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Colistin resistance genes *mcr-1* to *mcr-5*, including a case of triple occurrence (*mcr-1, -3* and -5), in *Escherichia coli* isolates from faeces of healthy pigs, cattle and poultry in Belgium, 2012–2016

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ABSTRACT

Colistin is a last-resort antimicrobial used to treat infections caused by multidrug-resistant Gram-negative bacilli (MDR-GNB). The emergence of colistin resistance, particularly linked to mobile genetic elements including the *mcr* genes, is a major threat to the management of MDR-GNB infections. The aim of this study was to assess the presence of *mcr* genes in a collection of 40 colistin-resistant commensal *Escherichia coli* isolated from healthy pigs, cattle and poultry in Belgium between 2012 and 2016. All isolates carried at least one *mcr* gene. The genes *mcr-1* to *-5* were observed in this collection. Different replicons associated with *mcr* genes were identified, including IncHI2/IncHI2A associated with *mcr-1*, IncX4 associated with *mcr-1* and *mcr-2*, and ColE10 associated with *mcr-4*. While the occurrence of multiple *mcr* genes in a single isolate has rarely been reported elsewhere, a triple occurrence (*mcr-1, -3* and *-5*) was found in this study. All isolates were MDR and carried between one and nine different replicons. Seventeen different sequence types were observed among the 40 *E. coli* isolates. In conclusion, this study revealed the presence of a reservoir of mobile colistin resistance genes (*mcr-1* to *-5*) observed during at least 5 years (2012–2016) in the commensal gut flora of pigs, cattle and poultry in Belgium.

1. Introduction

Antimicrobial resistance is a major problem in human and animal health. Surveillance and control of antimicrobial use are important priorities for many health authorities worldwide. Currently, few last-resort antimicrobials are available to treat bacterial infections caused by multidrug-resistant micro-organisms [1]. Among them, colistin is a cationic polypeptide belonging to the polymyxin family that displays broad-spectrum activity against Gram-negative bacteria [2]. This antimicrobial agent interacts by binding the lipid A of lipopolysaccharides (LPS) in the outer membrane of Gram-negative bacteria [3].

Discovered in 1947, colistin was first used in the 1950s to treat infections caused by Gram-negative bacteria in humans. However, because of its nephrotoxicity and neurotoxicity, colistin was abandoned during the 1970s. With the emergence of multidrug-resistant bacteria, colistin was re-used as a last-resort antibiotic in the mid-1990s [4]. Until recently, resistance to colistin was thought to be merely caused by point mutations in the chromosome, among others mutations in the *pmrABCE* genes encoding LPS biosynthetic enzymes [5]. However, in 2015, transferability of the plasmid-borne polymyxin resistance gene *mcr-1* was demonstrated for the first time in an *Escherichia coli* isolate in China [6]. Before 2015, colistin was classified by the European Medicines Agency (EMA) as a low-risk antibiotic for resistance gene transfer [7]. Since the discovery of the plasmid-borne *mcr* genes, the EMA has ranked the resistance transferability risk as high [7]. Today, colistin is classified by the World Health Organization (WHO) as a 'highest priority critically important antimicrobial for human medicine'[1] .

In the veterinary field, colistin has been used intensively mainly to prevent or treat intestinal infections in food-producing animal [5]. To maintain the therapeutic efficacy of colistin, in 2016 the Committee for Medicinal Products for Veterinary Use (CVMP) established some recommendations for EU member states to limit the use of colistin at 1.0 mg/kg biomass for countries with moderate consumption (e.g. Belgium) [7]. With this goal in mind, the

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use of colistin (in mg of colistin per kg of biomass) in the Belgian veterinary sector constantly decreased between 2012 and 2018 [8]. Currently in Belgium, the veterinary guidelines established by the Antimicrobial Consumption and Resistance in Animals Organisation (AMCRA) recommend the use of colistin as a second (instead of first) choice antibiotic for the treatment of the following indications: (i) poultry with colibacillosis; (ii) cattle with diarrhoea and septicaemia; and (iii) pigs with diarrhoea (including weaning diarrhoea) and salmonellosis. The occurrence of colistin resistance in commensal *E. coli* isolated from food-producing animals in Belgium remained low (<6.5%) between 2012 and 2016 [9] (Supplementary Fig. S1).

The first mobile colistin resistance gene, *mcr-1*, was discovered in *E. coli* isolates of animal and human origin [6] and encodes a phosphoethanolamine transferase enzyme. Retrospective studies demonstrated that this transferable gene was circulating for more than 30 years as attested to by its finding in *E. coli* isolates dating back to the 1980s [10].

Since the discovery of *mcr-1*, nine other *mcr* genes have been reported in various species of Enterobacteriaceae including *E. coli*, *Salmonella enterica* serovar Typhimurium and *Klebsiella pneumoniae* [11–19] in pigs, bovines and poultry.

Plasmid-borne *mcr* genes are an international problem as they can be horizontally transferred between bacteria and can rapidly spread globally [4]. It is therefore important to assess whether colistin-resistant bacteria carry *mcr* genes and could therefore transfer their colistin resistance. This study investigated the presence of *mcr* genes as well as the associated resistance profiles and plasmid replicons in a Belgian collection of colistin-resistant *E. coli* isolated from 2012–2016 from healthy food-producing animals. The genetic background, i.e. sequence type (ST), of the investigated isolates was also characterised to assess the genetic relatedness of the *mcr*-carrying isolates.

2. Materials and methods

2.1. Strain collection

All culturable *E. coli* isolates resistant to colistin ($n = 40$) [minimum inhibitory concentration (MIC) >2 mg/L according to the European Committee on Antimicrobial Susceptibility Testing epidemiological cut-off (EUCAST ECOFF) from 2019] collected from faecal material of healthy food-producing animals [broiler chickens ($n = 1$), fattening pigs ($n = 4$), meat cattle younger than 7 months ($n = 3$) and veal calves ($n = 32$)] between 2012 and 2016 in Belgium during the nationwide antimicrobial resistance surveillance programme of commensal indicator bacteria of the Federal Agency for the Safety of the Food Chain (FASFC) were analysed. Sampling was performed by inspectors from the FASFC [9]. Faecal material was inoculated on MacConkey agar medium without antibiotics until 2014 and on MacConkey agar with or without 1 mg/L cefotaxime in 2015 and 2016 [9]. Resistance to colistin and to other antibiotics was assessed by the broth microdilution method. All isolates were stored at –80 °C for further analysis.

2.2. Antimicrobial susceptibility testing

MICs for 14 antimicrobials (Supplementary Table S1), including colistin (concentration range tested 1–16 mg/L), were determined by the broth microdilution method with EUVSEC plates (Sensititre™, Thermo Fisher Scientific, Waltham, MA, USA) [9]. After 18–24 h of incubation, plates were read with a Sensititre™ Vizion™ instrument (Thermo Fisher Scientific) using Sensivision software (MCS Diagnostics BV, Swalmen, The Netherlands). MICs were interpreted according to EUCAST ECOFFs (June 2019 release).

2.3. Definitions

Isolates with an MIC strictly higher than the EUCAST ECOFF are referred to as 'resistant' rather than 'microbiologically antimicrobial-resistant' organisms for brevity. Extended-spectrum β-lactamase (ESBL) and AmpC phenotypes were assigned as recommended by the European Food Safety Authority (EFSA) (Supplementary Fig. S2) [20]. Multidrug resistance was defined as non-susceptibility to at least three different antimicrobial classes [21].

2.4. Whole-genome sequencing analysis

Genomic DNA was extracted using a DNeasy® Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions for Gram-negative bacteria. DNA purity and concentration were assessed with a NanoDrop™ 1000 spectrophotometer (Isogen Life Science, Utrecht, The Netherlands).

Next-generation sequencing was performed with a MiSeq sequencing platform (Illumina Inc., San Diego, CA, USA). An Illumina Nextera XT DNA Library Preparation Kit (Illumina Inc.) was used to prepare the sequencing libraries, followed by sequencing with a 250-bp paired-end protocol (MiSeq v3 chemistry) according to the manufacturer's instructions. Raw sequenced reads were trimmed with Trimmomatic v.0.38.0 with default settings [22]. SPAdes v.3.12.0 was used to proceed to the assembly with default settings [23]. The occurrence of antimicrobial resistance genes was investigated using ResFinder 4.0 for *E. coli* with default settings [24]. Chromosomal mutations were analysed using PointFinder with default settings [25]. The presence of plasmid replicons was determined using PlasmidFinder v.2.1 with default settings [26]. The decision tree from Barbau-Piednoir et al. [27] was used as reference to determine the pathotype of the isolates. Virulence genes were determined using VirulenceFinder 2.0 for *E. coli* with default settings [28]. Multilocus sequence typing (MLST) was performed using the Institut Pasteur *E. coli* MLST database and online tool [29,30] (genomes have been submitted under BioProject no. **PRJNA670414**). The minimum spanning tree was generated using BioNumerics 8.0 (bioMérieux SA, Marcy-l'Étoile, France). MLST profiles were uploaded into BioNumerics, an MLST comparison was performed, and settings were adapted to show STs and branch distances.

2.5. Identification of plasmids

Contigs carrying the *mcr* genes were BLASTed to look for sequences with 100% coverage and >99% identity. For any results matching these criteria, the corresponding GenBank accession numbers were BLASTed against all contigs of the investigated isolates. If the complete sequence of a plasmid was found in the contigs of an isolate, we considered that this plasmid was present in the isolate.

3. Results

3.1. Phenotypic characterisation of the isolates

Colistin resistance of all 40 *E. coli* isolates originating from healthy food-producing animals investigated in this study was re-tested and confirmed upon strain cultivation. Besides, resistance to other antibiotics was also observed in these isolates, as follows: ampicillin (40/40, 100%); tetracycline (39/40, 97.5%); sulfamethoxazole (39/40, 97.5%); trimethoprim (35/40, 87.5%); ciprofloxacin (31/40, 77.5%); nalidixic acid (29/40, 72.5%); chloramphenicol (25/40, 62.5%); gentamicin (19/40, 47.5%); cefotaxime (13/40, 32.5%); ceftazidime (12/40, 30.0%); azithromycin (5/40, 12.5%); and tigecycline (2/40, 5.0%) (Fig. 1). No resistance to

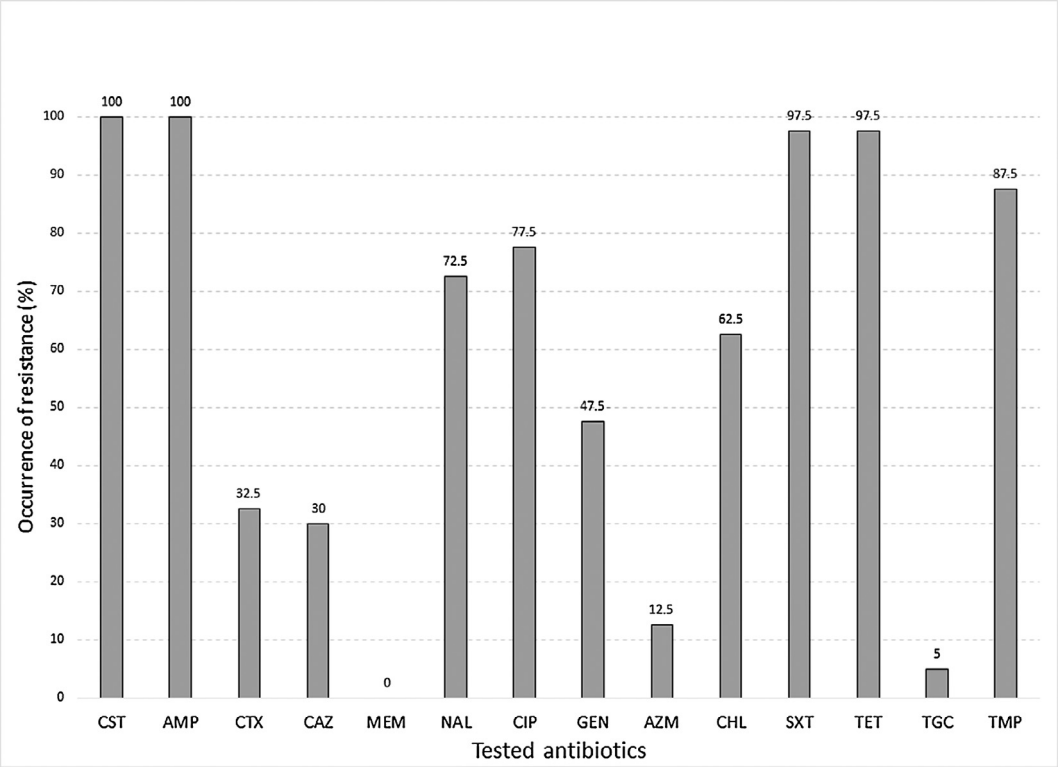

Fig. 1. Occurrence of resistance to the tested antibiotics among the 40 studied colistin-resistant commensal *Escherichia coli* isolates. AMP, ampicillin; AZM, azithromycin; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; CST, colistin; CTX, cefotaxime; GEN, gentamicin; MEM, meropenem; NAL, nalidixic acid; SXT, sulfamethoxazole; TET, tetracycline; TGC, tigecycline; TMP, trimethoprim.

meropenem was observed. MICs of all tested antibiotics for the 40 tested isolates are presented in Supplementary Table S1.

Among the 40 colistin-resistant isolates, 9 (22.5%) had an ESBL phenotype and 3 (7.5%) had an ESBL+AmpC phenotype (data not shown). All isolates showed a multidrug-resistant profile, with 33 isolates (82.5%) being resistant to at least six different classes of antibiotics (including colistin) (Supplementary Table S1). Furthermore, among 13 isolates resistant to third-generation cephalosporins, 11 were resistant to fluoroquinolones and to either gentamicin (4 isolates) or azithromycin (1 isolate) or both gentamicin and azithromycin (2 isolates) (Supplementary Table S1).

3.2. Genotypic characterisation of the isolates

All *E. coli* isolates carried at least one *mcr* gene. The *mcr-1* gene was found in 31 isolates (*mcr-1.1* variant only), *mcr-4* was found in 8 isolates (*mcr-4.6* variant only), and *mcr-2* (*mcr-2.1* variant), *mcr-3* (*mcr-3.2* variant) and *mcr-5* (*mcr-5.1* variant) were each observed in one isolate. Among the 31 *mcr-1*-positive isolates, 1 was isolated from a broiler chicken, 3 from fattening pigs and 27 from cattle (24 samples from veal calves and 3 from meat cattle younger than 7 months). BLAST analysis allowed the identification of four different known plasmids carrying *mcr-1* in 5 isolates [**KY075652.1** (n = 1), **KY075653.1** (n = 2), **KY075660.1** (n = 1) and **MK574666.1** (n = 1)] (Table 1) and to determine that one *mcr-1* was integrated into the chromosome of another isolate (1511425 1STR1). For the remaining 25 *mcr-1* genes, no known plasmid could be identified. However, all of the *mcr-1* genes showed the same genetic surrounding (Fig. 2a), even the *mcr-1* integrated into the chromosome (Fig. 2b). The *mcr-2* gene was observed in an isolate from a fattening pig. This gene was carried on the same IncX4 plasmid discovered previously in Belgium (GenBank **LT598652.1**) [11]. The *mcr-4* gene occurred in isolates isolated from veal calves (n = 8). Overall, this latter gene was observed in 22.9% (8/35) of the colistin-resistant *E. coli* isolates collected from cattle. According to BLAST results, all *mcr-4* genes in this study were found on the same ColE10 plasmid discovered by Carattoli et al. in 2017 (GenBank **MF543359.1**) [13]. No mutations known to confer colistin resistance were found in the *pmr* genes.

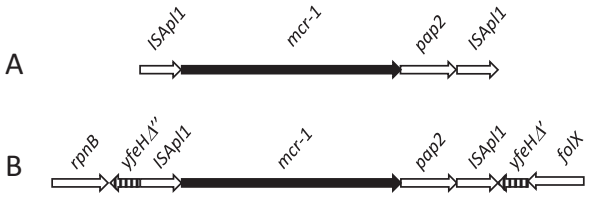

Fig. 2. Genetic map of the *mcr-1* genes. Antimicrobial resistance genes are represented by black arrows and other genes by white arrows. (A) Genetic context of all *mcr-1* genes of this study. (B) Genetic map showing the *mcr-1* gene inserted into the chromosome. The *yfeH* gene is drawn with black vertically hatched arrows, indicating that it is cut in two parts; the Δ′ and Δ′′ represent the 5′ and 3′ element of the gene, respectively.

Although samples were collected from healthy animals, virulence genes were investigated to determine the pathogenic group of isolates, if any. No pathogenic isolates were found except one (1203686 1STR1) belonging to the enteropathogenic *E. coli* (EPEC) group.

PlasmidFinder analysis identified 36 different replicons among the 40 *E. coli* isolates [Col(BS512), Col(MG828), Col(pHAD28), Col(Ye4449), Col156, Col440II, ColE10, IncFIA, IncFIA(HI1), IncFIB, IncFIB(AP001918), IncFIB(H89-PhagePlasmid), IncFIB(K), IncFIC(FII), IncFII, IncFII(29), IncFII(pAMA1167-NDM-5), IncFII(pCoo), IncFII(pHN7A8), IncFII(pRSB107), IncFII(Yp), IncHI1A, IncHI1B(R27), IncHI2, IncHI2A, IncI1-I(Gamma), IncI2, IncN, IncN3, IncQ1, IncR,

Table 1
mcr profile, sequence type (ST), plasmid replicons and antimicrobial resistance genes (ARGs) of the 40 colistin-resistant *Escherichia coli* isolates

Year	Strain no.	*mcr* profile	Animal origin	ST [a]	Identified *mcr*-carrying plasmid [b]	Plasmid replicons [c]	Other ARGs/mutations [d]
2012	1203686 1STR1	*mcr-1.1*	Broiler chicken	367	ND	IncFII(29), IncI1-I(Gamma), IncI2, IncX4, p0111	*aadA1, aadA2b, aph(3′)-Ia, bla*$_{TEM-1B}$*, cmlA1, lnu(F), mdf(A), sul3, tet(A), gyrA* p.S83L
2012	1211226	*mcr-1.1*	Veal calf	2[A]	ND	IncFIB(AP001918), IncFII, IncHI2, IncHI2A	*aadA1, aph(3′′)-Ib, aph(6)-Id, bla*$_{TEM-1B}$*, dfrA1, mdf(A), mph(B), sul1, sul3, tet(M)*
2012	1217177	*mcr-1.1*	Veal calf	471	ND	Col440II, IncFIA, IncFIB(AP001918), IncFII, IncQ1, IncX1, p0111	*aac(3)-IIa, aadA1, aadA2b, aph(3′)-Ia, aph(3′′)-Ib, aph(6)-Id, bla*$_{TEM-1A}$*, cmlA1, dfrA1, mdf(A), sul1, sul2, sul3, tet(B), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I, *parE* p.S458A
2012	1217795	*mcr-4.6*	Veal calf	960	**MF543359.1**	ColE10, IncX5, p0111	*bla*$_{TEM-1A}$*, mdf(A)*
2012	1220582	*mcr-1.1*	Veal calf	716	ND	IncFIA, IncFIB(AP001918), IncFII(pRSB107), IncQ1, IncX1, IncX4	*aadA1, aph(3′)-Ia, aph(3′′)-Ib, aph(6)-Id, bla*$_{TEM-1B}$*, catA1, dfrA1, mdf(A), sul2, tet(B), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.E84K
2012	1223744	*mcr-4.6*	Veal calf	88	MF543359.1	ColE10, Col(Ye4449), IncFII, IncHI2, IncHI2A, IncI1-I(Gamma), IncQ1, IncX1	*aac(3)-IV, aadA1, aadA2b, aadA5, aph(3′)-Ia, aph(3′′)-Ib, aph(4)-Ia, aph(6)-Id, bla*$_{TEM-1B}$*, catA1, cmlA1, dfrA17, floR, mdf(A), sul1, sul2, sul3, tet(A), tet(C), tet(M), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I
2013	1303300 STR1	*mcr-1.1*	Meat cattle aged <7 months	471	ND	Col440II, IncFIA(HI1), IncFII, IncHI1A, IncHI1B(R27), IncQ1, IncX1, IncX4, p0111	*aadA1, aph(3′)-Ia, aph(3′′)-Ib, aph(6)-Id, bla*$_{TEM-1B}$*, catA1, dfrA1, lnu(G), mdf(A), sul1, sul2, sul3, tet(B), tet(M), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I, *parE* p.S458A
2013	1311456 STR1	*mcr-1.1*	Meat cattle aged <7 months	310[A]	ND	IncFIA, IncFIB(AP001918), IncFII(pAMA1167-NDM-5), IncHI2, IncHI2A, IncQ1, IncX4	*aadA1, aph(3′)-Ia, aph(3′′)-Ib, aph(6)-Id, bla*$_{OXA-1}$*, bla*$_{TEM-1B}$*, catA1, dfrA1, mdf(A), sul1, sul2, sul3,* ***tet(A)****, tet(B), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I
2013	1301649 STR1	*mcr-4.6*	Veal calf	132[A]	**MF543359.1**	ColE10, Col(Ye4449), IncFIA, IncFIB, IncFII, IncN, IncX5	*aadA1, aph(3′′)-Ib, aph(6)-Id, bla*$_{TEM-1B}$*, dfrA1, mdf(A), mph(B), sul1, tet(A), tet(M)*
2013	1302187 STR1	*mcr-1.1*	Veal calf	471	**KY075652.1**	Col440II, IncFIB(AP001918), IncN, IncQ1, IncX1, IncX4	*aadA1, aph(3′)-Ia, aph(3′′)-Ib, aph(6)-Id, bla*$_{TEM-1B}$*, dfrA1, floR, lnu(G), mdf(A), mph(B), sul1, sul2, tet(A), tet(M), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I, *parE* p.S458A
2013	1307926 STR1	*mcr-1.1*	Veal calf	471	ND	IncFIA, IncFIB(AP001918), IncFII, IncQ1, IncX1, p0111	*aac(3)-IIa, aadA1, aadA2b, aph(3′)-Ia, aph(3′′)-Ib, aph(6)-Id, bla*$_{TEM-1A}$*, cmlA1, dfrA1, mdf(A), sul2, sul3, tet(B), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I, *parE* p.S458A
2013	1309946 STR1	*mcr-1.1*	Veal calf	87	ND	IncX1	*aac(3)-IIa, aadA1, aph(3′)-Ia, aph(3′′)-Ib, aph(6)-Id, bla*$_{TEM-1A}$*, catA1, dfrA1, mdf(A), mph(B), sul1, tet(A), tet(M), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I
2013	1309964 STR1	*mcr-4.6*	Veal calf	960	**MF543359.1**	Col440II, ColE10, IncFIA(HI1), IncFIB(AP001918), IncFIB(K), IncFII, IncI1-I(Gamma)	*aadA1, aph(3′)-Ia, bla*$_{TEM-1A}$*, dfrA1, mdf(A), sul1, sul3, tet(A), tet(M), gyrA* p.S83L
2013	1309979 STR1	*mcr-1.1*	Veal calf	960	ND	Col(MG828), IncHI2, IncHI2A, IncQ1, IncX5	*aac(3)-IV, aadA1, aph(3′)-Ia, aph(3′′)-Ib, aph(4)-Ia, aph(6)-Id, bla*$_{TEM-1B}$*, catB3, dfrA1, dfrB1, floR, mdf(A), sul1, sul2, tet(A)*
2013	1311787 STR1	*mcr-1.1*	Veal calf	471	**KY075653.1**	Col440II, IncFIB(AP001918), IncQ1, IncX4	*aadA1, aph(3′)-Ia, aph(3′′)-Ib, aph(6)-Id, bla*$_{TEM-1A}$*, dfrA1, lnu(G), mdf(A), mph(B), sul1, sul2, tet(A), tet(M), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I, *parE* p.S458A

(*continued on next page*)

Table 1 (*continued*)

Year	Strain no.	*mcr* profile	Animal origin	ST [a]	Identified *mcr*-carrying plasmid [b]	Plasmid replicons [c]	Other ARGs/mutations [d]
2013	1314414 STR1	*mcr-1.1*	Veal calf	471	ND	IncFIB(AP001918), IncFII, IncHI2, IncHI2A, p0111	*aac(3)-IIa, aadA1, aadA2b, aph(3'')-Ib, aph(6)-Id,* bla_{TEM-1A}*, cmlA1, dfrA1, dfrA12, mdf(A), mph(B), sul1, sul3, tet(B), tet(M), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I, *parE* p.S458A
2013	1316991 STR1	*mcr-4.6*	Veal calf	960	**MF543359.1**	Col440II, ColE10, IncFIB(AP001918), IncI1-I(Gamma), IncR	*aadA1, aph(3')-Ia,* bla_{TEM-1A}*, dfrA1, mdf(A), sul1, sul3, tet(A), tet(M), gyrA* p.S83L
2014	1402931 1STR1	*mcr-4.6*	Veal calf	132[A]	**MF543359.1**	Col440II, ColE10, IncFIA, IncFIB(AP001918), IncX1	*aadA1, aph(3'')-Ib, aph(6)-Id,* bla_{TEM-1B}*, dfrA1, mdf(A), mph(B), sul1, tet(M)*
2014	1403964 1STR1	*mcr-1.1*	Veal calf	471	ND	IncFIA, IncFIB(AP001918), IncFII, IncHI2, IncHI2A, IncQ1, IncX1, p0111	*aac(3)-IIa, aadA1, aph(3')-Ia, aph(3'')-Ib, aph(6)-Id,* bla_{TEM-1A}*, dfrA1, mdf(A), sul1, sul2, tet(B), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I, *parE* p.S458A
2014	1405327 1STR1	*mcr-1.1*	Veal calf	471	ND	IncFIA, IncFIB(AP001918), IncN3, IncQ1, IncX1, p0111	*aac(3)-IIa, aadA1, aadA2b, aph(3')-Ia, aph(3'')-Ib, aph(6)-Id,* bla_{TEM-1A}*, cmlA1, dfrA1, mdf(A), sul1, sul2, sul3, tet(B), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I, *parE* p.S458A
2014	1411979 1STR1	*mcr-4.6*	Veal calf	960	**MF543359.1**	Col(BS512), ColE10, IncFIA(HI1), IncFIB(K), IncFII(Yp), IncHI1A, IncHI1B(R27), p0111	*aadA1, aadA2b, aph(3')-Ia,* bla_{TEM-1A}*, catA1, cmlA1, dfrA1, mdf(A), sul1, sul3, tet(B), tet(M)*
2015	1509795 1STR1	*mcr-1.1*	Fattening pig	342[A]	**KY075660.1**	Col440II, Col(BS512), IncN, IncR, IncX1, IncX4	*aadA24,* $bla_{CTX-M-1}$*, lnu(G), mdf(A), mph(A), qnrS1, tet(B)*
2015	1506551 1STR1	*mcr-1.1, -3.2, -5.1*	Veal calf	2	**MK574666.1** (*mcr-1*), **MF461273.1**[*] (*mcr-3*), ND (*mcr-5*)	Col156, IncFIB(AP001918), IncHI2, IncHI2A (*mcr-1*), IncQ1, IncX1, IncY	*aac(3)-IId,* ***aac(3)-IV****, aadA2, aadA22, aadA5, aph(3')-Ia, aph(3'')-Ib,* ***aph(4)-Ia****, aph(6)-Id,* $bla_{CTX-M-55}$*,* bla_{TEM-1B}*, catA1, catA2, dfrA12, dfrA17, floR, mdf(A), mph(A), mph(B), sul1, sul2, sul3, tet(B), tet(M), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.A56T, *parC* p.S80I
2015	1506932 1STR1	*mcr-1.1*	Veal calf	2[A]	ND	Col440II, IncFIB(AP001918), IncHI2, IncHI2A, IncI1-I(Gamma), IncX5	*aadA1, aph(3')-Ia, aph(3'')-Ib, aph(6)-Id,* bla_{TEM-1}*, dfrA1, mdf(A), sul1, sul3,* ***tet(A)****, gyrA* p.S83L
2015	1506934 1STR1	*mcr-1.1*	Veal calf	471	ND	Col(pHAD28), IncFIA, IncFIB(AP001918), IncFII, IncX1, IncX4, p0111	*aac(3)-II, aadA1, aadA2b, aph(3')-Ia, aph(3'')-Ib, aph(6)-Id,* bla_{TEM-1A}*, cmlA1, dfrA1, mdf(A), sul2, sul3, tet(B), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I
2015	1508479 1STR2	*mcr-1.1*	Veal calf	66	ND	IncFIB(AP001918), IncFII, IncHI2, IncHI2A, IncI1-I(Gamma), IncQ1	*aac(3)-IIa, aac(3)-IV, aadA1, aph(3')-Ia, aph(3'')-Ib, aph(4)-Ia, aph(6)-Id,* $bla_{CTX-M-1}$*,* bla_{TEM-1A}*, catA1, dfrA1, mdf(A), sul1, sul2, sul3, tet(31), tet(A), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I, *ampC* n.–42C>T
2015	1508900 1STR1	*mcr-1.1*	Veal calf	741	ND	IncFIB(AP001918), IncFIB(K), IncHI2, IncHI2A, IncQ1	*aac(3)-IV, aph(3')-Ia, aph(3'')-Ib, aph(4)-Ia, aph(6)-Id,* $bla_{CTX-M-15}$*,* bla_{TEM-1A}*, dfrA14, floR, mdf(A), qnrS1, sul2, tet(A), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I
2015	1508900 2STR1	*mcr-4.6*	Veal calf	88	**MF543359.1**	ColE10, IncFIB(H89-PhagePlasmid), IncFII, IncI1-I(Gamma), IncX1	*aac(3)-IIa, aadA1, aadA2b, aph(3')-Ia, aph(3'')-Ib, aph(6)-Id,* bla_{SHV-2}*,* bla_{TEM-1A}*, catA1, cmlA1, dfrA1, dfrA7, erm(B), mdf(A), mph(B), qnrB19, qnrB5, qnrB81, sul1, sul3, tet(A), tet(M), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I

(*continued on next page*)

Table 1 (*continued*)

Year	Strain no.	*mcr* profile	Animal origin	ST [a]	Identified *mcr*-carrying plasmid [b]	Plasmid replicons [c]	Other ARGs/mutations [d]
2015	1511425 1STR1	*mcr-1.1*	Veal calf	58	ND	Col(MG828), IncFIB(AP001918), IncFIC(FII), IncI1-I(Gamma)	*aac(3)-IIa, aadA1, bla*$_{CTX-M-55}$, *dfrA14, floR, mdf(A), sul3, tet(A), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I, *parE* p.S458T
2015	1511914 2STR1	*mcr-1.1*	Veal calf	66	ND	IncFIB(AP001918), IncFIC(FII), IncHI2, IncHI2A, IncI1-I(Gamma), IncQ1	*aac(3)-IIa, aac(3)-IV, aadA1, aph(3′)-Ia, aph(3′′)-Ib, aph(4)-Ia, aph(6)-Id, bla*$_{CTX-M-1}$, *bla*$_{TEM-1A}$, *catA1, dfrA1, mdf(A), sul1, sul2, sul3, tet(31), tet(A), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I, *ampC* n.–42C>T
2016	U1603383 001	*mcr-2.1*	Fattening pig	638[A]	**LT598652.1**	IncI1-I(Gamma), IncX4, p0111	*aadA1, aadA2b, bla*$_{SHV-12}$, *bla*$_{TEM-1B}$, *cmlA1, dfrA10, mdf(A), sul1, sul3, tet(A)*
2016	U1603544 001	*mcr-1.1*	Fattening pig	86[A]	**KY075653.1**	IncFIB(AP001918), IncFII, IncI1-I(Gamma), IncQ1, IncX1, IncX4	*aph(3′′)-Ib, aph(6)-Id, bla*$_{TEM-52C}$, *dfrA5, mdf(A), sul2, tet(A)*
2016	U1605318 001	*mcr-1.1*	Fattening pig	88	ND	IncFIB(AP001918), IncFII(29), IncHI2, IncHI2A, IncQ1	*aac(3)-IIa, aadA1, aph(3′)-Ia, aph(3′′)-Ib, aph(6)-Id, bla*$_{TEM-1A}$, *dfrA1, mdf(A), sul1, sul2, sul3, tet(A)*
2016	U1603296 001	*mcr-1.1*	Meat cattle aged <7 months	2	ND	IncFIA, IncFIB(AP001918), IncFII(pCoo), IncHI2, IncHI2A, IncQ1	*aadA1, ant(2′′)-Ia, aph(3′)-Ia, aph(3′′)-Ib, aph(6)-Id, bla*$_{TEM-1B}$, *dfrA1, dfrA36, floR, mdf(A), sul1, sul2, sul3,* ***tet(A)****, tet(B), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I
2016	U1601509 001	*mcr-1.1*	Veal calf	471	ND	IncFII, IncHI1A, IncHI1B(R27), IncI1-I(Gamma), IncN3, IncX1, p0111	*aadA1, ant(2′′)-Ia, aph(3′)-Ia, aph(3′′)-Ib, aph(6)-Id, bla*$_{TEM-1B}$, *catA1, dfrA1, mdf(A), sul1, sul3, tet(A), tet(B), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I
2016	U1602624 001	*mcr-1.1*	Veal calf	31	ND	IncFIA, IncFIB(AP001918), IncFII(pCoo), IncHI2, IncHI2A, IncQ1	*aac(3)-IIa, aac(3)-IId, aadA1, aadA2b, aph(3′)-Ia, aph(3′′)-Ib, aph(6)-Id, bla*$_{TEM-1B}$, *catA1, cmlA1, dfrA1, dfrA12, mdf(A), mph(A), sul1, sul2, sul3, tet(A), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I
2016	U1602935 001	*mcr-1.1*	Veal calf	471	ND	IncFIA, IncFIB(AP001918), IncFII, IncHI2, IncHI2A, IncX1, p0111	*aadA1, aadA2b, aph(3′′)-Ib, aph(6)-Id, bla*$_{TEM-1A}$, *cmlA1, dfrA1, mdf(A), mph(B), sul1, sul3, tet(A), tet(B), tet(M), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I, *parE* p.S458A
2016	U1605233 001	*mcr-1.1*	Veal calf	731[A]	ND	IncFII(pHN7A8), IncHI2, IncHI2A, IncI1-I(Gamma), IncQ1	*aph(3′)-Ia, aph(3′′)-Ib, aph(6)-Id, bla*$_{TEM-1B}$, *mdf(A), sul2, tet(A), tet(B), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I
2016	U1605255 001	*mcr-1.1*	Veal calf	731[A]	ND	Col(MG828), IncHI2, IncHI2A, IncI1-I(Gamma), IncQ1	*aph(3′)-Ia, aph(3′′)-Ib, aph(6)-Id, bla*$_{TEM-52C}$, *mdf(A), sul2, tet(A), tet(B), gyrA* p.S83L, *gyrA* p.D87N, *parC* p.S80I
2016	U1607591 001	*mcr-1.1*	Veal calf	2	ND	IncFIB(AP001918), IncFII(pCoo), IncHI2, IncHI2A, IncR	*aadA1, aph(3′′)-Ib, aph(6)-Id, bla*$_{CTX-M-1}$, *bla*$_{TEM-1B}$, *cmlA1, dfrA1, dfrA15, mdf(A), mph(A), qnrS1, sul1, sul3, tet(A)*

ND, not determined.
[a] Sequence types (STs) with 'A' at the end indicate the nearest ST.
[b] See Section 2.5, 'Identification of plasmids'.
[c] Underlined replicons are associated with the identified plasmids and/or the contig carrying the *mcr* gene.
[d] ARGs in bold were found on the same contig as the *mcr* gene (for the triple occurrence case, ARGs are on the same contig as the *mcr-5* gene).
* Indicates that the GenBank accession number does not refer to a complete plasmid sequence.

IncX1, IncX4, IncX5, IncY and p0111]. The isolates carried between one and nine of these replicons (Table 1).

Apart from *mcr* genes, antimicrobial resistance gene analysis revealed the presence of resistance genes/mutations against aminoglycosides, β-lactams, lincosamides, macrolides, phenicols, quinolones, sulphonamides, tetracyclines and trimethoprim in the genomes of this collection of isolates (Table 1).

MLST analysis showed various sequence types or close variants thereof: ST471 ($n = 11$); ST2 ($n = 5$); ST960 ($n = 5$); ST88 ($n = 3$); ST66 ($n = 2$); ST132 ($n = 2$); ST731 ($n = 2$); and ST31, ST58, ST86,

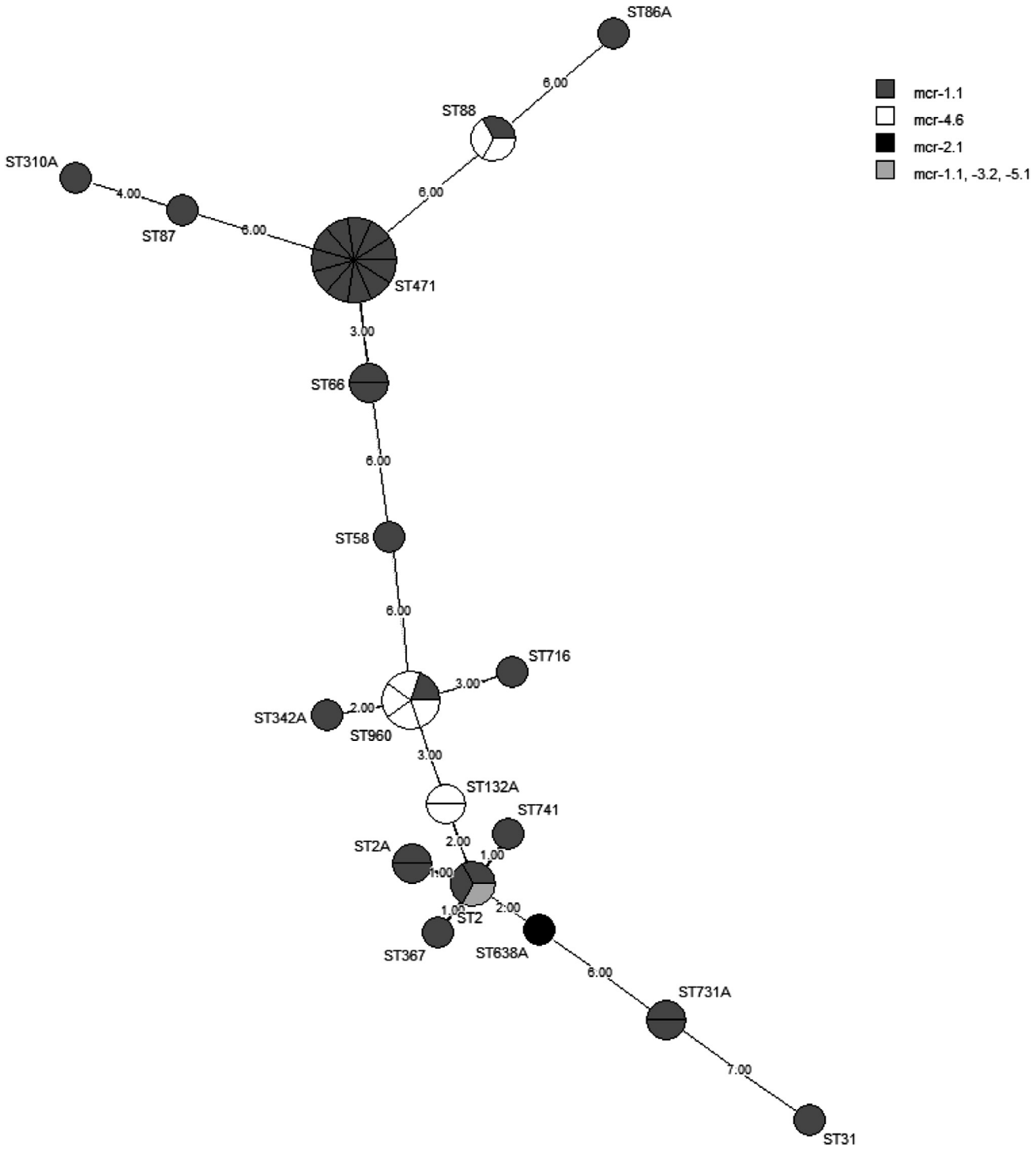

Fig. 3. Minimum spanning tree based on multilocus sequence typing (MLST) results. Branch lengths are indicated by the number of allele differences out of the eight loci. Sequence types (STs) are written close to the nodes. *mcr* profiles are represented by different filling patterns. Sequence types with 'A' at the end indicate the nearest ST.

ST87, ST310, ST342, ST367, ST638, ST716 and ST741 (n = 1 isolate each) (Fig. 3; Table 1).

3.3. Identification of a triple occurrence of mcr genes

Interestingly, *mcr-3* and *mcr-5* were observed together with *mcr-1* in one isolate collected from a bovine animal (veal calf) in 2015 (Supplementary Table S1). The isolate was resistant to 12 of the 14 tested antimicrobials (Table 1; Supplementary Table S1), as follows: colistin (*mcr-1.1, mcr-3.2, mcr-5.1*); ampicillin ($bla_{\text{CTX-M-55}}$, $bla_{\text{TEM-1B}}$); cefotaxime ($bla_{\text{CTX-M-55}}$); ceftazidime ($bla_{\text{CTX-M-55}}$); nalidixic acid [*gyrA* (p.D87N), *gyrA* (p.S83L), *parC* (p.A56T)]; ciprofloxacin [*parC* (p.A56T), *gyrA* (p.S83L)]; gentamicin [*aac(3)-IId, aac(3)-IV*]; azithromycin [*mph(A)*]; chloramphenicol (*catA1, catA2, floR*); sulfamethoxazole (*sul1, sul2, sul3*); tetracycline [*tet(B), tet(M)*]; and trimethoprim (*dfrA12, dfrA17*) (susceptible to tigecycline and meropenem). The isolate carried a total of 27 resistance genes (Table 1) and seven different replicons [Col156, IncFIB(AP001918), IncHI2, IncHI2A, IncQ1, IncX1 and IncY] (Table 1).

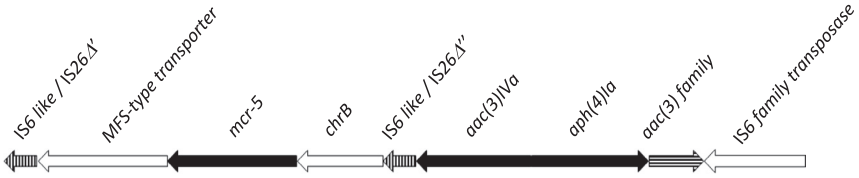

Fig. 4. Genetic map of the *mcr-5* locus (7354 pb). The IS*6*-like/IS*26* element are drawn with black vertically hatched arrows; the Δ′ and Δ′′ represent the 5′ and 3′ element of the IS*6*-like element, respectively. The grey horizontally hatched arrow represents a gene with the missing C-terminus. Antimicrobial resistance genes are represented by black arrows and other genes by white arrows.

The three *mcr* genes in this isolate were identified on three different contigs. BLAST analysis showed that *mcr-1* was carried on an IncHI2/IncHI2A plasmid identified as pDJB-3 (GenBank accession no. **MK574666.1**). The contig carrying *mcr-3* did not hit any known plasmid from the NCBI database but did so for a cluster (together with other contigs from the isolate) containing *mcr-3* (GenBank **MF461273**) with >99% homology. This cluster is part of the unpublished plasmid pZR10 from a Chinese *E. coli*. Finally, the contig carrying *mcr-5* also did not hit any known plasmid. This contig appears to result from a recombination in the IS*6*-like element disrupted by the fragment containing the MFS-type transporter, *mcr-5* and *chrB* genes (Fig. 4).

4. Discussion

This retrospective study conducted on 40 colistin-resistant commensal *E. coli* isolates of animal origin from 2012–2016 demonstrated the presence of *mcr-1* to *-5* in Belgium. In this study, 100% of the isolates carried at least one mobilisable *mcr* gene. Among the *mcr* genes observed, *mcr-1* was the most frequent and was observed in all of the investigated animal species. The same *mcr-1* variant was already reported in Belgium in previous studies [2,31–34]. Our study suggests that this gene was circulating during each year of the 5-year period (2012–2016). The *mcr-2* gene was already reported in Belgium on the same IncX4 plasmid in a previous study on *E. coli* isolates from piglets and calves with diarrhoea [11] and appears to be mostly confined to Belgium [31] and rarely reported elsewhere [35–37]. All occurrences of the *mcr-4* gene were from veal calf isolates and it was the second most frequent *mcr* gene observed in this study. This gene was found 4 years in a row (2012–2015), indicating that it was not associated with a unique event but circulated for several years. To the best of our knowledge, this is the first time that *mcr-4* was detected in bovine isolates and this gene has been rarely reported elsewhere [38]. Noteworthy, all *mcr-4* found in bovine isolates from our study were carried on the same ColE10 pMCR plasmid found in *E. coli* from piglets with post-weaning diarrhoea in Belgium and Spain in 2015 and 2016 [13], indicating that strains carrying this plasmid circulated in different animal hosts (cattle and pigs). To our knowledge, this is also the first time that *mcr-3* and *mcr-5* have been reported in bacteria isolated in Belgium, indicating that each *mcr-1* to *-5* were observed at least once in Belgium. Moreover, the isolates were collected from different geographical areas across the country (data not shown) and were characterised by various STs. Most of these STs were distantly linked (Fig. 3) and, among the closer STs (ST2, ST2A, ST132A, ST367, ST741 and ST638A), four different *mcr* profiles were observed, indicating that *mcr* genes were not linked to a single clonal lineage of *E. coli*. Our results showed that the *mcr* genes are present in different lineages of *E. coli*, mostly (39/40) in non-pathogenic isolates, in different plasmids (for *mcr-1*) hosted by isolates carrying various antimicrobial resistance genes. These numerous and various antimicrobial resistance genes highlighted the high potential of colistin resistance to be co-selected by several other antibiotics. Indeed, all of the isolates were multidrug-resistant and 33 were resistant to at least six different classes of antibiotics.

Multiple occurrences of *mcr* genes was detected in one isolate from a veal calf, which harboured three *mcr* genes (*mcr-1, -3* and *-5*). Multiple *mcr* gene occurrences have been rarely reported so far [39,40]. Moreover, this isolate was resistant to 12 of the 14 antibiotics tested in this study (susceptible to meropenem and tigecycline), including resistance to several antibiotics ranked critical for human health according to current policies (cephalosporins and quinolones). No transferable quinolone resistance genes were detected in this isolate. However, if such a multidrug-resistant profile occurred in a human pathogenic strain, it would represent a potential healthcare issue as the therapeutic options would be limited. No resistance against carbapenems was observed, although such co-resistance (carbapenem + colistin) was reported in an Italian isolate carrying $bla_{\text{OXA-181}}$ and *mcr-1* [41].

In conclusion, the search for *mcr* genes in this collection highlighted that *mcr-1* to *-5* were present in *E. coli* strains from Belgian food-producing animals between 2012 and 2016. Considering that all of the investigated Belgian isolates carried the *mcr* gene(s), this study revealed the presence of a reservoir of mobile resistance colistin genes observed during at least 5 years (2012–2016) in the commensal gut flora of pigs, cattle and poultry. Our results show the importance to add screening for *mcr* genes and other antimicrobial resistance genes to antimicrobial resistance monitoring to monitor the presence and potential spread of plasmid-borne colistin resistance genes. Such genetic screening has already been recommended several times [6,11–14,16,18,19]. Surveillance and awareness programmes, together with ad hoc policies restricting antibiotics use, are crucial to prevent the emergence and spread of such antimicrobial resistance genes.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2021.106350.

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