tet*(A), *aadA2*, *aac(3)-VIa*	IS*30*-like element ISApl1 family transposase	2	261 285	5
EC-MCR30	*E. coli*	ST624	1.1	IncHI2, IncHI2A[a]			2	261 102	5

Continued

Table 1. *Continued*

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
					cmlA1, *aadA1*[c], *sul3*, *aph(3')-Ia*, bla_{TEM-1}, *tet*(A), *aadA2*, *aac(3)-VIa*	IS*30*-like element ISApl1 family transposase			
EC-MCR31	*E. coli*	ST624	1.1	IncHI2, IncHI2A[a]	*aadA2*, *cmlA1*, *aadA1*[c], *sul3*, *aph(3')-Ia*, bla_{TEM-1}, *tet*(A), *aac(3)-VIa*	IS*30*-like element ISApl1 family transposase	2	260 457	[5]
EC-MCR32	*E. coli*	ST624	1.1	IncHI2, IncHI2A[a]	*cmlA1*[a], *aadA1*[a,c], *sul3*, *aph(3')-Ia*, bla_{TEM-1}[a], *tet*(A), *aadA2*, *aac(3)-VIa*	IS*30*-like element ISApl1 family transposase	2	261 285	[5]
EC-MCR33	*E. coli*	ST1564	1.1	IncX4	—	—	4	33 303	[5]
EC-MCR34	*E. coli*	ST117	1.1	none	—	IS*30*-like element ISApl1 family transposase	1	chromosomal	
EC-MCR35	*E. coli*	ST2079	1.1	IncHI2, IncHI2A[a]	*tet*(A), *sul1*, *aadA1*[a,c], *dfrA1*[a], *aph(6)-Id*, *aph(3'')-Ib*[a], *sul3*, *cmlA1*[a], *aadA2*, *catA1*[a]	IS*30*-like element ISApl1 family transposase	2	248 481	[6]

[a]Identity or alignment length is not 100%.
[b]Substitution in second base pair of first starting codon.
[c]Resistance gene detected twice.

Except for the IncX4, *mcr1.1* plasmids, all *mcr1.1* genes, plasmid or chromosomal, were flanked by IS*30* transposases (Table 1). All the IncX4 *mcr-1* plasmids shared, on average, 0.93 (standard deviation 0.08) of their k-mer content and did not contain any additional resistance genes. As a result, these plasmids were highly similar in size (average 33 kb, range 23 kb to 35 kb, Table 1). Additionally, the IncX4 plasmids found in this study were also highly similar to plasmids present in public databases, which originated from clinical isolates (Figure 1). Furthermore, the bacterial hosts of these IncX4 plasmids showed various STs (Table 1), indicating the widespread nature of this plasmid, most likely driven by conjugation. All IncX4 *mcr1.1*-containing plasmids carried a *virB* type IV secretion system, required for conjugation (data not shown). The AWGS0007 *mcr1.1* plasmid (IncB/O/K/Z) encoded an IncI-1-type conjugal transfer protein TrbA. All other plasmids carried specific incompatibility group-associated conjugation machinery (data not shown). The IncHI2+IncHIA2 plasmids showed high k-mer similarity among each other (mean 0.72, standard deviation 0.12), but less than the IncX4 plasmids. These were generally much larger in size, ranging from 151 kb to 267 kb and additionally encoded a heterogenous set of AMR genes. It should be noted that no plasmids with an IncI origin of replication (ORI) containing *mcr-1.1* were encountered in the strains in this study (Table 1, Figure 1) and only one IncI plasmid outside this study (NZ.CP02554.1) was derived from food origin.[1]

None of the *mcr-1* carrying plasmids in our study carried other genes encoding ESBL resistance. Three isolates contained resistance genes (TEM-52c, SHV-12 and CTX-M-1, respectively), however, these resistance genes were not present on the *mcr-1.1* plasmid. Additionally, one *mcr-1.1* plasmid encoded virulence factors, as it contained five genes of the aerobactin gene cluster (NZ.CP029748.1).

One novel *mcr-1*-containing plasmid was found (EC-MCR07) with a size of 93 kb, which also encoded the sulphonamide resistance gene *sul2*. This plasmid shared barely any sequence similarity as, on average, only a fraction of 0.02 (standard deviation 0.069) of all k-mers were shared with the other plasmids. This was the only plasmid with an IncB/O/K/Z ORI.

The two strains with a chromosomal *mcr-1.1* gene (EC-MCR23 and EC-MCR34) had no other known resistance genes within the same chromosomal region (within 50 kb, data not shown), indicating the mobilization of colistin resistance as a sole passenger of its mobile genetic element IS*30*. We observed multiple different isolates from retail meat with similar plasmids, which might be caused by the spread of these plasmids within the farms or by individual introduction since these are common plasmids. In addition, similar isolates with identical plasmids are found, which could indicate a batch effect.

Conclusions

In this study we aimed to elucidate the plasmid backbones from *mcr-1*-containing plasmids obtained from retail chicken in the Netherlands. In the strains collected here, *mcr-1* resided often but not always in various plasmids, indicating the high mobility nature of this gene in *E. coli* as a host. Most plasmid backbones found in this study were also found in human clinical isolates. This indicates

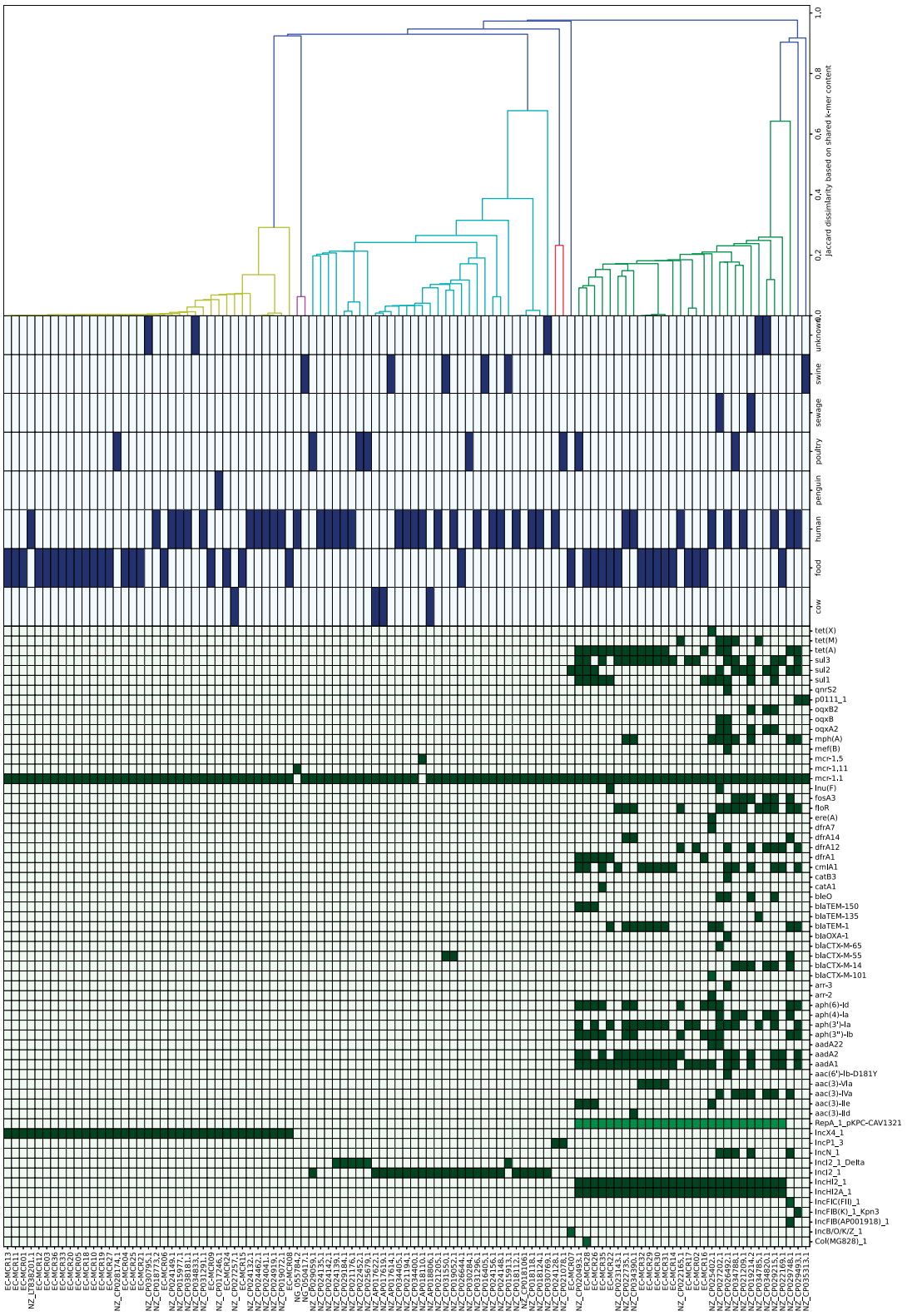

Figure 1. Heatmap and dendrogram showing all plasmids analysed in this study. The dendrogram represents the similarity among plasmid sequences based on the Jaccard dissimilarity of 31-mers of each plasmid. Coloured cells in the heatmap indicate either the presence of this gene or the origin of replication 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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