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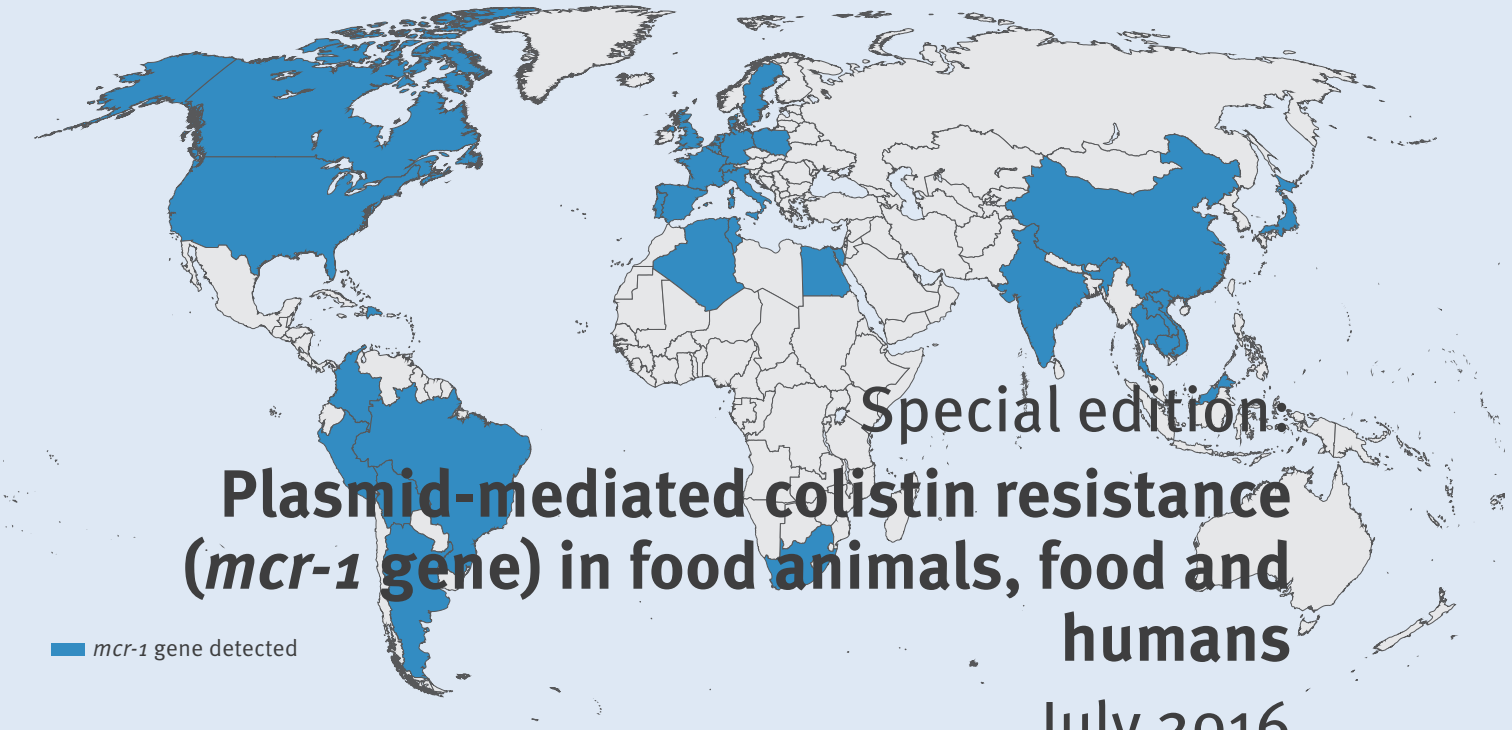

Special edition:

Plasmid-mediated colistin resistance (*mcr-1* gene) in food animals, food and humans

July 2016

Featuring

- **Plasmid-mediated colistin resistance (*mcr-1* gene): three months later, the story unfolds**
- **Silent dissemination of colistin-resistant *Escherichia coli* in South America could contribute to the global spread of the *mcr-1* gene**
- **Identification of a novel plasmid-mediated colistin-resistance gene, *mcr-2*, in *Escherichia coli*, Belgium, June 2016**

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Editorial team

Based at the European Centre for
Disease Prevention and Control (ECDC),
171 65 Stockholm, Sweden

Telephone number

+46 (0)8 58 60 11 38

E-mail

eurosurveillance@ecdc.europa.eu

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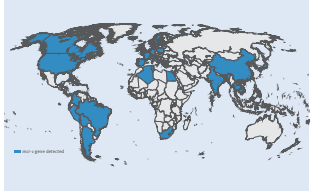

Source: Xavier BB, Lammens C, Ruhal R, Kumar-Singh S, Butaye P, Goossens H, Malhotra-Kumar S. Identification of a novel plasmid-mediated colistin-resistance gene, mcr-2, in Escherichia coli, Belgium, June 2016. Euro Surveill. 2016;21(27):pii=30280.

Plasmid-mediated colistin resistance (*mcr-1* gene): three months later, the story unfolds

RL Skov [1], DL Monnet [2]
1. Department of Microbiology and Infection Control, Statens Serum Institut, Copenhagen, Denmark
2. Office of Chief Scientist, European Centre for Disease Prevention and Control, Stockholm, Sweden

Correspondence: Robert L. Skov (rsk@ssi.dk)

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On 18 November 2015, Liu et al. reported the first description of plasmid-mediated colistin resistance (*mcr-1* gene) in food animals, food and humans in China [1]. In this issue, Kluytmans-van den Bergh et al. report on their finding of the *mcr-1* gene in *Escherichia coli* isolates from three (1.5%) of 196 samples of chicken meat collected at Dutch supermarkets, one in 2009 and two in 2014 [2]. This was done by whole genome sequencing of all *E. coli* isolates and then screening for the presence of the *mcr-1* gene by comparing the assembled sequences with sequence data from two databases. The same study did not find any *mcr-1*-positive isolate among 2,275 extended-spectrum beta-lactamase-positive *Escherichia coli* (screening and clinical isolates) sampled in humans between 2009 and 2015. The exact origin of the sampled chicken meat was not known, with the two samples from 2014 being labelled 'non-Dutch, European'. The fact that the genomes of the two isolates from 2014 differed by only three loci and were from the same lot of chicken meat strongly suggest cross-contamination from a common source.

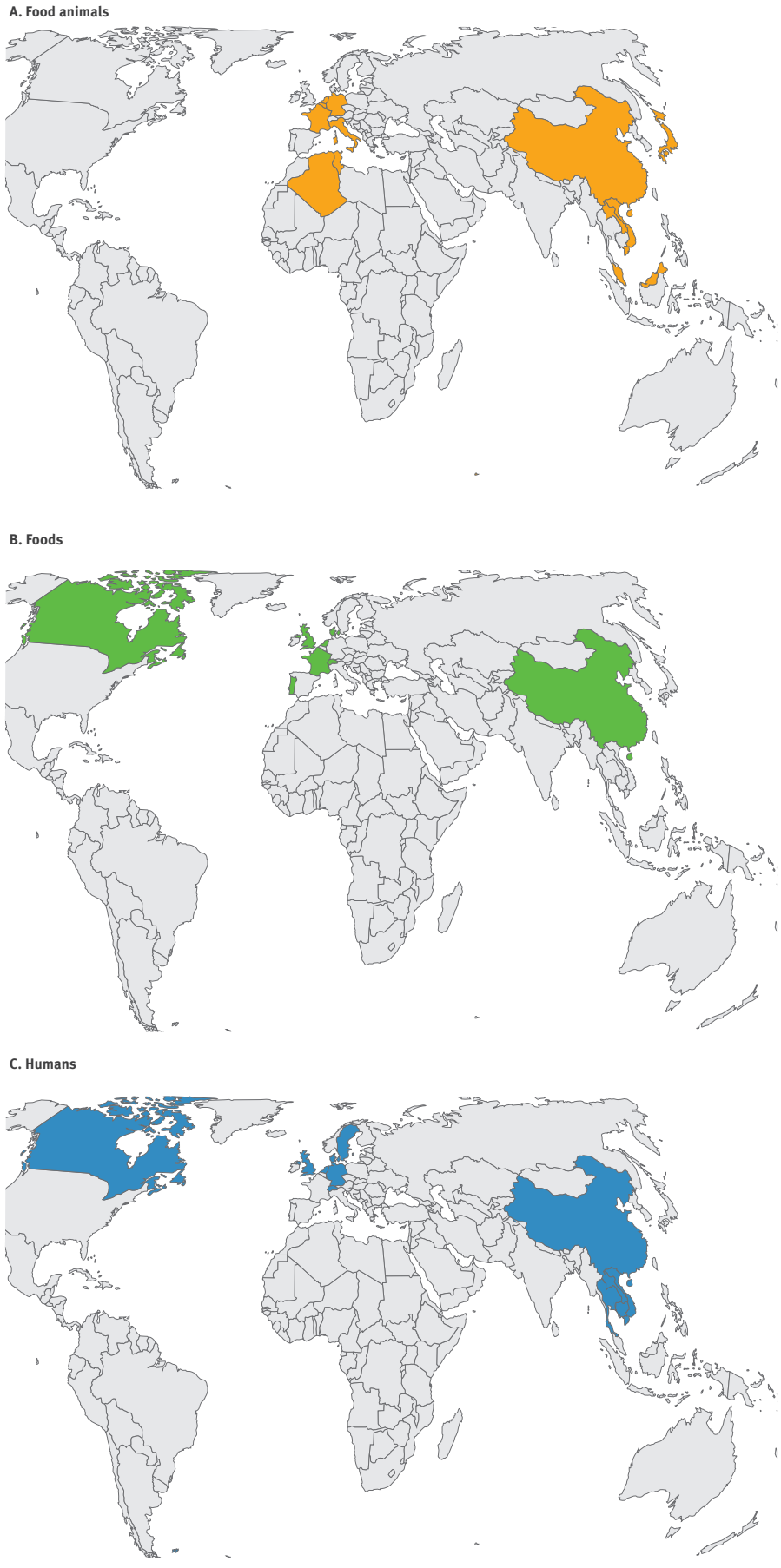
This study adds to the already long list of articles on plasmid-mediated colistin resistance published in this and other journals [3-30] (Figure and Table). Within just three months of the first description, we learned that the *mcr-1* gene (i) had spread to most continents (Figure), (ii) had been found in bacteria isolated from various food animals, from the environment including river water, from various types of meat and vegetables, and from infected patients and asymptomatic human carriers including international travellers, (iii) had been found in various bacterial species, mostly *E. coli*, and on several different plasmids, and (iv) was highly transferrable with in vitro transfer rates as high as 10^{-1}. The fact that we have gained much additional information in such a short time highlights the strength of whole genome sequencing and publicly available sequence databases.

Another important piece of information is that the *mcr-1* gene has been present, though not detected, for a long time. Shen et al. reported an *mcr-1*-positive isolate from chickens in China dating back to the 1980s [21]. In Europe, the oldest isolate reported so far is an *E. coli* from a diarrhoeic veal calf in France in 2005 [10]. The earliest reported isolate from humans is a *Shigella sonnei* from Vietnam in 2008. Trends are available in one study from China and show that the proportion of *mcr-1*-positive isolates in *E. coli* from chickens has been increasing sharply since 2009 [21]. For most studies, it is impossible to calculate the prevalence of *mcr-1*-positive isolates because detection of the *mcr-1* gene was only performed on colistin-resistant isolates. In France, systematic screening of all isolates from the routine European Union surveillance of antimicrobial resistance in zoonotic commensal bacteria showed that prevalence of the *mcr-1* gene ranged from 0.5% in *E. coli* from pigs to 5.9% in *E. coli* from turkeys in the period 2013 and 2014 [16].

Plasmid-mediated colistin resistance lies at the interface between animal health and human health. Polymyxins, and in particular colistin, have been used, both in human and veterinary medicine, for more than 50 years, although their parenteral usage in humans has been limited because of concerns about nephrotoxicity and neurotoxicity. In veterinary medicine, colistin is widely used, especially for controlling diarrhoeal diseases in pig and poultry production [31]. However, its use varies widely between countries; in Europe, from 0 mg (Finland, Iceland, Norway) to more than 20 mg (Italy, Spain) per kg animal biomass were used in 2013 [32]. Data from other parts of the world are more scarce, however Liu et al. reported that the market value for colistin for veterinary usage increased from USD 8.7 billion (EUR 8.0 billion) in 1992 to a projected USD 43 billion (EUR 39.6 billion) in 2018, with China being the largest user of a projected 12,000 tonnes in 2015 [1]. The Committee for Medicinal Products for Veterinary

Figure

Geographic distribution of the *mcr-1* gene (as of 1st March 2016)



Countries shown in colour have reported at least one isolate with the *mcr-1* gene [1-30].

Table a

Characteristics of *mcr-1*-positive isolates from food animals, the environment, food and humans, 1980s–2015 (as of 1st March 2016)

Source	Year	Country	Type of specimen/ animal /infection	Origin/ travelled region	Isolates n (%)	Species	Extended-spectrum beta-lactamase (ESBL)	Carbapenemase	Reference
Food animals	1980s–2014	China	Chickens	[a]	104	*E. coli*	NA	NA	[21]
	2005–2014	France	Veal calves	[a]	106	*E. coli*	CTX-M-1 (n = 7)	No	[10]
	2008–10	Japan	Pigs	[a]	2	*E. coli*	NA	NA	[23]
	2010–2011	Germany	Pigs	[a]	3	*E. coli*	CTX-M-1 (n = 3)	No	[7]
	2010-2015	The Netherlands	Chickens, veal calves, turkeys	[a]	4 (< 1%)	*E. coli*	NA	NA	[5]
	2011	France	Pigs	[a]	1 (< 1%)	*E. coli*	NA	NA	[16]
	2011–12	Belgium	Pigs	[a]	6	*E. coli*	No	No	[13]
	2011–12	Belgium	Veal calves	[a]	7	*E. coli*	No	No	[13]
	2012	Laos	Pigs	[a]	3	*E. coli*	NA	NA	[30]
	2012	China	Pigs	[a]	31 (14%)	*E. coli*	NA	NA	[1]
	2012–13	Japan	Cattle	[a]	4	*E. coli*	CTX-M-27	No	[23]
	2013	Japan	Pigs	[a]	1	*Salmonella* Typhimurium	NA	NA	[23]
	2013	China	Pigs	[a]	68 (25%)	*E. coli*	NA	NA	[1]
	2013	Malaysia	Chickens	[a]	3	*E. coli*	NA	NA	[17]
	2013	Malaysia	Pigs	[a]	1	*E. coli*	NA	NA	[17]
	2013	France	Pigs	[a]	1 (< 1%)	*E. coli*	No	No	[16]
	2013	France	Chickens	[a]	3 (2%)	*E. coli*	No	No	[16]
	2013	France	Chickens (farm)	[a]	1	*Salmonella* 1,4 [5],12:i:-	NA	NA	[26]
	2014	France	Broilers	[a]	4 (2%)	*E. coli*	No	No	[16]
	2014	France	Turkeys	[a]	14 (6%)	*E. coli*	CMY-2	No	[16]
	2014	Italy	Turkeys	[a]	1	*E. coli*	No	No	[4]
	2014	China	Pigs	[a]	67 (21%)	*E. coli*	NA	NA	[1]
	2014	China	Chickens	[a]	1	*E. coli*	CTX-M-65	NDM-9	[27]
	2014–15	Vietnam	Pigs	[a]	9 (38%)	*E. coli*	CTXM-55	No	[14]
	2015	Tunisia	Chickens	France /Tunisia	37 (67%)	*E.coli*	CTX-M-1	NA	[9]
	2015	Algeria	Chickens	[a]	1	*E. coli*	NA	NA	[30]
Environment	2012	Switzerland	River water	[a]	1	*E. coli*	SHV-12	NA	[29]
	2013	Malaysia	Water	[a]	1	*E. coli*	NA	NA	[17]
Food	2009	The Netherlands	Chicken meat	Unknown	1	*E. coli*	CTX-M-1	No	[2]
	2009-2016	The Netherlands	Retail meat (mostly chicken and turkey)	Dutch fresh meat and imported frozen meat	47 (2%)	*E. coli*	NA	NA	[5]
	2010	Canada	Ground beef	Unknown	2	*E. coli*	No	No	[15]
	2011	Portugal	Food product	NA	1	*Salmonella* Typhimurium	CTX-M-32	No	[25]
	2011	China	Chicken meat	[a]	10 (5%)	*E. coli*	NA	NA	[1]
	2011	China	Pork meat	[a]	3 (6%)	*E. coli*	NA	NA	[1]
	2012–2014	Denmark	Chicken meat	Germany	5	*E. coli*	CMY-2, SHV-12	No	[11]
	2012	France	Chicken meat, guinea fowl pie	NA	2	*Salmonella* Paratyphi B	NA	NA	[26]
	2013	France	Pork sausage	NA	1	*Salmonella* Derby	NA	NA	[26]
	2013	China	Chicken meat	[a]	4 (25%)	*E. coli*	NA	NA	[1]
	2013	China	Pork meat	[a]	11 (23%)	*E. coli*	NA	NA	[1]
	2014	China	Chicken meat	[a]	21 (28%)	*E. coli*	NA	NA	[1]
	2014	China	Pork meat	[a]	29 (22%)	*E. coli*	NA	NA	[1]
	2014	The Netherlands	Chicken meat	Europe, non-Dutch (n = 1), origin unknown (n = 1)	2	*E. coli*	SHV-12	No	[2]
	2014	Switzerland	Vegetables	Thailand, Vietnam	2	*E. coli*	CTX-M-55, CTX-M-65	No	[29]
	2012–2015	United Kingdom	Poultry meat	European Union, non-United Kingdom	2	*Salmonella* Paratyphi B var Java	NA	NA	[19]

NA: not available; *E. coli*: *Escherichia coli*; *K. pneumoniae*: *Klebsiella pneumoniae*.

[a] Same as reporting country.

Table b

Characteristics of *mcr-1*-positive isolates from food animals, the environment, food and humans, 1980s–2015 (as of 1st March 2016)

Source	Year	Country	Type of specimen/ animal /infection	Origin/ travelled region	Isolates n (%)	Species	Extended-spectrum beta-lactamase (ESBL)	Carbapenemase	Reference
Humans	2008	Vietnam	Dysentery	Vietnam	1	*Shigella sonnei*	NA	NA	[24]
	Before 2010	China	Faecal carriage	[a]	27 (7%)	NA	NA	NA	[12,20]
	2011	Canada	Gastrostomy tube	Egypt (previous healthcare)	1	*E. coli*	NA	OXA-48	[15]
	2011	The Netherlands	Bloodstream infection	[a]	1 (0.08%)	*E. coli*	NA	NA	[5]
	2012–2013	The Netherlands	Faecal carriage	China (n = 2), South America (n = 2), Tunisia, South-East Asia	6	*E. coli*	CTX-M-1, CTX-M-14, CTX-M-15, CTX-M-55 (2), CTX-M-65	No	[3]
	NA	Sweden	Faecal carriage	Asia	2	*E. coli*	NA	NA	[8]
	2012	Thailand	Faecal carriage	[a]	2	*E. coli*	NA	NA	[30]
	2012	Laos	Faecal carriage	[a]	6	*E. coli*	NA	NA	[30]
	2012	Cambodia	Faecal carriage	[a]	1	*E. coli*	CTX-M-55	No	[22]
	2012–2015	United Kingdom	Salmonellosis	Asia (n = 2)	8	*Salmonella* Typhimurium	No	No	[19]
	2012–2015	United Kingdom	Salmonellosis	Asia	1	*Salmonella* Paratyphi B var Java	No	No	[19]
	2012–2015	United Kingdom	Salmonellosis	[a]	1	*Salmonella* Virchow	No	No	[19]
	2012–2015	United Kingdom	NA	NA	3	*E. coli*	CTX-M-type	No	[19]
	2014	Germany	Wound infection (foot)	NA	1	*E. coli*	No	KPC-2	[7]
	2014	China	Inpatient	[a]	13 (1%)	*E. coli*	NA	NA	[1]
	2014–2015	China	Bloodstream infection	[a]	2	*E. coli*	CTX-M-1	No	[6]
	2015	Denmark	Bloodstream infection	[a]	1	*E. coli*	CTX-M-55, CMY-2	No	[11]
	2015	Switzerland	Urinary tract infection	NA	1	*E. coli*	No	VIM	[18]
	2015	China	Inpatient	[a]	3 (< 1%)	K. *pneumoniae*	NA	NA	[1]
	2015	China	Surgical site infection, peritoneal fluid	[a]	2	K. *pneumoniae*	CTX-M-1	NDM-5	[6]
	2015	China	Faecal carriage (children)	[a]	5 (2%)	*E. coli*	CTX-M-15	No	[28]

NA: not available; *E. coli*: *Escherichia coli*; *K. pneumoniae*: *Klebsiella pneumoniae*.

[a] Same as reporting country.

Use (CVMP) of the European Medicines Agency (EMA) reviewed all veterinary medicinal products containing colistin oral use and recommended variations to the terms of their marketing authorisations, for example that the indication is restricted to enteric infections caused by non-invasive *E. coli* susceptible to colistin and that presence of the disease in the herd should be established before metaphylactic treatment [33]. This opinion of the CVMP was converted into a Decision by the European Commission on 16 March 2015 [34], and a similar review is currently being performed for combination products containing colistin. In addition, in view of the recent developments with plasmid-mediated colistin resistance and at the request of the European Commission, the Antimicrobial Advice ad hoc Expert Group of the EMA is currently working on an update of its 2013 advice on the "use of colistin products in animals within the European Union: development of resistance and possible impact on human and animal health" [35].

In human medicine, colistin is increasingly used parenterally for the treatment of patients infected with highly resistant bacteria such as carbapenem-resistant *Enterobacteriaceae* and *Acinetobacter* spp. for which other treatment options are limited. In addition it is used topically by inhalation, especially in cystic fibrosis patients, as well as part of the regimen for selective decontamination of the digestive tract and of the oropharynx. As a result, consumption of polymyxins, mainly colistin, in European healthcare increased by 50% between 2010 and 2014, although with wide variation in the consumption rate depending on the country [36]. In some European countries, this has resulted in increasing percentages of isolates and outbreaks of *Enterobacteriaceae*, mainly *Klebsiella pneumoniae*, that

are resistant to both carbapenems and colistin, the latter because of chromosomal point mutations [37,38].

In 2012, consumption of polymyxins, mainly colistin, was on average more than 600 times higher in food animals than in humans for those 19 Member States in the European Union and European Economic Area that reported complete data both for food animals and for humans and after controlling for biomass (analysis of data from the first joint report by the European Centre for Disease Prevention and Control (ECDC), the European Food Safety Agency (EFSA) and EMA on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals [39], data not shown). The fact that plasmid-mediated colistin resistance originated from animals combined with the much larger use of colistin in animals than in humans, has contributed to the perception that the problem needs to be tackled first in veterinary medicine. As documented by Kluytmans-van den Bergh et al., *mcr-1*-positive isolates have so far only been found sporadically in humans in Europe [2]. This could be due to absence of selection in a non-favourable environment as indicated by the fact that all travellers that were tested positive for *mcr-1* upon return were negative after one month [3]. However, the presence of plasmid-mediated colistin resistance in foods and asymptomatic human carriers combined with increasing colistin use in European hospitals may be a game changer. In addition, *mcr-1*-positive isolates often carry multiple resistance genes, including genes encoding for an extended-spectrum beta-lactamase or a carbapenemase (Table), and may thus be selected by usage of most antibiotics. Ultimately, if index cases are not detected early and proper control measures are not implemented, Europe may face hospital outbreaks of infections for which there will be little, and possibly no, antibiotic treatment options.

Hospitals must be aware of this new threat to patient safety and may want to consider a few practicable and proportionate preparedness options. Clinical microbiology laboratories should consider testing for colistin susceptibility more frequently, within their available resources, for example in situations involving multidrug-resistant Gram-negative bacteria, isolates from patients that receive or have received colistin, or isolates from patients transferred from or recently hospitalised in a foreign country. It should be noted that disk diffusion is not a reliable test for colistin susceptibility, which should rather be assessed by a method measuring the minimum inhibitory concentration [40]. Enhanced infection control precautions, including patient isolation, should be considered already at the suspicion of colistin resistance and not await confirmation from a reference laboratory. Finally, measures aiming at strengthening infection prevention and control (hospital hygiene) as well as a more prudent use of antibiotics are essential to prevent and control antimicrobial resistance in general, and should be considered for plasmid-mediated colistin resistance.

There is no doubt that more information will surface in the coming months. In the meantime, increased awareness and preparedness may prevent spread of *mcr-1*-positive *Enterobacteriaceae* in hospitals and other healthcare settings in Europe and elsewhere.

Conflict of interest

None declared.

Authors' contributions

RSK and DLM both compiled the data and wrote the manuscript.

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Identification of a novel plasmid-mediated colistin-resistance gene, *mcr-2*, in *Escherichia coli*, Belgium, June 2016

BB Xavier [1,2,3], C Lammens [1,2,3], R Ruhal [1,2,3], S Kumar-Singh [1,3,4], P Butaye [5,6,7], H Goossens [1,2,3], S Malhotra-Kumar [1,2,3]

1. Laboratory of Medical Microbiology, Wilrijk, Belgium
2. Vaccine & Infectious Disease Institute, Wilrijk, Belgium
3. University of Antwerp, Wilrijk, Belgium
4. Molecular Pathology group, Cell Biology and Histology, Wilrijk, Belgium
5. Ghent University, Faculty of Veterinary Medicine, Ghent, Belgium
6. CODA-CERVA, Brussels, Belgium
7. Ross University School of Veterinary Medicine, Basseterre, Saint Kitts and Nevis

Correspondence: Surbhi Malhotra-Kumar (surbhi.malhotra@uantwerpen.be)

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We identified a novel plasmid-mediated colistin-resistance gene in porcine and bovine colistin-resistant *Escherichia coli* that did not contain *mcr-1*. The gene, termed *mcr-2*, a 1,617 bp phosphoethanolamine transferase harboured on an IncX4 plasmid, has 76.7% nucleotide identity to *mcr-1*. Prevalence of *mcr-2* in porcine colistin-resistant *E. coli* (11/53) in Belgium was higher than that of *mcr-1* (7/53). These data call for an immediate introduction of *mcr-2* screening in ongoing molecular epidemiological surveillance of colistin-resistant Gram-negative pathogens.

Following the report of of *mcr-1* detection in China in November 2015 [1], we screened 105 colistin-resistant *Escherichia coli* (colistin minimum inhibitory concentration (MIC) 4–8 mg/L [2]) isolated during 2011–12 from passive surveillance of diarrhoea in 52 calves and 53 piglets in Belgium [3]. *mcr-1* was detected in 12.4% (n = 13) of the *E. coli* isolates, of which six and seven were from calves and piglets, respectively [3,4]. In the present study, we analysed porcine and bovine colistin-resistant *Escherichia coli* isolates that did not show presence of *mcr-1* and identified a novel plasmid-mediated colistin resistance-conferring gene, *mcr-2*.

Identification of *mcr-2* in colistin-resistant *E. coli* isolates not harbouring *mcr-1*

Of 92 porcine and bovine colistin-resistant *Escherichia coli* isolates not harbouring *mcr-1*, 10 were randomly selected for further analysis. Plasmid DNA was isolated (PureLink HiPure Plasmid Miniprep Kit, Invitrogen, Carlsbad, CA, United States), sequenced by Illumina (2 x 250 bp) (Nextera XT sample preparation kit, MiSeq), de novo assembled and annotated using SPAdes (v3.8.1) and RAST [5,6]. Plasmids from three of the 10 *E. coli* isolates showed the presence of a gene for a putative membrane protein, which was identified as a phosphoethanolamine transferase (sulfatase) using pfam and Interproscan protein databases [7,8] The *mcr-2* gene, as we termed it, is 1,617 bp long, nine bases shorter than *mcr-1* (1,626 bp), and shows 76.75% nt identity to *mcr-1* (supplementary material [9]).

The entire *mcr-2* gene was amplified (PCR primers: MCR2-F 5' TGGTACAGCCCCTTTATT 3'; MCR2-R 5'GCTTGAGATTGGGTTATGA 3'), cloned (vector pCR 2.1, TOPO TA Cloning kit, Invitrogen) and electroporated into DH-5 α *E. coli*. Transformants exhibited colistin MICs of 4–8 mg/L (E-test, bioMerieux, Marcy l'Etoile, France), which were reconfirmed by macrobroth dilution (European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [2]).

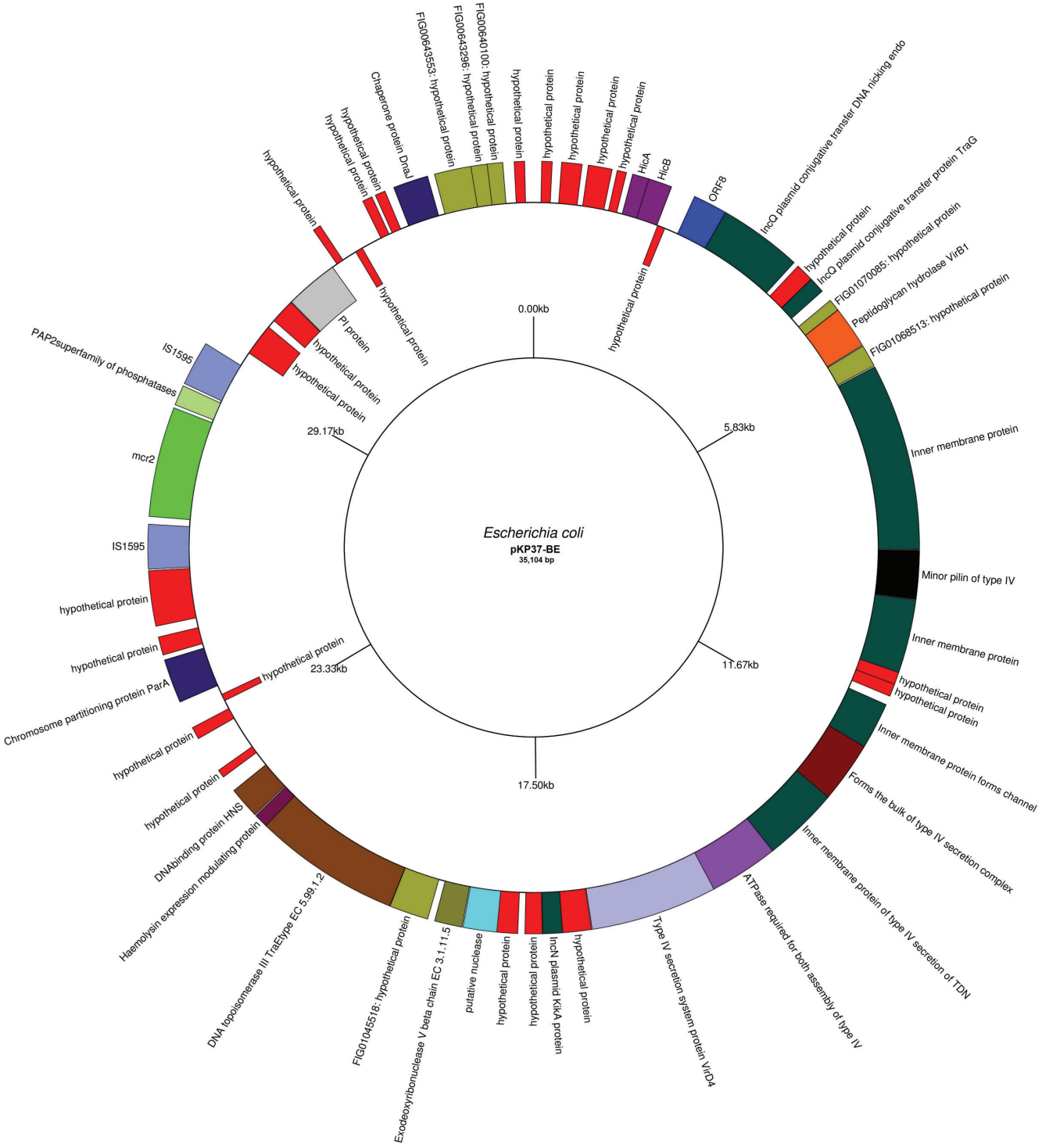
mcr-2 is harboured on IS1595 with likely origins in *Moraxella* spp.

mcr-2-harbouring plasmids from all three *E. coli* isolates were analysed. The mobile element harbouring *mcr-2* was identified as an IS element of the IS1595 superfamily, which are distinguished by the presence of an ISXO2-like transposase domain [10].

We also identified a 297 bp open reading frame downstream of *mcr-2* on this element, which encodes a PAP2 membrane-associated lipid phosphatase with 41% identity to *Moraxella osloensis* phosphatidic acid phosphatase (71% query coverage). Interestingly, a blastn search of the IS1595 backbone, after removal of the *mcr-2* and *pap2* phosphatase gene sequences, identified a single hit to *Moraxella bovoculi* strain 58069 (GenBank accession number CP011374) genomic region

Figure 1

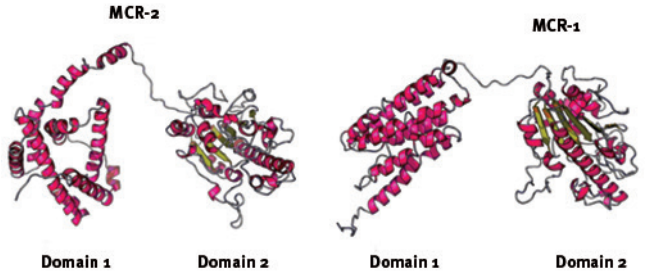
Genetic organisation and structure of the *mcr-2*-harbouring plasmid pKP37-BE from a colistin-resistant *Escherichia coli* isolate not harbouring *mcr-1*, Belgium, June 2016



The plasmid map was generated using GenomeVx [23].

Figure 2

MCR-2 and *MCR-1* predicted tertiary structures



RaptorX [24] was used to generate the structures. For both MCR-2 and MCR-1, domain 1 was predicted to be a transporter and domain 2 a phosphoethanolamine transferase (sulfatase).

(1,531,602 to 1,532,255 bp) with 75% identity and 100% query coverage.

mcr-2 is harboured on an IncX4 incompatibility-type plasmid in *E. coli* ST10

The three *mcr-2* plasmid-harbouring *E. coli* isolates belonged to ST10 (n = 2, porcine) and ST167 (n = 1, bovine). All three plasmids belonged to IncX4 incompatibility type; all three *mcr-2* genes showed 100% homology.

Plasmid pKP37-BE isolated from one of the porcine ST10 *E. coli* isolates was found to have a size of 35,104 bp, 41.3% GC content and 56 protein-encoding gene sequences (RAST) (Figure 1); European Nucleotide Archive accession numbers PRJEB14596 (study) and LT598652 (plasmid sequence).

Apart from IS1595, pKP37-BE did not carry any other resistance genes and the plasmid backbone was highly similar to *Salmonella enterica* subsp. *enterica* serovar Heidelberg plasmid pSH146_32 (GenBank accession number JX258655), with 98% identity and 90% query coverage. Several *Salmonella*-associated virulence genes were found on pKP37-BE, including *virB/D4* that encodes a type 4 secretion system [11].

Conjugation experiments using a rifampicin-resistant *E. coli* recipient (A15) showed an approximately 1,200-fold higher transfer frequency of the *mcr-2*-harbouring pKP37-BE (1.71 × 10−3) compared with the *mcr-1* harbouring IncFII plasmid, pKP81-BE (1.39 × 10−6) [4]. Both *mcr-1* and *mcr-2* transconjugants exhibited colistin MICs of 4–8 mg/L (macrobroth dilution).

Structure predictions and phylogenetic analyses of the MCR-2 protein

MCR-2 protein was predicted to have two domains, with domain 1 (1 to 229 residues) as a transporter and domain 2 (230 to 538 residues) as a transferase domain (Figure 2).

The best template for domain 1 was 4HE8, a secondary membrane transport protein with a role in transferring solutes across membranes [12]. The best-fit template for domain 2 was 4kav (p = 4.13 e-13), a lipooligosaccharide phosphoethanolamine transferase A from *Neisseria meningitides*, also previously shown to be the best-fit template for MCR-1 [1]. 4kav belongs to the YhjW/YjdB/YijP superfamily and its role in conferring polymyxin resistance has been experimentally validated [13]. Overall, the un-normalised global distance test (uGDT) was 318 (GDT: 58) and all 538 residues were modelled (Figure 2).

MCR-1 and MCR-2 proteins showed 80.65% identity (supplementary material [9]). In addition, MCR-2 showed 64% identity to the phosphoethanolamine transferase of *Moraxella osloensis* (WP_062333180) with 99% sequence coverage, and 65%, 65%, and 61% identity to that of *Enhydrobacter aerosaccus* (KND21726), *Paenibacillus sophorae* (WP_063619495) and *Moraxella catarrhalis* (WP_003672704), respectively, all with 97% query coverage.

We also carried out blastp searches of the two domains of MCR-2 separately. The identity level of domain 1 between MCR-1 and MCR-2 was low (72%) compared with that for domain 2 (87.4%). Other blastp hits for the domain 2 transferase were *Enhydrobacter aerosaccus* and *Moraxella osloensis* (69% identity; 100% query coverage) followed by *Paenibacillus sophorae* (68% identity; 100% query coverage) and *Moraxella catarrhalis* (68% identity; 99% query coverage). Phylogenetic analysis showed that MCR-2 might have originated from *Moraxella catarrhalis* (56% bootstrap value) (Figure 3).

PCR-based screening identified a higher prevalence of *mcr-2* than of *mcr-1* in porcine *E. coli* in Belgium

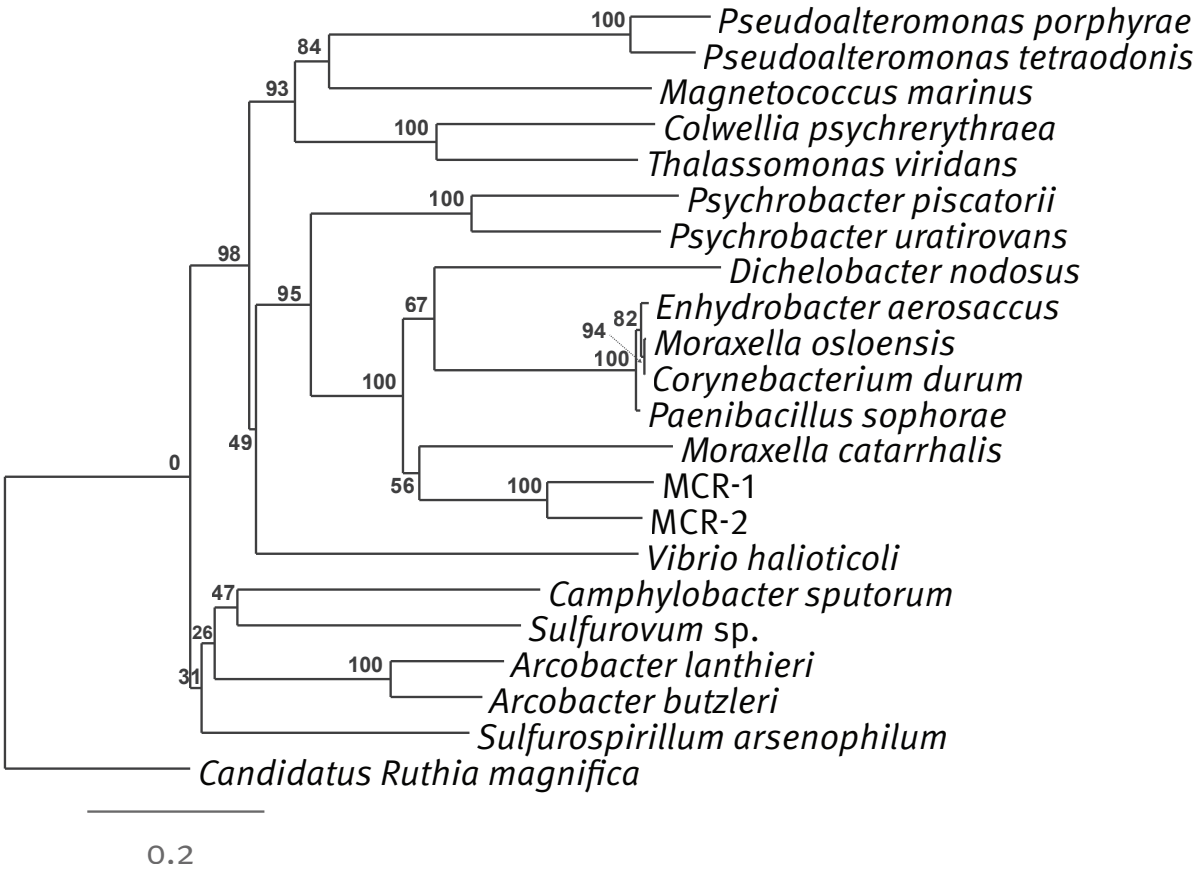
We screened our entire collection of porcine and bovine colistin-resistant *E. coli* isolates (n = 105) using an *mcr-2*-specific PCR approach using primers MCR2-IF 5' TGTTGCTTGTGCCGATTGGA 3' and MCR2-IR 5' AGATGGTATTGTTGGTTGCTG 3', and the following cycling conditions: 33 cycles of 95 °C × 3 min, 65 °C × 30 s, 72 °C × 1 min, followed by 1 cycle of 72 °C × 10 min. We found *mcr-2* in 11/53 porcine and 1/52 bovine colistin-resistant *E. coli* isolates (an overall prevalence of 11.4%).

Discussion

Identification of plasmid-mediated colistin resistance represents a paradigm shift in colistin-resistance mechanisms, which until recently were restricted to chromosomal mutations and vertical transmission. Since *mcr-1* conferring plasmid-mediated colistin resistance was first detected in China, *mcr-1* has been identified in 30 countries across five continents [14-17] (Figure 4).

Figure 3

Phylogenetic analysis of the entire MCR-2 protein sequence



Maximum likelihood tree generated by bootstrap analysis using 1,000 replicates. The analysis was carried out using CLC Genomics workbench v9.0.1 (clcbio, Qiagen) in-built tool for this evolutionary relationship with other related sequences. Branch length is proportional to the number of substitutions per site. Bootstrap values are indicated in the nodes.

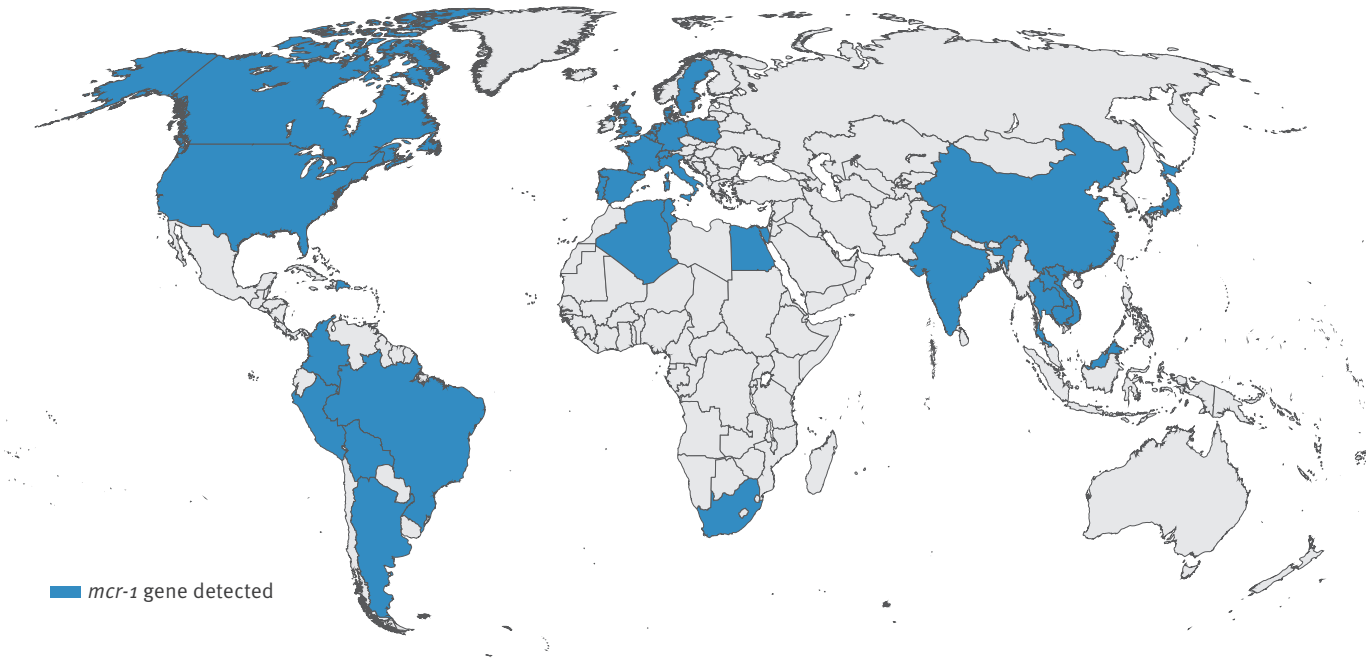
Our analysis identified a novel plasmid-mediated phosphoethanolamine transferase-encoding gene, *mcr-2*, which was detected at an even higher prevalence than that of *mcr-1* among colistin-resistant porcine *E. coli* in our study. We were, however, limited by small sample numbers. It should also be noted that the calves and piglets were from different regions of the country (calves from Wallonia and piglets from Flanders).

Phylogenetic analysis of MCR-2 provided strong evidence that this protein was distinct from MCR-1, and that it might have originated from *Moraxella catarrhalis*. The latter set of data are further strengthened by the fact that *mcr-2* is co-harboured with a lipid phosphatase gene that shows highest homology to a phosphatase from *Moraxella* spp., and that the genetic element IS1595 harbouring these two genes might itself have originated from *Moraxella* spp. While *Moraxella* spp. are not polymyxin producers, this bacterial genus is known to be intrinsically resistant to polymyxins [18] and potential intergeneric transfer of *mcr-2* from co-habiting *Moraxella* spp. of animal, human or environmental origin is therefore highly likely. Phosphoethanolamine transferases are housekeeping enzymes that catalyse the addition of the phosphoethanolamine moiety to the outer 3-deoxy-D-manno-octulosonic acid (Kdo) residue of a Kdo(2)-lipid A [19]. The fact that we did not identify any chromosomal mutations in the known colistin resistance-conferring genes in our *E. coli* isolates (by whole genome sequencing, data not shown) additionally supports the role of the acquired phosphoethanolamine transferase in conferring colistin resistance.

Finally, the high transfer frequency of the *mcr-2*-harbouring IncX4 plasmid might underlie the higher prevalence of *mcr-2* in our porcine isolates. In the three *mcr-2* harbouring isolates analysed, IS1595 showed presence of direct repeats and a complete *tnpA* gene, while inverted repeats were not found (data not shown). However, the carrier plasmid IncX4 is itself highly transmissible, showing 10^2–10^5-fold higher transfer frequencies than, for instance, epidemic IncFII plasmids, as shown previously [20] as well as in our own transconjugation experiments. Importantly, a lack of fitness-burden of IncX4 carriage on bacterial hosts [20]

Figure 4

Countries (n = 30) reporting presence of *mcr-1* in samples of animal, environmental or human origin (data collected till 27 June 2016)



Adapted from [15]; updated using data from [14,16,17,25-27].

makes this plasmid replicon a highly effective vehicle for dissemination of *mcr-2*. IncX4 plasmids have also been previously shown to harbour *mcr-1* [21] as well as extended spectrum beta-lactamase genes, $bla_{CTX\text{-}M}$ [20]. Interestingly, the pKP37-BE backbone, which likely originated from *Salmonella* spp., harboured a battery of virulence genes including the *virB4/D4* genes encoding a type-IV secretion system that has been shown to mediate downregulation of host innate immune response genes and an increased bacterial uptake and survival within macrophages and epithelial cells [11]. Outer membrane modifications leading to colistin resistance have been shown to attenuate virulence [22]: whether these co-harboured virulence genes are able to compensate the pathogenic abilities of colistin-resistant *E. coli* remains to be explored.

Taken together, these data call for immediate inclusion of *mcr-2* screening in ongoing molecular epidemiological surveillance to gauge the worldwide dissemination of *mcr-2* in both human and animal colistin-resistant Gram-negative bacteria of medical importance.

* Authors' correction

The number of countries in which *mcr-1* has been identified was updated to 32 and supporting references were added on 11 July 2016. The references in the article were renumbered accordingly.

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The complete plasmid sequence of pKP37-BE was deposited at the European Nucleotide Archive accession numbers PRJEB14596 (study) and LT598652 (plasmid sequence).

Conflict of interest

None declared.

Authors' contributions

This study was designed by SMK. Isolates were collected by PB. Experimental work was done by BBX and CL. Data was analysed and interpreted by BBX, RR, SKS, HG and SMK. The manuscript was drafted by BBX, SKS and SMK, and was reviewed by all authors.

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MCR-1 in multidrug-resistant and copper-tolerant clinically relevant *Salmonella* 1,4,[5],12:i:- and *S.* Rissen clones in Portugal, 2011 to 2015

J Campos [1], L Cristino [2], L Peixe [1], P Antunes [1,2]
1. UCIBIO/REQUIMTE, Department of Biological Sciences, Microbiology laboratory, Pharmacy Faculty, University of Porto, Porto, Portugal
2. Faculty of Nutrition and Food Sciences, University of Porto, Porto, Portugal

Correspondence: Patricia Antunes (patriciaantunes@fcna.up.pt)

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The *mcr-1* gene was found in 11 isolates from a Portuguese *Salmonella* collection (n = 1,010; 58 serotypes; 2002–15) of clinical samples, foodstuff, food-animals and water. *Mcr-1* has been located on different plasmids (IncX4/IncHI2) in pig-associated multidrug-resistant, copper-tolerant S.1,4,[5],12:i:-/ST34 and *S.* Rissen/ST469 clones from human and pork products since at least 2011. Our data highlight dissemination of *mcr-1* by successful resistant clones in Europe and raise questions about the efficacy of copper-based interventions to reduce colistin use.

Since the description of plasmid-mediated colistin resistance encoded by the *mcr-1* gene in *Enterobacteriaceae* from multiple sources in China [1] and its worldwide dissemination mostly in animal sources [2], the use of polymyxins (colistin) in food-producing animals has been questioned in Europe because it may have an impact on human health [3]. Nevertheless, data on the transmission of *mcr-1*-mediated colistin resistance particularly by clonal expansion are lacking [3,4]. In fact, the *mcr-1* gene has been found in zoonotic food-borne bacteria such as *Salmonella* [2] but the presence of this gene in particular successful resistant clones has not been demonstrated [3]. In this study, we report the presence of the *mcr-1* gene in pig-associated clinically relevant *Salmonella* serotypes and clones recovered from human clinical samples and pork products in Portugal, collected as early as 2011.

Laboratory investigation

We analysed a total of 1,010 *Salmonella* isolates of 58 serotypes from several sources (human clinical cases, food products, food-animal production settings and aquatic environments) and regions of Portugal, collected between 2002 and 2015 (Table 1). The isolates were screened for the *mcr-1* gene by PCR and sequencing, using primers CLR5-F (5´-CGGTCAGTCCGTTTGTTC-3´) [1] and Mcr1-Rv2 (5´-CCAGCGTATCCAGCACATTT-3´) [this study].

The 1,010 isolates comprised the most frequent worldwide *Salmonella* serotypes (n = 256 *S.* Typhimurium and n = 34 *S.* Enteritidis), but also emerging serotypes (n = 436 *S.* 1,4,[5],12:i:- and n = 93 *S.* Rissen) or serotypes less frequently detected in European surveillance studies (n = 191 isolates from 54 different serotypes). They included all isolates previously characterised [5,6] and recent ones from ongoing surveillance studies (data not shown) covering all serotypes, sampling dates, sources, regions, antibiotic susceptibility phenotypes/genotypes and PFGE types. The isolates positive for *mcr-1* by PCR were further tested for susceptibility to colistin by the proposed broth microdilution method [7] and interpreted according to the European Committee on Antimicrobial Susceptibility Testing [8]. Isolates were also subjected to standard conjugation assays using the recipient strain *Escherichia coli* HB101 [6]. Replicon typing, pMLST, hybridisation experiments (I-CeuI/S1-PFGE nuclease) [5,9] and detection of the insertion sequence element IS*Apl1* was performed in *Salmonella* strains and transconjugants. The presence and location of IS*Apl1* was determined using primers ISApl1-Fw (5´-GTCGCTTTGGACATTGGGAA-3´) and ISApl1-Rv (5´-GATTGATGTCTTGGTGCTTCGG-3´) designed as part of this study, and CLR5-R (5´-CTTGGTCGGTCTGTAGGG-3´) [1]. Clonal relatedness of *Salmonella* strains was assessed by *Xba*I PFGE [5,6] and MLST [10].

Detection of *mcr-1* gene in pig-associated clinically-relevant clones

The *mcr-1* gene was detected in 11 (1.1%) of the 1,010 Portuguese *Salmonella* isolates, recovered from human clinical sources and pork food products from across the country (Table 1, Table 2). This gene had 100% homology with the first published *mcr-1* sequence in an

Table 1

Salmonella isolates from different sources by year and presence of the *mcr-1* gene, Portugal, 2002–2015 (n = 1,010)

Source (number of isolates)	Years	Isolates tested for *mcr-1* (serotype/number of isolates)a	*mcr-1*-positive isolates (serotype/number of isolates)[a]
Human clinical cases (n = 522)	2002–10	258	0
	2011–12	155 (*S.* 1,4,[5],12:i:-/n = 75)	4 (*S.* 1,4,[5],12:i:-)
	2013–15	109	0
Food products (n = 413)			
Pork (n = 296)	2002–13	44	0
	2014–15	252 (*S.* 1,4,[5],12:i:-/n = 130; *S.* Rissen/n = 23)	7 (*S.* 1,4,[5],12:i:-/n = 5; *S.* Rissen/n = 2)
Other[b] (n = 117)	2002–15	117	0
Food production animals (n = 58)			
Pigs/piggeries (n = 54)	2006–08	54	0
Aquacultures (n = 4)	2010–12	4	0
Aquatic environment (n = 17)	2002–11	17	0

[a] The serotypes of *Salmonella* isolates are presented only for those among which *mcr-1*-positive ones were detected.

[b] Other studied food products comprised: poultry, beef, cow, quail, clam and cooked meals.

Escherichia coli strain from China (GenBank accession number: KP347127) [1], which was further described in diverse other *Enterobacteriaceae* including sporadic *Salmonella* isolates from European countries (France, the Netherlands, Spain, the United Kingdom) [2,11-14]. In most of these studies, detection of *mcr-1* gene was only performed in colistin-resistant isolates. This impairs the determination of its real prevalence because the gene may be silent, as described in one *E. coli* strain [15]. All our isolates carrying the *mcr-1* gene presented a minimum inhibitory concentration (MIC) of 4–8 mg/L for resistance to colistin (Table 2).

During the study period (2002 to 2015), *Salmonella* isolates harbouring the *mcr-1* gene were only recovered between 2011 and 2015 and originated from human clinical sources (0.8%, n = 4/522) and pork products, mostly from slaughterhouses, (2.4%, n = 7/296) (Table 1). Colistin has been widely used in veterinary medicine, particularly in food-producing animals, primarily in pigs [16,17]. The available data from 2004 to 2006 had already shown high use of colistin for food-producing animals in Portugal [18], which is one of the European countries with highest consumption of polymyxins that has been increasing in the last years (2011–13) [3,19]. Taking into account the current picture of colistin use in Portugal, the detection of *mcr-1* in the most recent collections and in pork products is of concern. Nevertheless, data on chronology, current prevalence of the *mcr-1* gene and its evolution in bacteria from animals, food and humans are lacking [3].

The 11 *mcr-1*-positive *Salmonella* isolates belonged to the serotypes *S.* 1,4,[5],12:i:- and *S.* Rissen (Table 2), which have been strongly associated with pig production and caused human infections in Europe [5,6,20-22] including in Portugal [23]. In both cases, we found them associated with particular successful multidrug-resistant (MDR) clonal lineages, either of the *S.* Rissen/ST469 clone or the *S.* 1,4,[5],12:i:-/ST34 European clone that is currently spreading epidemically in European countries [5,20,22] and has been dominant in our *Salmonella* collection for the last years [5,6,20; unpublished data]. A previous report on *mcr-1* in the clinically relevant *Salmonella* serotype *S.* Typhimurium/ST34 was associated with travel to South-East Asia [13].

Of note, all *S.* 1,4,[5],12:i:- and *S.* Rissen *mcr-1*-carrying isolates were co-resistant to antibiotics used in a human and/or veterinary context and carried diverse metal tolerance genes, remarkably those conferring tolerance to copper (all carrying *pcoD+silA* on the chromosome) (Table 2), a feed additive mostly used for pigs or piglets in Europe. The fact that these successful clones presented higher tolerance to copper, as previously demonstrated [6,20], can contribute to their selection and wider expansion with potential repercussions for *mcr-1* transmission.

Location of *mcr-1* gene in diverse plasmid backbones

The *mcr-1* gene was located on two plasmid types, IncX4 (n = 5; 35 kb; 4 transferable) and IncHI2 (n = 6), either of ST4 subtype (n = 3; 200–300 kb; all transferable) or non-typeable (n = 3; 120–125 kb; all non-transferable) and mostly associated with the IS*Apl1* transposable element (Table 2). IncHI2/ST4 and IncX4 plasmids have been widely implicated in the spread of *mcr-1* gene in diverse *Salmonella* serotypes and other *Enterobacteriaceae* in European and non-European countries, both from human and animal sources [2,12-14]. Transferability of the *mcr-1* gene was achieved from *S.* Rissen (n = 1) and *S.* 1,4,[5],12:i:- (n = 6) isolates and was associated with a 32–64-fold increase in the colistin MIC and, in some isolates, with acquisition of

Table 2

Characterisation of *Salmonella* isolates recovered from clinical and food samples and carrying the *mcr-1* gene, Portugal, 2011–2015 (n = 11)

Serotype[a] (number of isolates)	Source-origin (number of isolates)	Clone designation; ST(eBG); PFGE-type[b] (number of isolates, source)	Year/ Regions	Antibiotic resistance phenotype/genotype[c] (number of isolates)	Metal tolerance genes (number of isolates)[d]	Plasmid-mediated colistin resistance *mcr-1*		
						Colistin MIC-mg/L, donor (transconjugant/ number of isolates)[e]	Plasmid type carrying *mcr-1* gene (pMLST, Kb)[f] (number of isolates)	IS*Apl1* upstream of *mcr-1* (number of isolates)
1,4,[5],12:i:- (n = 9)	Clinical faeces/blood (n = 4)	European clone; ST34(eBG1); C (n = 1, 1 hospital), E (n = 3, 2 hospitals)	2011–12 North	AMP, (GEN), STR, SUL, TET/ bla_{TEM}, [*aac(3)-IV*], strA-*strB*, *sul2*, *tet*(B) (n = 4)	*pcoD* + *silA* + *merA* + (*terF*) (n = 4)	4–8 (4–8/n = 1)	X4 (35) (n = 1) HI2 (NT, 120–125) (n = 3)	No (n = 1) Yes (n = 3)
	Pork carcass (n = 4)	European clone; ST34(eBG1); A (n = 1, 1 slaughterhouse), B (n = 2, 2 slaughterhouses), F (n = 1, 1 slaughterhouse)	2014–15 North, Centre	AMP, (CLO), (CIP, PEF), STR, SUL, TET, (TMP)/ bla_{TEM}, (*floR*)/(*catA-cmlA*), (*aadA1/aadA2*) –*strA-strB*, *sul1-sul3/sul2*, *tet*(A)/*tet*(B), (*dfrA1/ dfrA12*) (n = 4)	*pcoD* + *silA* + (*merA*) + (*terF*) (n = 4)	4–8 (4–8/n = 4)	X4 (35) (n = 2) HI2 (ST4, 230–300) (n = 2)	No (n = 2) Yes (n = 2)
	Pork meat (n = 1)	European clone; STNew/ Single locus variant of ST34; B (n = 1, 1 meat production unit)	2015 South	AMP, STR, SUL, TET/ bla_{TEM}, *strA-strB*, *sul2*, *tetB* (n = 1)	*pcoD* + *silA* + *merA* + *terF* (n = 1)	4 (4/n = 1)	HI2 (ST4, 200) (n = 1)	No (n = 1)
Rissen (n = 2)	Pork carcass (n = 2)	ST469(eBG66); N (n = 2, 2 slaughterhouses)	2014–15 North	AMP, CLO, STR, SUL, (TET), TMP/ bla_{TEM}, *cmlA*, *aadA1*, *sul1-sul3*, [*tet*(A)], *dfrA1* (n = 2)	*pcoD* + *silA* + *merA* (n = 2)	4 (4/n = 1)	X4 (35) (n = 2)	No (n = 2)

AMP: ampicillin; CIP: ciprofloxacin; CLO: chloramphenicol; GEN: gentamicin; MIC: minimum inhibitory concentration; PEF: pefloxacin; pMLST: plasmid multilocus sequence type; STR: streptomycin; SUL: sulfamethoxazole; TET: tetracycline; TMP: trimethoprim.

[a] The serotypes of *Salmonella* isolates were determined by classical serotyping, performed at the National Centre of Salmonella (INSA, Lisbon, Portugal) and/or PCR assay for determination of *S.* 4,[5],12:i:- [5].

[b] PFGE types are designated by capital letters and include previously described types [5,6] and types described for the first time in this study. The human clinical isolates (n = 4 from four patients) were recovered from three hospitals, and pork products (n = 7) were recovered from six slaughterhouses and one meat production unit.

[c] Antimicrobial susceptibility was evaluated by disc diffusion assay. Variable antibiotic resistance phenotypes and genotypes are presented between brackets; Antibiotic resistance patterns and genes transferred by conjugation are underlined; In two *S.* 1,4,[5],12:i:- isolates, transfer of genes *strA-strB* and/or bla_{TEM} was observed; Some genes were included on class 1 integrons (1,700bp (*dfrA1-aadA1*) or 2,000bp (*dfrA12-orfF-aadA2*)); Integrons were located on the chromosome in *S.* Rissen (n = 2) and on the IncHI2/ST4 plasmid in *S.* 1,4,[5],12:i:- isolates (n = 2).

[d] Screening for genes encoding tolerance to metals were done by PCR [20]. Metal tolerance genes that were not observed in all the isolates are presented between brackets; Metal tolerance genes transferred by conjugation are underlined. All *pcoD* + *silA* genes were chromosomally located.

[e] Recipient strain used in conjugation assays: *Escherichia coli* HB101 (azide sodium, resistant to streptomycin and kanamycin); colistin MIC = 0.125 mg/L).

[f] Plasmid types carrying the *mcr-1* gene transferred by conjugation are underlined.

resistance to other antibiotics and metals tolerance genes (Table 2). The fact that successful MDR *S.* 1,4,[5],12:i:- and *S.* Rissen clones have the ability to acquire plasmids carrying the *mcr-1* gene is of concern because colistin resistance may contribute to their further expansion, particularly in the pig reservoir. In addition, those strains could act as reservoir of *mcr-1*-carrying plasmids with a broad host range enhancing colistin resistance transmission for other clinically relevant bacteria sharing the same ecological niche.

Conclusions

This study has evidenced the acquisition of *mcr-1*-carrying plasmids by two clinically relevant MDR and copper-tolerant clones of *S.* 1,4,[5],12:i:- and *S.* Rissen, strongly associated with pork food products and which were dominant in the collection studied. The detection of *S.* 1,4,[5],12:i:- from human infections, already in 2011, is also of note, suggesting long-term dissemination of this resistance gene in humans in Portugal. Finally, the detection of *mcr-1* in copper-tolerant clones raises questions about the efficacy of recently suggested metal-based interventions (e.g. copper) to reduce the use of colistin and contain *mcr-1* dissemination [3].

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Conflict of interest

None declared.

Authors' contributions

JC, LP and PA designed the study and analysed epidemiological, microbiological and molecular data, JC and LC performed the phenotypic and molecular assays, JC and PA wrote the first draft of the manuscript, PA and LP participated in the coordination and concept of the manuscript and revised the final version.

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Silent dissemination of colistin-resistant *Escherichia coli* in South America could contribute to the global spread of the *mcr-1* gene

MR Fernandes [1], Q Moura [2], L Sartori [1], KC Silva [3], MP Cunha [3], F Esposito [1], R Lopes [2], LK Otutumi [4], DD Gonçalves [4], M Dropa [5], MH Matté [5], DF Monte [6], M Landgraf [6], GR Francisco [7], MF Bueno [7], D de Oliveira Garcia [7], T Knöbl [3], AM Moreno [3], N Lincopan [1,2]

1. Department of Clinical Analysis, School of Pharmacy, Universidade de São Paulo, São Paulo, Brazil
2. Department of Microbiology, Institute of Biomedical Sciences, Universidade de São Paulo, São Paulo, Brazil
3. School of Veterinary Medicine, Universidade de São Paulo, São Paulo, Brazil
4. Department of Veterinary Preventive Medicine, School of Veterinary Medicine, Universidade Paranaense, Paraná, Brazil
5. Public Health Laboratory, School of Public Health, Universidade de São Paulo, São Paulo, Brazil
6. Food and Experimental Nutrition Department, School of Pharmacy & Food Research Center, Universidade de São Paulo, São Paulo, Brazil
7. Center of Bacteriology, Instituto Adolfo Lutz, São Paulo, Brazil

Correspondence: Nilton Lincopan (lincopan@usp.br)

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During a Brazilian multicentric antimicrobial resistance surveillance study, colistin resistance was investigated in 4,620 Enterobacteriaceae isolated from human, animal, food and environmental samples collected from 2000 to 2016. We present evidence that *mcr-1*-positive *Escherichia coli* has been emerging in South America since at least 2012, supporting a previous report on the possible acquisition of *mcr-1*-harbouring *E. coli* by European travellers visiting Latin American countries.

We present evidence that *mcr-1*-harbouring *Escherichia coli* has been occurring in food-producing animals in Brazil since at least 2012.

Screening Enterobacteriaceae isolates for potential colistin resistance and the *mcr-1* gene

Between 2000 and 2016, a total of 4,620 Enterobacteriaceae isolates were collected in Brazil, as part of different surveillance projects on carbapenemase- and/or extended-spectrum beta-lactamases (ESBL)-producing Gram-negative bacteria important to human and veterinary medicine [1-4]. Within this Brazilian multicentric antimicrobial resistance surveillance study, we hereby also investigate colistin resistance.

The 4,620 isolates were screened using MacConkey agar plates supplemented with colistin (2 mg/L). A total of 515 isolates, which had grown on the screening plates were obtained. These originated from food-producing animals (227 isolates), chicken feed (4 isolates), companion (9 isolates) and non-companion animals (24 isolates), humans (137 isolates), food (102 isolates) and the environment (12 isolates). The 515 isolates were further tested for susceptibility to colistin by agar dilution and/or broth microdilution method, whereby a minimum inhibitory concentration (MIC) >2 mg/L was considered indicative of colistin resistance according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [5]. Isolates were also subjected to polymerase chain reaction (PCR) to check whether respective strains harboured the *mcr-1* gene [6], which if present was sequenced (Table).

The *mcr-1* gene was detected in 16 commensal *E. coli* strains exhibiting colistin MICs from 1 to 16 mg/L (MIC_{50} = 8 mg/L). Two of the *mcr-1*-positive *E. coli* strains were found in faecal samples collected in 2012 from healthy pigs in farms located in Santa Catarina and Minas Gerais states. One of these two isolates was susceptible for colistin (MIC = 1mg/L). The remaining 14 *mcr-1*-harbouring *E. coli* strains originated from faecal samples of healthy chickens, which had been gathered in 2013 from farms located in Paraná, São Paulo and Minas Gerais states. All 14 isolates from chickens had a MIC ≥ 8 mg/L.

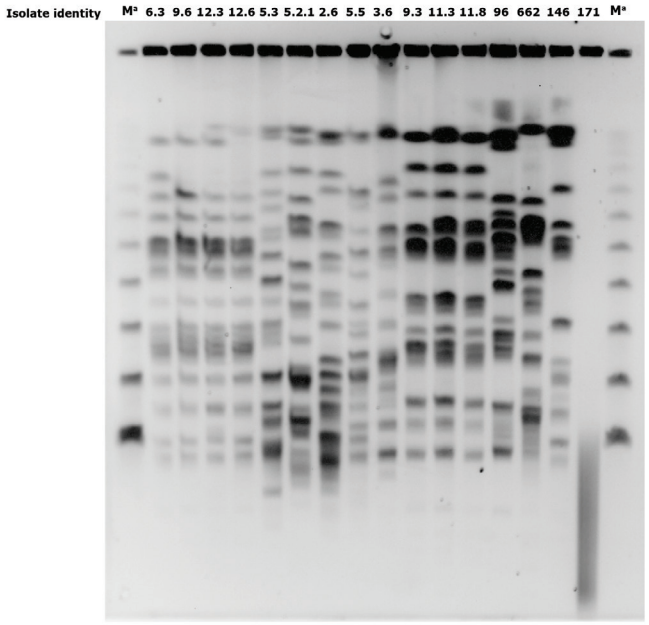
Relationships between *mcr-1*-positive isolates, and testing for extended-spectrum beta-lactamases

The sequences of the 16 *mcr-1*-positive *E. coli* strains were phylogenetically analysed [7], revealing that 11

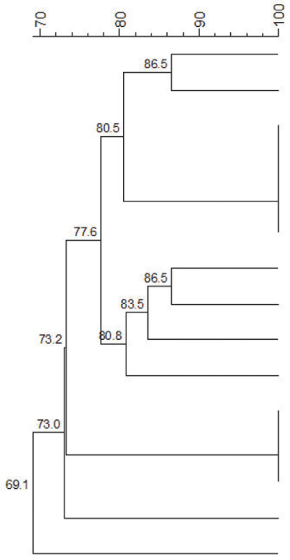
Figure 1

Pulsed-field gel electrophoresis (PFGE) and antimicrobial resistance chraracteristics of *mcr*-1-positive *Escherichia coli* strains isolated from faeces of healthy livestock, Brazil, 2012–2013

A. *XbaI* PFGE of MCR-1-positive *E. coli* strains isolated from faeces of healthy livestock



B. Relationship between isolates obtained after *XbaI* PFGE and antimicrobial resistance



Isolate	Source	Year	State[b]	Resistance profile (Kirby – Bauer)[c]	Colistin MIC (mg/L)[d]	β-lactamase	Phylogroup	PFGE cluster[e]
5.3	Chicken	2013	PR	CRO, CTX, CTF, CPM, TET	8	CTX-M-1	A	A
662	Chicken	2013	SP	–	8	–	A	B
12.3	Chicken	2013	PR	CRO, CTX, TET	16	CTX-M-8	A	C
12.6	Chicken	2013	PR	–	8	–	A	C
6.3	Chicken	2013	PR	CRO, CTX	8	CTX-M-8	A	C
9.6	Chicken	2013	PR	CRO, CTX, CTF, CPM, ENO	8	CTX-M-8	A	C
2.6	Chicken	2013	PR	CRO, CTX, TET	8	CTX-M-15	A	D
3.6	Chicken	2013	PR	TET	8	–	B1	E
5.5	Chicken	2013	PR	CRO, CTX, CTF, CPM, TET	8	CTX-M-15	A	F
146	Swine	2012	SC	AMC, FOX, CLO, SXT, TET	1	CMY-2	A	G
11.3	Chicken	2013	PR	CRO, CTX, CTF, CPM, CIP, ENO	8	CTX-M-8	B1	H
11.8	Chicken	2013	PR	CRO, CTX, CTF, CPM, CIP, ENO	8	CTX-M-8	B1	H
9.3	Chicken	2013	PR	CRO, CTX, CPM, CIP, ENO	8	CTX-M-8	B1	H
96	Chicken	2013	MG	ENO, CIP	8	–	A	I
5.2.1	Chicken	2013	PR	ENO	8	–	A	J
171	Swine	2012	MG	CLO, SXT	8	–	B1	nt

MIC: minimum inhibitory concentration; nt: non typeable by PFGE.

GenBank accession number for mcr-1 genes identified in this study: KU750813, KU928239–42, KU935441–9, KX01152–1.

a The marker (M) used was the Lambda ladder 0.05–1Mb, Bio-Rad. Separation of fragments was carried out at 6V/cm at 14°C for 20h, with linear pulse time of 3.51s to 30.82s.

b The states were as follow: MG: Minas Gerais state (South-east Brazil); PR: Paraná state (South); SC: Santa Catarina state (South); SP: São Paulo (South-east).

c The antimicrobial susceptibility was evaluated by disc diffusion assay. Extended-spectrum beta-lactamase (ESBL) production was investigated by using a double-disc synergy test (DDST) [5,23,24]. AMC: amoxicillin/clavulanic acid; CAZ: ceftazidime; CFX: cefoxitin; CIP: ciprofloxacin; CLO: chloramphenicol; CPM: cefepime; CRO: ceftriaxone; CTF: ceftiofur; CTX: cefotaxime; ENO: enrofloxacin; FOS: fosfomycin; GEN: gentamicin; SXT: trimethoprim/sulfamethoxazole; TET: tetracycline.

d MICs were determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [5,25]. Colistin resistance was defined as a colistin MIC› 2 mg/L, according to EUCAST clinical breakpoints [5].

e PFGE patterns were analysed using the Dice similarity with coefficient optimisation set at 1% and tolerance at 2% (BioNumerics software; Applied Maths, Kortrijk, Belgium).

Figure 2

Geographical distribution of *mcr*-1-positive *Escherichia coli* isolates reported from South America, 2012–2016

A light grey colour is used for Brazil, where this study was conducted. The dark grey colour indicates countries (Bolivia, Colombia and Peru) visited between November 2012 and November 2013, by unrelated Dutch travellers, for whom acquisition of faecal colonisation and carriage with MCR-1 and extended-spectrum beta-lactamase (ESBL)-producing E. coli was shown one to two weeks after their return to the Netherlands [12]. A dark grey colour is used for Ecuador, where subsequent to the identification of a human mcr-1-positive isolate, a sequence was deposited in GenBank in March 2016 (GenBank accession number: KU886144.1).

Table

Results of screening *Enterobacteriaceae* isolates from different sources by culture with colistin and presence of the *mcr*-1 gene in the screened isolates, Brazil, 2000–2016 (n = 4,620 isolates screened)

Source[a]		Years of isolate collection	Enterobacteriaceae isolates tested *n*	Enterobacteriaceae isolates with growth on screening plates (2 mg/L colistin) *n*[b]	Isolates positive for *mcr-1* *N (% of isolates screened)*[c]
Food-producing animals	Chicken	2003–2015	280	113	14 (5.0)
	Swine	2012–2014	113	79	2 (1.8)
	Cattle	2014–2015	158	22	0 (0)
	Goat	2013	7	1	0 (0)
	Ostriches	2015	9	2	0 (0)
	Buffalo	2010	36	10	0 (0)
Chicken feed	–	2000–2014	8	4	0 (0)
Companion animals	Cats	2013	4	0	0 (0)
	Dogs	2013	51	9	0 (0)
Non-Companion animals	Horse	2013	13	3	0 (0)
	Rodents	2013–2014	14	13	0 (0)
	Turtle	2015	21	8	0 (0)
	Urban pigeons	2015–2016	36	0	0 (0)
	Urban waterfowl	2012–2014	75	0	0 (0)
Human infection/ colonisation	–	2004–2016	3,591	137	0 (0)
Food	Chicken meat	2013	42	22	0 (0)
	Swine meat	2012–2014	113	79	0 (0)
	Cabbage	2016	2	0	0 (0)
	Lettuce	2016	2	0	0 (0)
	Spinach	2016	1	1	0 (0)
Environment	Lake	2012–2013	20	2	0 (0)
	River	2011	3	3	0 (0)
	Sewage	2009–2013	21	7	0 (0)
Total	**–**	**–**	**4,620**	**515**	**16 (0.3)**

[a] Isolates originated from previous surveillance studies of carbapenemase- and/or extended-spectrum beta-lactamases (ESBL)-producing Gram-negative bacteria in food, food-producing animals (faecal samples from healthy animals), chicken feed, companion and non-companion animals (faecal samples from healthy animals), environment and human patients from healthcare settings (27 faecal samples from colonised individuals and 3,564 clinical cultures from infections), all collected in Brazil between 2000 and 2016 [1-4].

[b] Isolates were screened for potential colistin resistance using MacConkey agar plates supplemented with colistin (2 mg/L).

[c] Enterobacteriaceae isolates with growth on screening plates were subjected to *mcr-1* polymerase chain reaction and sequencing [6].

strains belonged to the phylogroup A and five to the phylogroup B1. Clonal relatedness of the strains were further determined by *XbaI* pulsed-field gel electrophoresis (PFGE) (www.cdc.gov/pulsenet/). PFGE differentiated *mcr-1*-positive *E. coli* isolates into 10 distinct pulsotypes (named A to J), which clustered into two major groups, C (n = 4) and H (n = 3) (Figure 1).

The 16 *mcr-1*-positive isolates were additionally tested for the production of extended-spectrum beta-lactamases (ESBLs) by using a double-disc synergy test (DDST) as well as for the presence of ESBL- and plasmid-mediated AmpC (pAmpC) beta-lactamase genes [1,6].

Most (n= 9) *mcr-1*-positive isolates exhibited resistance to human and/or veterinary cephalosporins. In this regard, such isolates harboured $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-8}}$ and/or $bla_{\text{CTX-M-15}}$ ESBL genes, and one isolate carried the pAmpC $bla_{\text{CMY-2}}$ gene. On the other hand, all isolates carrying the *mcr-1* gene belonged to low-virulence *E. coli* phylogroups (i.e. A and B1 as described above).

Discussion

The plasmid-mediated colistin (polymyxin E) resistance mechanism MCR-1 was first described in Enterobacteriaceae isolated from animals, food and human beings in China [6]. Since, and as summarised by Skov and Monnet [8], MCR-1 has also been reported to occur in other countries in Asia, Europe and North America. Recent descriptions from Egypt [9], Italy [10]

and Spain [11] further denote dissemination of the mechanism, while identifications of *mcr-1* positive strains in imported food, urban rivers and travellers [12-16] highlight the potential for MCR-1 to continue spreading. In addition, co-production of ESBLs or carbapenemases by *mcr-1*-harbouring Enterobacteriaceae has now been documented [12,13,15-18].

We report *mcr-1*-positive *E. coli* isolates from food-producing animals in the southern (Santa Catarina and Paraná states) and south-eastern (São Paulo and Minas Gerais states) regions of Brazil (Figure 2). Interestingly, in most of these isolates (9 of 16), *E. coli* strains co-produced CTX-M-type ESBLs.

Our findings moreover suggest that *mcr-1*-harbouring *E. coli* strains have been present in South America since at least 2012, supporting the results of a previous study on the possible acquisition of *mcr-1*-carrying *E. coli* by European travellers visiting this continent (Figure 2) [12]. In this previous prospective study, the carriage of multiresistant bacteria after travel (COMBAT) consortium had shown that unrelated Dutch travellers to Bolivia, Colombia and Peru between November 2012 and November 2013 had become carriers of/colonised with MCR-1 and ESBL-producing *E. coli* one to two weeks after their return to the Netherlands [12].

Recently the *mcr-1* gene has also been identified in another Latin American country, Ecuador, whereby a respective sequence from a human clinical *E. coli* isolate was submitted to GenBank (GenBank accession number: KU886144.1) in March 2016. Therefore, hospital laboratories worldwide should be aware of the possibility of MCR-1 in Enterobacteriaceae isolates resistant to polymyxins from patients living in or returning from Latin American countries.

That *E. coli* with plasmid-mediated MCR-1 are found in Brazil is also relevant for medical centres in this country, where the emergence and dissemination of multidrug-resistant pathogens, which is associated with high rates of treatment failure, have led to high use of polymyxins, mainly in intensive care units [19]. There, this class of antimicrobial agents represents the main therapeutic option for treating severe 'superbug' infections, particularly *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* producing SPM-1, OXA-23 or KPC-2 carbapenemases, which are highly prevalent in most Brazilian hospitals [19]. On a positive note however, our study did not find *mcr-1*-positivity in any of the human isolates screened, which is consistent with the very low background carriage of MCR-1 in humans, as described previously [6,12-14].

Our result that the *mcr-1* gene occurs in Brazilian livestock is a cause for concern in terms of the global contribution of Brazil to national and international movement of people and products, as this could contribute to the acceleration of the worldwide spread of the *mcr-1* gene. Indeed, with a population of 205 million inhabitants, Brazil has continental proportions and is the biggest country in Latin America. Furthermore, in the agribusiness it is the third producer of chicken meat (only after the United States and China) and the largest exporter of this product [20]. In this regard, colistin sulphate is widely used in animal feed as a growth promoter in Brazilian livestock, mainly in pigs and poultry, supporting a link between the agricultural use of colistin and colistin resistance [21].

Finally, the identification of a colistin-susceptible *E. coli* strain carrying the *mcr-1* gene, in this study, suggests that *mcr-1*-positive isolates may be difficult to detect if the *mcr-1* gene is only tested for in colistin resistant isolates. This may contribute to the silent dissemination of *mcr-1* harbouring strains. In fact, many MCR-1 producers are known to exhibit low level of resistance to colistin (i.e. 4–16 mg/L) [6,8-14,16,22].

In summary, since MCR-1-producing strains have already become established in South America, we emphasise the need for continuous local surveillance programmes to identify the risk to human health. To reduce this risk, the authors suggest that colistin should only be used for treatment of clinical infectious diseases and no longer for animal production, in order to prevent the wide spread of MCR-1-producing bacteria, achieving the principles of responsible use of antibiotics.

Erratum

The term '*mcr-1*' had been mistyped as '*mrc-1*' on several occasions and this was corrected on 02 May 2016.

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Conflict of interest

None declared.

Authors' contributions

MRF, QM, LS, FE, RL, LKO, DDG, MD, MHM, DFMM, ML, DdOG, TK and AMM collected the data and samples, MRF, QM, LS, KCS, MPVC, FE, RL, MD, GRF, MFCB and NL performed the microbiological and molecular analysis, MRF, QM, KCS, FE, MD, DdOG, TK and NL participated in drafting the manuscript, NL coordinated and edited the manuscript.

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Detection of *mcr-1* colistin resistance gene in polyclonal *Escherichia coli* isolates in Barcelona, Spain, 2012 to 2015

N Prim [1], A Rivera [1], J Rodríguez-Navarro [1], M Español [1], M Turbau [2], P Coll [1,3], B Mirelis [1,3]
1. Microbiology Department, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain
2. Emergency Department, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain
3. Universitat Autònoma de Barcelona, Barcelona, Spain

Correspondence: Núria Prim (nprim@santpau.cat)

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Colistin resistance was detected in 53 of 10,011 *Escherichia coli* (0.5%) by prospective phenotypic testing of consecutive clinical isolates in a single hospital in Barcelona, Spain (2012–15). The *mcr-1* gene was retrospectively identified by PCR and sequencing in 15 of 50 available isolates. Each isolate had a unique PFGE pattern except for two. This clonal diversity supports the hypothesis of horizontal dissemination of the *mcr-1* gene in the local study population.

Following the report on the plasmid-mediated colistin resistance gene *mcr-1* in China [1], several authors have reported the detection of this gene in *Escherichia coli* isolates of animal origin [1-5]. Currently there have been few reports of detections in humans and these involve mainly multidrug-resistant (MDR) Gram-negative bacilli [3,5-7]. To date, *mcr-1* has been detected in at least five European countries in animals and humans, and often in association with recent travel to Asia [3,5-7]. In this context, we describe *mcr-1* detection in unselected clinical isolates of *E. coli* in Barcelona in samples from 2012 to 2015.

Laboratory investigation

A total of 10,011 *E. coli* were isolated between January 2012 and December 2015 from clinical specimens in our institution, a tertiary referral teaching hospital covering an area of 407,902 inhabitants in Barcelona, Spain. Only one isolate per patient was included. Isolates from colonisation screenings were not considered. Antibiotic susceptibility testing was performed by disc diffusion according to guidelines from the Clinical and Laboratory Standards Institute (CLSI) [8]. As a first approach to screen colistin resistance, a 10 µg disc of colistin was used. Isolates displaying an inhibition zone ≤ 12 mm (n = 61) were selected for further testing of minimal inhibitory concentration (MIC) by gradient diffusion (Etest, bioMérieux, France). Both diffusion methods were performed on Mueller Hinton agar (bioMérieux, France). MIC results of colistin were interpreted following the EUCAST breakpoints for *Enterobacteriaceae* [9]. Resistance to colistin was detected in 53 *E. coli* isolates (0.5%). Of these, 40 were isolated from urine specimens, eight from blood cultures and the remaining five from other clinical specimens. The average age of the patients with infections caused by colistin-resistant *E. coli* was 70.9 years (range: 6–99 years). The male:female ratio was 1:2.

By amplification and Sanger sequencing, we searched for the presence of the *mcr-1* gene in our collection of colistin-resistant *E. coli* isolates (only 50 isolates were available). The amplification of *mcr-1* was performed as described by Liu et al. [1]. This gene was detected in 15 isolates; the amplified fragments had 100% sequence homology with the previously described *mcr-1* [1].

The patients' average age was 62 years (range: 6–97), eight of them were male and seven were female. Patients were not epidemiologically linked (Table). One patient was referred from a nursing home, and nine had had at least one hospital admission during the previous year. No travel abroad was recorded in any of the patients. The rate of positivity corresponded to 0.15% of the total of *E. coli* isolates within the period studied. Seven *mcr-1*-harbouring isolates were not MDR according to international definitions [10]. Only two were extended-spectrum beta-lactamase carriers and one had an AmpC overproduction profile (Table). Tested by Etest, the MIC to colistin ranged from 4 mg/L to 12 mg/L. The *mcr-1*-positive isolates were typed by pulsed-field gel electrophoresis (PFGE); each isolate had a unique PFGE pattern except for two.

Discussion

Colistin is one of the last resorts to treat infections caused by MDR Gram-negative bacilli. Resistance to colistin is rarely reported in *E. coli*, especially in non-MDR isolates from humans [11]. Until recently, this resistance was considered to be based solely on

Table

Characteristics of *Escherichia coli* isolates harbouring *mcr-1* and epidemiological data of the patients, Barcelona, 2012–15 (n = 15)

Date of isolation	Isolation site	Classification of infectiona	Colistin MIC (mg/L)	Antimicrobial resistance pattern
27/12/2012	Blood	Community-acquired	8	AMP-SXT
09/01/2013	Sputum	Hospital-acquired (haematology)	4	AMP-CTX-CAZ-FEP CIP-SXT (ESBL)
26/02/2013	Blood	Community-acquired	4	AMP-SXT
01/03/2013	Blood	Hospital-acquired (oncology)	12[b]	AMP-GEN-TOB
07/03/2013	Blood	Healthcare-associated	6	AMP-CTX-CAZ-FEP (ESBL)
12/03/2013	Sputum	Healthcare-associated	12	AMP-AMC-CTX-CAZ-SXT
08/06/2013	Urine	Community-acquired	4	AMP-NAL
07/07/2013	Blood	Community-acquired	4[b]	AMP-GEN-TOB
01/11/2013	Sputum	Hospital-acquired (recovery room)	4	AMP-CIP
22/05/2014	Urine	Hospital-acquired (neurosurgery)	4	AMP-NAL-SXT
22/08/2014	Urine	Healthcare-associated	6	AMP-NAL-GEN-TOB-SXT
06/10/2014	Surgical wound	Healthcare-associated	8	AMP-CIP-GEN-TOB
14/03/2015	Urine	Healthcare-associated	4	AMP-CIP-GEN-TOB-SXT
29/03/2015	Urine	Hospital-acquired (cardiology)	4	AMP-CIP-GEN-SXT
16/06/2015	Urine	Healthcare-associated[c]	4	AMP-NAL

AMP: ampicillin; AMC: amoxicillin/clavulanic acid; CAZ: ceftazidime; CIP: ciprofloxacin; CTX: cefotaxime; FEP: cefepime; GEN: gentamicin; MIC: minimum inhibitory concentration; NAL: nalidixic acid; SXT: trimethoprim-sulfamethoxazole; TOB: tobramycin; ESBL: extended spectrum beta-lactamase.

[a] Classification of the infection according to the place of acquisition. When hospital-acquired, the hospital ward where the clinical specimen was taken is shown in brackets.

[b] Isolates sharing the same PFGE pattern.

[c] This patient was referred from a nursing home.

genomic mutations in several genes involved in the synthesis of lipopolysaccharide [12]. Since Liu et al. reported plasmid-mediated colistin resistance in *E. coli* isolates [1], the whole scenario has changed and the possibility of horizontal gene transfer needs to be considered. These plasmids carry the *mcr-1* gene coding for a phosphoethanolamine transferase, an enzyme related to changes in lipid A [1]. Despite the large amount of information on *mcr-1* obtained in only a few months, the real prevalence of this gene in clinical isolates is not yet known. Most reports are retrospective, mainly refer to faecal carriers and describe scattered colistin-resistant isolates randomly collected [3,5-7,13]. We here describe *mcr-1* prevalence in colistin-resistant clinical isolates of *E. coli*. As a limitation, no other mechanisms of colistin resistance were searched for in the present study. However, the high percentage of *mcr-1* among our colistin-resistant isolates is noteworthy.

The clonal diversity shown in the present report supports the hypothesis of horizontal dissemination of *mcr-1* gene-related colistin resistance in *E. coli* isolated from our urban patient population in Barcelona. Colistin is not always tested in non-MDR *E. coli* isolates of human origin. This may explain why the previous reports describing *mcr-1* in humans mainly referred to MDR *E. coli* isolates [3,5-7]. Technical variability among methods for colistin susceptibility testing is notorious [14]. Given the discrepancies between the international committees and the lack of colistin breakpoints for *Enterobacteriaceae* in CLSI, we considered it convenient to apply a screening method. Although disc diffusion is not recommended to test colistin susceptibility, it was useful for an initial screening followed by confirmation using a MIC method.

The fact that seven of 15 *mcr-1*-harbouring strains were not MDR may not seem clinically relevant. However, horizontal spread is important epidemiologically. Screening of colistin resistance in human isolates of *Enterobacteriaceae* should be encouraged in order to know the real extent of a problem that may get worse given the constant exchange of resistance genes across microbiomes (i.e. food animals, the environment and human populations). The broad veterinary use of colistin and the increasing reports of colistin resistance in *Enterobacteriaceae* isolates from food animals are a matter of concern [15]. Spain is one of the European countries with larger use of polymyxins in veterinary medicine [16]. This fact may correlate with the high rates of colistin-resistant *Salmonella* spp. isolates in farm animals previously reported in our country [17]. The use of colistin in humans varies depending on the type of institution involved and their corresponding antimicrobial policy. In our hospital, it has increased 14-fold (0.10 to 1.47 defined daily dose /100 occupied bed-days) from 2007 to 2014.

Considering that the emerging plasmid-mediated resistance to colistin has already spread across microbiomes and considering the selective pressure that the veterinary use of this antibiotic may exert, action is urgent at a global level. Otherwise we may soon face a situation without useful antibiotics to treat infections caused by MDR Gram-negative bacteria.

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Conflict of interest

None declared.

Authors' contributions

NP, AR and BM conceived and designed the study; NP and AR performed the antimicrobial susceptibility tests; JRN and ME performed the molecular assays; MT collected the epidemiological data; NP, AR, MT, PC and BM wrote the manuscript.

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Impact of food animal trade on the spread of *mcr-1*-mediated colistin resistance, Tunisia, July 2015

R Grami [1,3], W Mansour [2,3], W Mehri [4], O Bouallègue [3], N Boujaâfar [3], J Madec [1], M Haenni [1]
1. Unité Antibiorésistance et Virulence Bactériennes, ANSES Site de Lyon, Lyon, France
2. Institut Supérieur des Sciences Appliquées et de Technologie de Mahdia, Tunisia
3. Unité Résistances Bactériennes Emergentes et Sécurité des Soins, UR12SP37, Laboratoire de Microbiologie, Hôpital Universitaire Sahloul, Sousse, Tunisia
4. Commissariat Régional au Développement Agricole, Sousse, Tunisia

Correspondence: Marisa Haenni (marisa.haenni@anses.fr)

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We report a high prevalence of MCR-1 and CTX-M-1-producing *Escherichia coli* in three Tunisian chicken farms. Chickens were imported from France or derived from French imported chicks. The same IncHI2-type plasmid reported to carry those genes in cattle in France and in a food sample in Portugal was found in Tunisian chickens of French origin. This suggests a significant impact of food animal trade on the spread of *mcr-1*-mediated colistin resistance in Europe.

Horizontal transfer was found to play a major role in the spread of colistin resistance in *Enterobacteriaceae* when a plasmid-located *mcr-1* gene was reported to be circulating in livestock, foodstuff and human beings in China in late 2015 [1]. A few weeks later, *mcr-1* was recognised in Europe among extended-spectrum beta-lactamase (ESBL)- or AmpC-producing *Escherichia coli* isolated from chicken meat and humans [2]. In January 2016, the worldwide distribution of the *mcr-1* gene was highlighted [3,4].

The plasmid type first identified as a *mcr-1* vehicle in China was an IncI2-like plasmid, but several different *mcr-1*-positive plasmids have now been reported, including IncHI2-type plasmids. Indeed, Tse et al. reported *mcr-1* on an IncHI2-type plasmid in a *Salmonella enterica* isolate from a food sample in Portugal in 2011 [5]. Interestingly, IncHI2-type plasmids were also recognised to spread $bla_{CTX\text{-}M\text{-}1}$ and *mcr-1* in *E. coli* in food animals in France [6]. These data suggest a specific epidemiology of *mcr-1* plasmids in the European animal reservoir that pose a risk for humans. This prompted us to investigate 37 *E. coli* strains recovered from 29 Tunisian chickens imported from France or derived from French imported chicks and harbouring resistance to colistin and broad-spectrum cephalosporins.

Detection of the $bla_{CTX\text{-}M\text{-}1}$ and *mcr-1* genes in healthy chickens in Tunisia

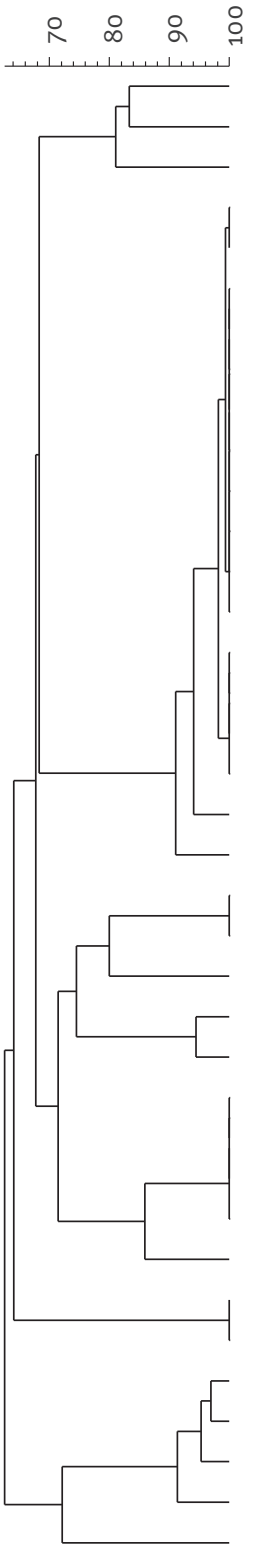
In July 2015, 52 randomly chosen healthy birds were collected on three different Tunisian farms: 10 on farm A, 12 on farm B and 30 on farm C with the initial purpose to investigate the prevalence of ESBL-positive chickens. A faecal sample of each individual was plated on MacConkey agar containing 4 mg/L cefotaxime and one colony per morphology was picked up. This resulted in the identification of 37 *E. coli* isolates harbouring resistance to broad-spectrum cephalosporins and originating from 29 birds (Table).

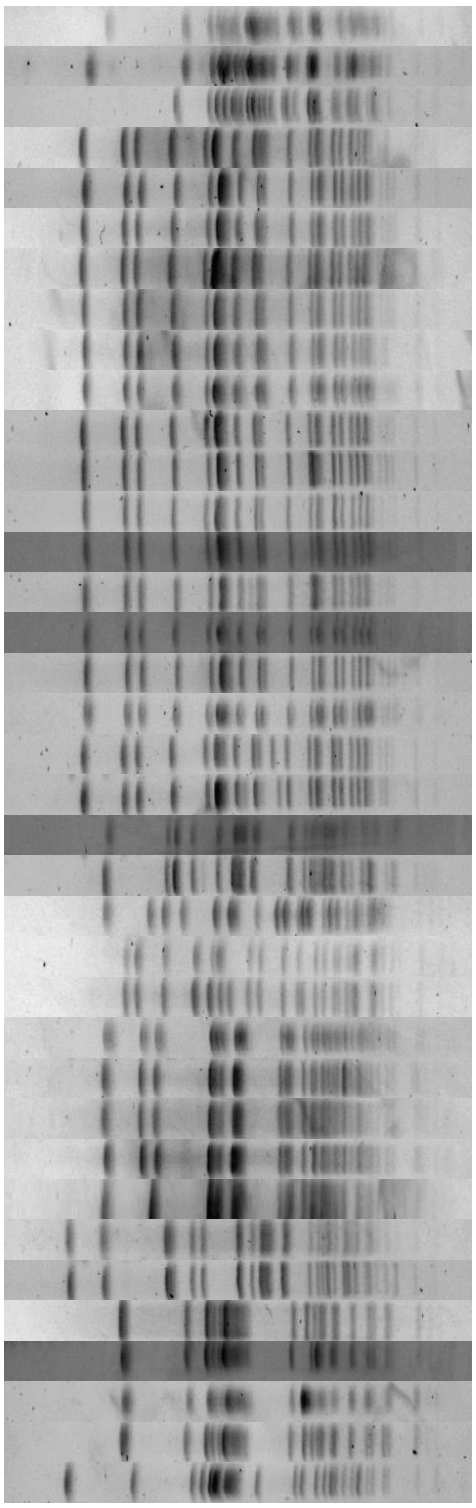
Those 29 birds were from farm A (2/10), farm B (2/12) and farm C (25/30). All 37 isolates produced an ESBL as attested by the synergy test, and the $bla_{CTX\text{-}M\text{-}1}$ gene was identified in all isolates by PCR and sequencing. All isolates expressed additional co-resistances to phenicols, tetracyclines, sulfonamides, trimethoprim, quinolones and fluoroquinolones as determined by disk diffusion against 32 antibiotics. Surprisingly, disk diffusion also revealed small colistin diameters (16–17 mm). We were in the course of investigating these non-susceptible isolates when the publication by Liu et al. [1] drew a different light on our results and prompted us to further investigate colistin resistance.

All isolates presented a minimum inhibitory concentration (MIC) of 2–6 µg/mL to colistin by E-test. PCR and sequencing using published primers [1] revealed the newly described *mcr-1* gene in all of the ESBL-positive *E. coli* with 100% homology to the published sequence (GenBank: KP347127.1). Isolates from farm A presented two closely related but not identical *Xba*I pulsed-field gel electrophoresis (PFGE) patterns (one band difference) belonging to cluster G, while isolates from farm B presented two distinct patterns belonging to the clusters F and K (Figure 1).

Figure 1

Pulsed-field gel electrophoresis-based dendrogram and *XbaI* macrorestrictions, Tunisia, July 2015 (n = 37)





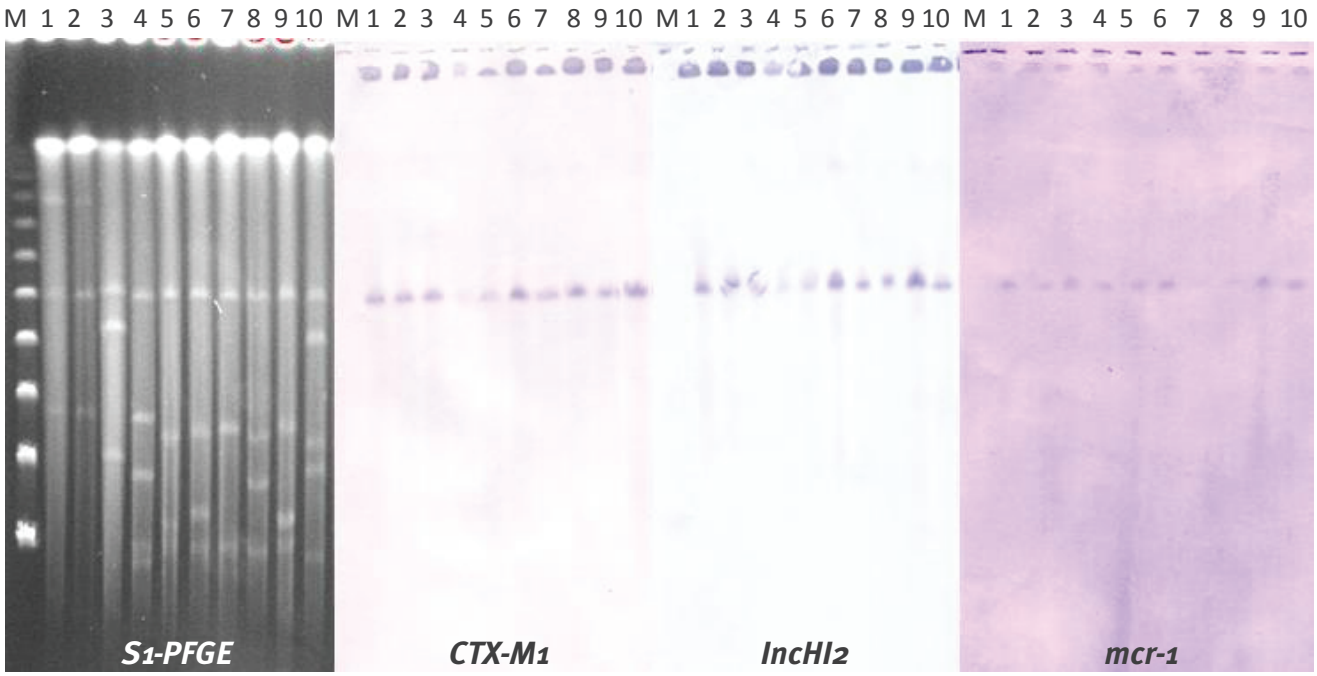
Isolate	Farm	MIC (µg/mL)	PFGE cluster
24B	C	3	A
33B	C	6	B
39B	C	3	C
19A	C	3	D
33A	C	3	D
14	C	6	D
18B	C	4	D
22	C	3	D
23	C	3	D
24A	C	3	D
26	C	4	D
32B	C	2	D
40	C	5	D
41	C	5	D
32A	C	3	D
42A	C	5	D
36A	C	3	D
39A	C	3	D
25A	C	6	D
30	C	4	D
21A	C	4	E
25B	C	3	E
10	B	6	F
MF5	A	3	G
MF8	A	5	G
15	C	3	H
16A	C	4	H
17A	C	3	H
20	C	4	H
18A	C	3	I
16B	C	3	J
31A	C	3	J
12	B	3	K
42B	C	3	K
36B	C	3	K
38B	C	4	K
38A	C	3	L

MIC: minimum inhibitory concentration; PFGE: pulsed-field gel electrophoresis.

Analysis was performed using the Dice coefficient with optimisation set at 0.5% and tolerance at 1.5%.

Figure 2

Southern blot hybridisations on S1 nuclease-pulsed-field gel electrophoresis gels using specific probes for the detection of $bla_{CTX\text{-}M\text{-}1}$, IncHI2 and *mcr-1*, Tunisia, July 2015 (n = 10)



PFGE: pulsed-field gel electrophoresis

M: size marker (Lambda ladder 0.05-1 Mb, Bio-Rad); Lane 1: isolate MF5; Lane 2: isolate MF8; Lane 3: isolate 10; Lane 4: isolate 12; Lane 5: isolate 14; Lane 6: isolate 16A; Lane 7: isolate 16B; Lane 8: isolate 18A; Lane 9: isolate 21A; Lane 10: isolate 24B.

All 37 isolates presented the same profile, so that only a subset of 10 isolates is presented here.

Isolates from farm C presented one main cluster (cluster D encompassing 17 isolates presenting patterns with › 90% similarity) and nine additional clusters (A–C, E, H–L) presenting patterns with ‹ 90% similarity. Antibiotics used were colistin, sulfonamides and enrofloxacin on farms A and C, and chloramphenicol and enrofloxacin on farm B.

Co-localisation of $bla_{CTX\text{-}M\text{-}1}$ and *mcr-1* on IncHI2-type plasmids

Replicon typing and hybridisation experiments proved that $bla_{CTX\text{-}M\text{-}1}$ and *mcr-1* co-localised in all isolates on a single and large (250–280 kbp) IncHI2-type plasmid (Figure 2).

According to the plasmid double locus sequence typing (pDLST) scheme [7], these IncHI2-type plasmids belonged to the ST4 subtype and presented positive amplification of the *hipA* gene and no amplification of the *smr092* and *smr0183* genes [7]. Interestingly, the IncHI2-type plasmids recently found in food animals in France also belonged to the very same ST4 subtype (data not shown) [6]. Hence, IncHI2-type plasmids were responsible for the spread of $bla_{CTX\text{-}M\text{-}1}$ and *mcr-1* in different chicken farms in Tunisia, in the bovine sector in France and in foodstuff in Portugal.

High prevalence of *mcr-1*-positive chickens on Tunisian farms

Data on *mcr-1* from the poultry reservoir are lacking except for one single case in Algeria [8]. However, *mcr-1* reports from chicken meat have been recurrent [1,2,9,10]. Here, farms A and C (counting 7,500 and 8,500 chickens, respectively) host grandparent flocks and import one-day-old chicks from France (Table). Farm B is located 80 km apart from the others and rears 200,000 broilers deriving from one-day-old chicks sold by a Tunisian hatchery also importing birds from France. Thus, the estimated true prevalence (with confidence intervals at 95%) of *mcr-1*-positive chickens reaches 20% (3–56%) on farm A, 17% (4–49%) on farm B and 83% (65–94%) on farm C. This last figure is even higher than recently reported from food animals in China [1].

Conclusion

From this study, we conclude that the live chicken population in Tunisia is heavily colonised by *mcr-1*-positive *E. coli* with subsequent possible contamination

Table

Epidemiological and molecular features of $bla_{CTX\text{-}M\text{-}1}$/mcr-1-positive *Escherichia coli*, Tunisia, July 2015 (n = 37)

Farm	Location	Number of birds on farm	Age of birds	Origin of the birds	Number of birds sampled	Number of ESBL-positive birds	Number of *mcr1* and bla_{CTXM_1}-positive *E. coli*	Plasmid type carrying bla_{CTXM_1} and *mcr1*
Farm A	Moknine	8,500	17–18 weeks	France	10	2	2	IncHI2/ST4
Farm B	Enfidha	200,000	35 days	Tunisia/France	12	2	2	IncHI2/ST4
Farm C	Moknine	7,500	62 weeks	France	30	25	33[a]	IncHI2/ST4

ESBL: extended-spectrum beta-lactamase.

[a] One colony per morphology was picked up, resulting in a higher number of *E. coli* isolates than the number of samples.

of chicken products [11,12]. Multilocus sequence typing (MLST) was not performed in this study since PFGE demonstrated the presence of numerous clusters of *E. coli* (A to L) so that the *mcr-1* dissemination was clearly a consequence of the spread of the unique IncHI2/ST4 plasmid in various *E. coli* backgrounds.

Contamination of both the poultry production pyramid and the food chain is undoubtedly of public health relevance. It is now crucial to determine the prevalence of the *mcr-1* gene in poultry and poultry meat as well as in other livestock (live animals or meat) in Tunisia and other African countries in order to estimate the risk to human health.

In addition, the finding of a single IncHI2-type plasmid spreading the $bla_{CTX\text{-}M\text{-}1}$/*mcr-1* genes in the food sector in different European and non-European countries makes us believe that global imports and exports of food animals and foodstuff are a major determinant of *mcr-1* dissemination. Global chicken meat production is forecast to dramatically increase in the future because of rising demands worldwide and subsequent rising production volumes in the major exporting countries. European countries already faced a major spread of ESBL/pAmpC genes in animals that subsequently became ESBL sources for humans, mostly as a result of poultry trades [13,14]. Worryingly, genes providing resistance to broad-spectrum cephalosporins and colistin have been shown to be tightly linked on the same plasmids, indicating that urgent international attention is necessary on the global market of veterinary drugs for food animals.

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Conflict of interest

None declared.

Authors' contributions

RG collected the isolates, collected the data and performed the molecular analysis. MH, WM, and JYM coordinated the work and participated to the data analysis. MH and JYM drafted the manuscript, WM and RG participated in the writing of the manuscript, and all authors have read and accepted the submitted manuscript.

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Prevalence of *mcr-1* in commensal *Escherichia coli* from French livestock, 2007 to 2014

A Perrin-Guyomard [1], M Bruneau [1], P Houée [1], K Deleurme [1], P Legrandois [1], C Poirier [1], C Soumet [1], P Sanders [1]
1. ANSES, Laboratoire de Fougères, Fougères, France

Correspondence: Agnès Perrin-Guyomard (agnes.perrin-guyomard@anses.fr)

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Colistin resistance was investigated in 1,696 isolates collected from 2007 to 2014 within the frame of the French livestock antimicrobial resistance surveillance programme. The *mcr-1* gene was detected in all commensal *Escherichia coli* isolates with a minimum inhibitory concentration to colistin above the 2 mg/L cut-off value (n=23). In poultry, *mcr-1* prevalence was 5.9% in turkeys and 1.8% in broilers in 2014. In pigs, investigated in 2013, this prevalence did not exceed 0.5%. These findings support that *mcr-1* has spread in French livestock.

We report *mcr-1* prevalence data in commensal *Escherichia coli* isolated from French livestock from 2007 to 2014.

Laboratory investigation

According to the European Union surveillance programme on antimicrobial resistance in zoonotic and commensal bacteria (directive 2003/99/EC) [1], a random sample of faecal (until 2013) or caecal (since 2014) content from the same epidemiological unit (defined as in [2]) of broilers, pigs and turkeys was taken at slaughter houses all over the country, in order to be representative of national productions. The sampling was proportional to the slaughter houses' annual throughputs and was spread over the year. The number of samples collected per animal species and year was calculated to be able to recover at least 170 *E. coli* isolates for each combination of bacterial species and animal production. Isolates were streaked on MacConkey medium, identified and tested for antimicrobial susceptibility by the broth microdilution method (Trek diagnostic systems) using a panel of 14 antimicrobial substances. The minimum inhibitory concentrations (MIC) obtained were compared with the epidemiological cut-off values of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [3]. The DNA of strains with a colistin MIC over 2 mg/L was extracted and the presence of *mcr-1* sought by polymerase chain reaction (PCR) [4].

Colistin resistance and presence of the *mrc-1* gene in isolates

Most (1,427/1,450; 98%) commensal *E. coli* strains isolated and tested from French livestock between 2007 and 2014 were susceptible to colistin (Table).

During the study period however, a total of 23 isolates were resistant to colistin at concentrations above the cut-off value of 2 mg/L, with MICs ranging from 4 to 16 mg/L. Interestingly, each individual *E. coli* isolate from French livestock with a MIC to colistin greater than the cut-off harboured the *mcr-1* gene. From 2011 to 2013, two strains resistant to colistin were isolated from healthy pigs. The prevalence of colistin resistance in broilers was 1.8% in 2014. In turkey production, monitoring commensal *E. coli* became mandatory at European level in 2014 and the prevalence of resistance to colistin was 5.9% that year. Co-resistance patterns were diverse, ranging from one to eight associated mechanism of resistance (data not shown). Nevertheless, in four of the 14 *mcr-1* positive turkey isolates, colistin resistance coincided with simultaneous resistance to ampicillin, quinolones, sulfamethoxazole, tetracycline and trimethoprim (data not shown). One single strain derived from turkeys was also resistant to cefotaxime and carrying the bla_{CMY-2} gene. Plasmid profiling in order to assess the transferability of these *mcr-1* genes from food producing animals to other hosts such as humans is under progress.

Discussion

For decades, colistin has been widely used in veterinary medicine against infections caused by *Enterobacteriaceae* in food-producing animals in Europe [5]. To offset limited data on colistin resistance in European livestock, this antibiotic was added in 2014 to the antimicrobial substances required to be tested under antimicrobial resistance programmes conducted by European Member States (decision 2013/652/EU [2]).

Table

Colistin resistant and mcr-1 positive commensal *Escherichia coli* strains from French livestock, France, 2007–2014

Year	Animals	*E. coli* strains tested for MIC N	*E. coli* strains resistant to colistin N	Proportion of *mcr-1* positive (n) among colistin-resistant *E. coli* strains (N) n/N	Prevalence of *mcr-1* positive *E. coli* strains % (95%CI)
2014	Turkeys	239	14	14/14	5.9 (2.9–8.8)
	Broilers	227	4	4/4	1.8 (0.1–3.5)
2013	Pigs	196	1	1/1	0.5 (0.0–1.5)
	Broiler	193	3	3/3	1.6 (0.0–3.3)
2012	Pigs	194	0	N.a.	N.a.
	Broiler	201	0	N.a.	N.a.
2011	Pigs	200	1	1/1	0.5 (0.0–1.5)
2007	Turkeys	ND[a]	ND[a]	0/246[a]	0 (0.0–1.2)
Total	**All**	**1,450**	**23**	**N.a.[a]**	**N.a.[a]**

CI: confidence interval; MIC: minimum inhibitory concentration; N.a.: not applicable; ND: not determined.

[a] As susceptibility to colistin was not tested in 2007, each isolate obtained in that year was tested for the presence of *mcr-1*.

In spite of this, prior to 2015, the mechanism of resistance to colistin was only known to involve chromosomal mutations, and so its spread was expected to be limited to vertical transmission [6]. In 2015 however, the first plasmid-mediated colistin resistance involving the *mrc-1* gene was discovered in China by Liu et al. [4]. Since, other reports detail retrospective detection of this gene in *E. coli* from animal origin. In Germany, the gene was found in three of the 129 whole-genome sequences of *E. coli* isolated from livestock since 2009 [7]. The *mrc-1* positive strains originated from swine and were sampled in 2010 and 2011. The *mcr-1* gene was also detected in five *E. coli* isolates from chicken meat of European origin imported in Denmark in 2012, 2013 and 2014 [8]. In Belgium, 13 of 105 colistin-resistant *E. coli* isolates collected in 2011 and 2012 from piglets and bovine calves with diarrhoea were positive for *mcr-1* [9]. Also, in France, extended-spectrum beta-lactamase (ESBL)-positive *E. coli* isolated from diarrhoeic bovine calves as early as 2005 were confirmed to be *mcr-1* positive [10] as well as four *Salmonella* isolates from 2012 to 2013 collected within the French agricultural food sector [11]. A number of these findings implicated pathogenic strains, isolated in the context of event-based surveillance networks or programmes.

Prompted by these reports of *mrc-1*-mediated colistin resistance, we investigated the prevalence of *mcr-1* in non-pathogenic *E. coli* isolated through the official European surveillance programme on antimicrobial resistance in French livestock. This programme is designed to be comparable between Member States but its power to detect emergent resistance is likely to be limited. In fact, after three years of continuous monitoring, starting from an initial theoretical point of 0.1% of resistant isolates, this programme cannot detect any changes if the overall increase is lower than 2% per year [2]. The fact that *mcr-1* emergence is detected through this surveillance programme supports the idea of a rapid spread of plasmid-mediated colistin resistance in French livestock.

The presence of co-resistances in strains harbouring the *mcr-1* gene could have contributed to select and enhance the rapid dissemination of the plasmid-mediated resistance to colistin jointly with antibiotic pressure by other antimicrobial use in food producing animals.

The dissemination of *mcr-1* in French livestock, either in a pathogenic or healthy context, raises the question of colistin use in animals. Colistin use should be now revisited in a double perspective: first, in a veterinary medicine perspective, that might suddenly start to face treatment failures in animal digestive disorders such as colibacillosis or salmonellosis; and second, in a human medicine perspective, in order to maintain the efficacy of a last-resort therapeutic option to counteract multidrug-resistant bacterial infections [5].

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Conflict of interest

None declared.

Authors' contributions

APG designed the study, analysed and interpreted data, drafted and coordinated the manuscript elaboration, MB analysed the data and contributed to the manuscript, PH, KD, PL, CP produced phenotypic and molecular data, CS contributed to the manuscript, PS contributed to the manuscript and given scientific advice.

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Detection of *mcr-1* encoding plasmid-mediated colistin-resistant *Escherichia coli* isolates from human bloodstream infection and imported chicken meat, Denmark 2015

H Hasman [1], AM Hammerum [1], F Hansen [1], RS Hendriksen [2], B Olesen [3], Y Agersø [2], E Zankari [2], P Leekitcharoenphon [2], M Stegger [1,4], RS Kaas [2], LM Cavaco [2], DS Hansen [3], FM Aarestrup [2], RL Skov [1]

1. 1. Department of Microbiology and Infection Control, Statens Serum Institut, Copenhagen, Denmark
2. National Food Institute, Technical University of Denmark, Lyngby, Denmark
3. Department of Clinical Microbiology, Herlev and Gentofte Hospital, Copenhagen University Hospital, Herlev, Denmark
4. Pathogen Genomics Division, Translational Genomics Research Institute (TGen), Flagstaff, Arizona, USA

Correspondence: Henrik Hasman (henh@ssi.dk)

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The plasmid-mediated colistin resistance gene, *mcr-1*, was detected in an *Escherichia coli* isolate from a Danish patient with bloodstream infection and in five *E. coli* isolates from imported chicken meat. One isolate from chicken meat belonged to the epidemic spreading sequence type ST131. In addition to IncI2*, an incX4 replicon was found to be linked to *mcr-1*. This report follows a recent detection of *mcr-1* in E. coli from animals, food and humans in China.

Very recently, in November 2015, *Liu et al.* reported the finding of a transferable plasmid-mediated colistin resistance gene, *mcr-1*, detected in *Escherichia coli* isolates from animals, food and patients in China. Moreover, they found *mcr-1* in *Klebsiella pneumoniae* isolates from patients [1]. Horizontal gene transfer represents a paradigm shift in colistin resistance, which until then only was found to be mediated by chromosomal mutations and thus spread by vertical transmission.

National surveillance of antimicrobial resistance in food animals, food and humans in Denmark using whole genome sequencing

Since 2012, the national surveillance of antimicrobial resistance in food animals, food and humans in Denmark (www.DANMAP.org) has used whole-genome sequence (WGS) analysis for detection of resistance genes and multilocus sequence typing (MLST) using the open-access bioinformatic web-tools ResFinder and MLST, respectively from www.genomicepidemiology.org for characterisation of extended spectrum beta-lactamase (ESBL)- and AmpC-producing *E. coli* isolates [2-4]. The *mcr-1* sequence from China was added on 24 November 2015 to the ResFinder database as soon as it was available from The National Center for Biotechnology Information (NCBI).

Investigation of presence of *mcr-1* in *E. coli* isolates from food animals, food and human bloodstream infections

The updated version of ResFinder was used to analyse the WGS data from ESBL- and AmpC-producing *E. coli* isolates from food animals and food for the years 2012 to 2014, as well as ESBL- and AmpC-producing *E. coli* isolates from human bloodstream infections, and carbapenemase-producing organisms (CPOs) from humans, from January 2014 to beginning of November 2015 (Table 1), for the presence of *mcr-1*. Furthermore, fluoroquinolone resistance determinants were investigated by searching manually for mutations in the GyrA, ParC and ParE. [5].

The *mcr-1* gene was detected in one *E. coli* isolate from a human bloodstream infection from 2015 and in five *E. coli* isolates obtained from chicken meat of European origin imported to Denmark from 2012, 2013 and 2014 (Table 2). None of the CPOs were positive for *mcr-1* (Table 1).

The patient infected with the *mcr-1*-positive *E. coli* was an elderly man with prostate cancer and repeated urinary tract infections with ESBL-producing *E. coli* resulting in four positive urine samples over five month prior to the bloodstream infection, all resistant to third generation cephalosporins, gentamicin, sulfamethoxazole, trimethoprim and ciprofloxacin (these isolates were not

Table 1

Numbers of extended spectrum beta-lactamase and AmpC-producing *E. coli* isolates obtained and analysed by WGS from chicken meat, humans and carbapenemase-producing isolates from humans tested for *mcr-1* using ResFinder, Denmark, November 2015 (n=914)

Isolate origin	No. of isolates analysed by WGS	No. of sequences positive for *mcr-1*
ESBL- and AmpC-producing *E. coli* isolates from Danish chicken meat (2012–2014)	125	0
ESBL- and AmpC-producing *E. coli* isolates from imported chicken meat (2012–2014)	255	5
ESBL- and AmpC-producing *E. coli* isolates from human bloodstream infections (January 2014– beginning of November 2015)	417	1
Carbapenemase-producing isolates from humans (January 2014–beginning of November 2015)	117	0

ESBL: extended spectrum beta-lactamase; No: number; WGS: whole-genome sequence.

Table 2

Genotypic characterisation of *mcr-1*-positive *E. coli* isolates, Denmark, November 2015 (n=6)

Isolate name	Origin	Year of detection	MLST	Resistance genes detected by ResFinder besides *mcr-1*	Detection of chromosomal mutations encoding resistance to quinolones
0412016126	Chicken meat	2012	ST359	*aadA1*, *aadA5*, *aph(3')-Ic*, bla_{CMY-2}, bla_{TEM-1B}, *dfrA1*,*strA*, *strB*, *sul1*, *sul2*, *tet*(B)	GyrA (S83L, D87N) ParC (E62K)
0412044854	Chicken meat	2012	ST48	*aadA1*, bla_{CMY-2}, bla_{TEM-1B}, *dfrA1*, *mph(B)*, *strA*, *strB*, *sul1*, *sul2*, *tet*(A)	GyrA (S83L)
0412049521	Chicken meat	2012	ST131	*aadA1*, bla_{SHV-12}, bla_{CMY-2}, *strA*, *strB*, *sul1*, *dfrA1*, *tet*(A)	ND
0413040864	Chicken meat	2013	ST1112	*aadA1*, *aadA2*, bla_{SHV-12}, *cmlA1*, *sul3*, *tet*(A)	ND
14042624	Chicken meat	2014	ST2063	*aadA1*, *aadA2*, bla_{SHV-12}, *cmlA1*, *sul3*	ND
ESBL20150072	Human, bloodstream infection	2015	ST744	*aadA5*, bla_{CMY-2}, $bla_{CTX-M-55}$, bla_{TEM-1B}, *catA1*, *dfrA17*, *floR*, *fosA*, *mph*(A), *rmtB*, *strA*, *strB*, *sul1*, *sul2*, *tet*(A)	GyrA (S83L, D87N) ParC (E62K)

MLST: multilocus sequence typing; ND: not detected.

investigated further). He had been treated empirically with piperacillin/tazobactam and subsequently meropenem after susceptibility testing of the bloodstream isolate, but not with colistin according to the available patient data.

Besides *mcr-1*, the human isolate from the Danish patient contained 15 different resistance genes including $bla_{CTX-M-55}$ and bla_{CMY-2} conferring resistance to extended-spectrum beta-lactam antibiotics as well as two GyrA mutations (S83L, D87N) and a ParC mutation (E62K) leading to high-level fluoroquinolone resistance (Table 2). The human *mcr-1* positive *E. coli* isolate belonged to ST744, a rare sequence type in both humans and animals in Denmark. The patient had not been travelling abroad recently and the origin of the isolate is unknown.

The bla_{CMY-2} gene was detected in three of the five *mcr-1*-positive chicken meat isolates. In addition, three of the chicken meat *E. coli* isolates carried bla_{SHV-12} conferring resistance to extended-spectrum beta-lactam antibiotics excluding cephamycins.

One of the *mcr-1* positive *E. coli* isolates from chicken meat belonged to ST131. The other chicken meat isolates belonged to sequence types not frequently found in Denmark (Table 2). The human MCR-1-producing *E. coli* isolate was only susceptible to piperacillin/tazobactam, carbapenems and tigecycline according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints [6], whereas the chicken meat isolates were less resistant (Table 3).

WGS analysis using the web-tool PlasmidFinder [9] identified an IncI2 replicon present in the human isolate as well as in three of the chicken meat isolates, but this replicon was not detected in the remaining two chicken meat isolates. De novo assembly using CLCbio Genomics Workbench (v8.5.1; Qiagen, Aarhus,

Table 3

Antimicrobial susceptibility profiles of the five *MCR-1*-producing *E. coli* isolates from chicken meat and the *MCR-1*-producing *E. coli* isolate from human bloodstream infection, Denmark November 2015

Origin	Human		Chicken meat									
Isolate name	ESBL20150072		14042624		0413040864		0412049521		0412016126		0412044854	
Antimicrobial agent	MIC (mg/L)	S/R	MIC (mg/L)	S/R	MIC (mg/L)	S/R	MIC (mg/L)	S/R	MIC (mg/L)	S/R	MIC (mg/L)	S/R
Polymyxins												
Colistin	>4	R	>4	R	>4	R	>4	R	>4	R	>4	R
Polymyxin B[a]	4	R	>4	R	4	R	>4	R	>4	R	4	R
Beta-lactam/beta-lactam inhibitor combinations												
Ticarcillin/clavulanic acid	128/2	R	>128/2	R	≤16/2	S	64/2	R	64/2	R	≤16/2	S
Piperacillin/tazobactam	≤8/4	S	≤8/4	S	≤8/4	S	≤8/4	S	≤8/4	S	≤8/4	S
Cephalosporins												
Cefotaxime	>32	R	8	R	8	R	8	R	8	R	4	R
Ceftazidime	>16	R	>16	R	8	R	>16	R	16	R	8	R
Cefepime	>2	R	≤2	S	≤2	S	≤2	S	≤2	S	≤2	S
Monobactams												
Aztreonam	>16	R	>16	R	>16	R	>16	R	8	R	8	R
Carbapenems												
Ertapenem	≤0.25	S	≤0.25	S	≤0.25	S	≤0.25	S	≤0.25	S	≤0.25	S
Meropenem	≤1	S	≤1	S	≤1	S	≤1	S	≤1	S	≤1	S
Imipenem	≤1	S	≤1	S	≤1	S	≤1	S	≤1	S	≤1	S
Doripenem	≤0.125	S	≤0.125	S	≤0.125	S	≤0.125	S	≤0.125	S	≤0.125	S
Aminoglycosides												
Gentamicin	>8	R	≤1	S	2	S	≤1	S	≤1	S	≤1	S
Tobramycin	>8	R	≤1	S	2	S	≤1	S	2	S	≤1	S
Amikacin	>4	R	≤4	S	≤4	S	≤4	S	≤4	S	≤4	S
Fluoroquinolones												
Ciprofloxacin	>2	R	≤0.25	S	≤0.25	S	≤0.25	S	>2	R	≤0.25	S
Levofloxacin	8	R	≤1	S	≤1	S	≤1	S	>8	R	≤1	S
Tetracyclines												
Doxycycline[b]	8	I	≤2	S	16	R	8	I	16	R	8	I
Minocycline[b]	4	S	≤2	S	4	S	≤2	S	16	R	≤2	S
Glycylcyclines												
Tigecycline	≤0.25	S	≤0.25	S	≤0.25	S	≤0.25	S	≤0.25	S	≤0.25	S
Folate pathway inhibitors												
Trimethoprim/ sulfamethoxazole	>4/76	R	≤0.5/9.5	S	≤0.5/9.5	S	>4/76	R	>4/76	R	>4/76	R

R/S according to the European Committee on Antimicrobial Susceptibility (EUCAST) clinical breakpoints [6].
I: intermediate; MIC: Minimal Inhibitory Concentration; R: resistant: S: sensitive.
[a] Breakpoint according to Société Francaise de Microbiologie [7]
[b] R/I/S according to The Clinical and Laboratory Standards Institute (CLSI) guidelines (M100-S25) [8].

Denmark) of the genomic data produced a direct link between the *mcr-1* gene and an IncX4 replicon in one of the two isolates not containing IncI2 replicons. An identical IncX4 replicon was detected in four of the chicken meat isolates including both isolates lacking an IncI2 replicon (but not in the human isolate).

Discussion and conclusion

Here we describe a MCR-1 producing *E. coli* isolate from a human infection coproducing both an ESBL (CTX-M-55) and an AmpC (CMY-2) cephalosporinase as well as five MCR-1 producing *E. coli* from chicken meat coproducing either and ESBL (SHV-12) or an AmpC (CMY-2) cephalosporinase, or both. Human and animal CTX-M-55-producing isolates are commonly reported from Asia [10,11], but are relatively rarely seen in Denmark. CTX-M-55-producing *E. coli* isolates were detected in only 3% of the *E. coli* from bloodstream infections in 2014 [4]. CMY-2-producing *E. coli* isolates have commonly been detected from chicken meat both in Denmark and other countries [2-4,12,13], but bla_{CMY-2} has been relatively rare in the Danish human bloodstream *E. coli* isolates. Similarly, only two of the 245 human bloodstream *E. coli* isolates from 2014 were

SHV-12-producing [4]. Based on antibiogram data it seems plausible that the bloodstream infection is related to the repeated urinary tract infections, but this will need to be confirmed by additional WGS analysis. At this point in time, the origin of the human isolate is unresolved.

MLST analysis did not show any close clonal relationship between any of the six isolates. However, one of the chicken meat isolates belonged to ST131. This sequence type is commonly associated with human *E. coli* urinary tract and blood infection isolates worldwide, but are rare in animal *E. coli* isolates [4,14,15]. The fact that a ST131 MCR-1-producing *E. coli* isolate was found is of special concern, since ST131 isolates have spread epidemically during the last decade [14,15] and the ability of *mcr-1* to be acquired by this sequence type has been demonstrated here.

The *mcr-1* gene was initially reported to be located on an IncI2 plasmid without other known resistance markers [1]. Here only four of the isolates were found to contain an IncI2 replicon, suggesting that the *mcr-1* gene was either located on the chromosome or on a plasmid type belonging to another group. In support of the latter is the fact that *de novo* assembly of the genomic data from one of the isolates produced a continuous DNA fragment containing both an IncX4 and the *mcr-1* gene, strongly suggesting that the *mcr-1* gene is not restricted to the IncI2 plasmid group, but additional studies are needed to clarify this further.

In conclusion, this study is to our knowledge, the first proof of colistin-resistant *mcr-1* positive *E. coli* outside China. The human isolate was only susceptible to very few antimicrobial classes such as carbapenems. Should an isolate like this acquire carbapenem resistance, it would leave very few, if any, suitable treatment options. Finally, our findings underline the importance of continuous microbiological surveillance programs and not the least the benefit of employing comprehensive WGS-based surveillance of antimicrobial resistance, as it allows for rapid re-analysis of large datasets *in silico* and thus make early detection and risk assessment possible when new resistance genes emerge.

*Authors' correction

Upon request of the authors, Christina Aaby Svendsen's name was corrected in the Acknowledgement section on 14 December 2015. In addition, the sentence "In addition to IncN2, an incX4 replicon was found to be linked to mcr-1." was corrected to read "In addition to IncI2, an incX4 replicon was found to be linked to mcr-1." on 16 December 2015 upon request of the authors.

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Conflict of interest

None declared.

Authors' contributions

HH and AMH collected the data and drafted the manuscript, HH, MS, PL, EZ and RK did the molecular analysis, FMA, FH, YA, RSH, LC, DSH, BO produced phenotypic data and participated in the coordination and concept of the manuscript, RLS coordinated and edited the manuscript.

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Research article

Presence of *mcr-1*-positive *Enterobacteriaceae* in retail chicken meat but not in humans in the Netherlands since 2009

MF Kluytmans–van den Bergh [1,2], P Huizinga [3], MJ Bonten [1,4], M Bos [5], K De Bruyne [6], AW Friedrich [7], JW Rossen [7], PH Savelkoul [8,9], JA Kluytmans [1,3]

1. Julius Centre for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht, The Netherlands
2. Amphia Academy Infectious Disease Foundation, Amphia Hospital, Breda, The Netherlands
3. Laboratory for Microbiology and Infection Control, Amphia Hospital, Breda, The Netherlands
4. Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands
5. Microbiome Ltd, Amsterdam, The Netherlands
6. Applied Maths NV, Sint-Martens-Latem, Belgium
7. Department of Medical Microbiology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
8. Department of Medical Microbiology, Maastricht University Medical Centre, Maastricht, The Netherlands
9. Department of Medical Microbiology and Infection Control, VU University Medical Center, Amsterdam, The Netherlands

Correspondence: Marjolein Kluytmans–van den Bergh (marjoleinkluytmans@gmail.com)

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Recently, the plasmid-mediated colistin resistance gene *mcr-1* was found in *Enterobacteriaceae* from humans, pigs and retail meat in China. Several reports have documented global presence of the gene in *Enterobacteriaceae* from humans, food animals and food since. We screened several well-characterised strain collections of *Enterobacteriaceae*, obtained from retail chicken meat and hospitalised patients in the Netherlands between 2009 and 2015, for presence of colistin resistance and the *mcr-1* gene. A total of 2,471 *Enterobacteriaceae* isolates, from surveys in retail chicken meat (196 isolates), prevalence surveys in hospitalised patients (1,247 isolates), clinical cultures (813 isolates) and outbreaks in healthcare settings (215 isolates), were analysed. The *mcr-1* gene was identified in three (1.5%) of 196 extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* isolates from retail chicken meat samples in 2009 and 2014. Two isolates were obtained from the same batch of meat samples, most likely representing contamination from a common source. No *mcr-1*-positive isolates were identified among 2,275 human isolates tested. All *mcr*-1-positive isolates were colistin-resistant (minimum inhibitory concentration (MIC) › 2 mg/L). Our findings indicate that *mcr*-1-based colistin-resistance currently poses no threat to healthcare in the Netherlands. They indicate however that continued monitoring of colistin resistance and its underlying mechanisms in humans, livestock and food is needed.

Introduction

The worldwide emergence of extended-spectrum beta-lactamases (ESBL) and carbapenemases has limited the available treatment options for infections with Gram-negative bacteria [1]. Colistin is considered to be an antibiotic of last resort for the treatment of infections with carbapenem-resistant bacteria, and its use in humans is increasing [1].

In November 2015, the presence of a plasmid-mediated colistin-resistance gene, *mcr-1*, in *Enterobacteriaceae* from food animals, food and patients in China was reported [2]. The *mcr-1* gene was detected in 21% of *Escherichia coli* isolates cultured from pigs at slaughter and in 15% of *E. coli* isolates cultured from retail meat between 2011 and 2014. In addition, the *mcr*-1 gene was present in 1.4% of *E. coli* isolates and 0.7% of *Klebsiella pneumoniae* isolates from clinical cultures from patients in two Chinese hospitals in 2014. Directly following this publication, the *mcr-1* gene was reported to be present in 0.2% of ESBL- and AmpC-producing *E. coli* isolates from human bloodstream infections, and in 2% of *E. coli* isolates cultured from imported chicken meat in Denmark since 2012 [3].Hereafter, several reports have documented the global presence of the *mcr-1* gene in *Enterobacteriaceae* cultured from humans, food animals and food [4-13].

Traditionally, colistin resistance was thought to be mediated by chromosomal mutations only, and to spread exclusively via clonal transmission of resistant isolates [14]. The emergence of plasmid-mediated

Table 1

Enterobacteriaceae isolates from retail chicken meat, rectal samples, clinical cultures and outbreaks by year of culture, type of isolate, and colistin-resistance, analysed by whole genome sequencing for the presence of the mcr-1 gene, the Netherlands, 2009–2015 (n = 2,471)

Isolate origin	Year	Type of isolate	Number of isolates	Number of colistin-resistant isolates	Number of *mcr-1*-positive isolates
Retail chicken meat (n = 196)					
Prevalence survey (n = 74)	2009	ESBL-producing *Escherichia coli*	68	NA[a]	1
		ESBL-producing *Klebsiella pneumoniae*	6	NA	0
Prevalence survey (n = 122)	2014	ESBL-producing *E. coli*	119	2	2
		ESBL-producing *K. pneumoniae*	3	0	0
Hospitalised patients, rectal samples (n = 1,247)					
Prevalence survey, 4 hospitals (n = 50)	2009	ESBL-producing *E. coli*	39	NA	0
		ESBL-producing *K. pneumoniae*	11	NA	0
Prevalence surveys, 1 hospital (n = 63)	2013–2014	ESBL-producing *E. coli*	54	0	0
		ESBL-producing *K. pneumoniae*	8	0	0
		ESBL-producing *K. oxytoca*	1	0	0
Prevalence surveys, 14 hospitals (n = 1,134)	2011–2014	ESBL-producing *E. coli*	821	2	0
		ESBL-producing *K. pneumoniae*	172	3	0
		ESBL-producing *K. oxytoca*	13	0	0
		ESBLproducing *Enterobacter cloacae*	77	2	0
		ESBL-producing *Citrobacter* spp.	38	1	0
		ESBL-producing *Morganella morganii*	6	6[b]	0
		Other ESBLproducing *Enterobacteriaceae*	7	0	0
Hospitalised patients, clinical cultures (n = 813)					
Blood cultures, 4 hospitals (n = 25)	2009	ESBL-producing *E. coli*	16	NA	0
		ESBL-producing *K. pneumoniae*	7	NA	0
		ESBL-producing *K. oxytoca*	2	NA	0
Blood cultures, 4 hospitals (n = 77)	2013–2014	ESBL-producing *E. coli*	67[c]	0	0
		ESBL-producing *K. pneumoniae*	8[c]	0	0
		ESBL-producing *K. oxytoca*	2	0	0
Clinical cultures, 14 hospitals (n = 711)	2011–2014	ESBL-producing *E. coli*	546	4	0
		ESBL-producing *K. pneumoniae*	101	2	0
		ESBL-producing *K. oxytoca*	5	0	0
		ESBL-producing *E. cloacae*	46	3	0
		ESBL-producing *Citrobacter* spp.	4	0	0
		ESBL-producing *M. morganii*	3	3[b]	0
		ESBL-producing *Proteus mirabilis*	2	2[b]	0
		ESBL-producing *P. vulgaris* group	1	1[b]	0
		Other ESBL-producing *Enterobacteriaceae*	3	0	0
Outbreaks in healthcare settings (n = 215)					
Several wards, including rehabilitation centre (n = 29)[d]	2012–2015	CTX-M-15 producing *K. pneumoniae*	29	0	0
Surgical ward (n = 14)	2014	*E. cloacae*	14	0	0
Intensive care unit (n = 86)	2009–2014	Colistin-resistant *E. cloacae*	86	86	0
Nursing home (n = 10)	2012	Colistin-resistant KPC-producing *K. pneumoniae*	10	10	0
ERCP related procedures (n = 50)	2014–2015	Colistin-resistant *K. pneumoniae*	50	43	0
Neonatal intensive care unit (n = 26)[d]	2014–2015	Colistin-resistant *Serratia marcescens*	26	26[b]	0

ERCP: endoscopic-retrograde cholangio-pancreaticography; ESBL: extended-spectrum beta-lactamase; KPC: *Klebsiella pneumoniae* carbapenemase; NA: not available.

[a] The *mcr-1*-positive isolate was tested colistin-resistant with Etest.

[b] Intrinsic resistance.

[c] Two *E. coli* isolates and one *K. pneumoniae* isolate were not available for whole genome sequencing.

[d] Outbreak and subsequent surveillance.

Colistin resistance was defined as a colistin minimum inhibitory concentration (MIC) > 2 mg/L, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints [26].

Table 2

Characteristics of the *mcr-1*-positive *Escherichia coli* isolates from retail chicken meat, the Netherlands, 2009–2015

Isolate	Origin	Date of purchase	Supermarket	MLST	Serotype	Resistance genes	Plasmid replicons
213	Chicken meat	14 October 2009	A	ST2079	O8:H19	*aadA1, aadA2, aadA3, aph(3')-Ia, aph(3")-Ib, aph(3')-Ic, aph(6)-Id, bla*$_{CTX-M-1}$, *bla*$_{TEM-1B}$, *tet*(A), *mcr-1, lnu*(F), *cmlA1, catA1, sul2, sul3, dfrA5*	FIB, FII, HI2, HI2A, I1, I2, P, po111
14M009386[a]	Chicken meat	29 January 2014	B	ST117	O159:H4	*aadA1, bla*$_{SHV-12}$, *mcr-1, sul1, sul3*	FIB, FII
14M009387[a]	Chicken meat	29 January 2014	B	ST117	O159:H4	*aadA1, bla*$_{SHV-12}$, *mcr-1, sul1, sul3*	FIB, FII

MLST: multilocus sequence typing.

[a] Isolate 14M009386 and 14M009387 were cultured from different meat samples with the same lot number.

colistin resistance enables the much more efficient horizontal transfer of colistin resistance genes to other bacteria, making *mcr-1* a potential threat to public health. The aim of this study was to screen several well-documented strain collections of *Enterobacteriaceae*, obtained from retail chicken meat and hospitalised patients in the Netherlands since 2009, for the presence of colistin resistance and the *mcr-1* gene.

Methods

Strain collections

A total number of 2,471 *Enterobacteriaceae* isolates were analysed for the presence of colistin resistance and the *mcr-1* gene. Isolates originated from prevalence surveys in retail chicken meat (196 isolates), prevalence surveys in hospitalised patients (1,247 isolates), clinical cultures (813 isolates) and several outbreaks in healthcare settings (215 isolates), all collected in the Netherlands between 2009 and 2015.

Retail chicken meat

Two ESBL-producing *Enterobacteriaceae* (ESBL-E) prevalence surveys in Dutch retail chicken meat were performed in 2009 and in 2014 [15,16]. A total number of 196 ESBL-E isolates were obtained, 74 isolates from 71 ESBL-E-positive meat samples in 2009 (89 samples cultured), and 122 isolates from 86 ESBL-E-positive meat samples in 2014 (101 meat samples cultured).

Hospitalised patients, rectal samples

The retail chicken meat surveys in 2009 and 2014 were accompanied by hospital-wide prevalence surveys in patients who were admitted to four hospitals in the region where the chicken meat was bought [15,16]. In 2009, ESBL-E rectal carriage was detected in 45 (5.1%) of 876 patients, who carried 50 ESBL-E isolates. Two repeated prevalence surveys in one of the four hospitals in 2013 and 2014, yielded 63 ESBL-E isolates obtained from 63 (5.9%) ESBL-E carriers among 1,065 patients cultured [17].

A multi-centre cluster-randomised study comparing contact isolation strategies for known ESBL-E carriers was performed in 14 Dutch hospitals between 2011 and 2014 (SoM study) [18]. All consecutive adult patients with a routine clinical culture with ESBL-E were placed on contact precautions and enrolled in the study (=index patient). Ward-based ESBL-E prevalence surveys were performed one week after enrolment of the index patient. Perianal swabs were obtained from 10,691 patients and identified 992 (9.3%) ESBL-E carriers, from whom 1,134 ESBL-E isolates were cultured.

Hospitalised patients, clinical cultures

In 2009, 2013 and 2014, all consecutive ESBL-E isolates from blood cultures were prospectively collected in the four hospitals that participated in the ESBL-E rectal carriage prevalence surveys [15,16]. A total number of 102 ESBL-isolates from blood cultures were obtained, 25 isolates from 23 patients with an ESBL-E-positive blood culture in 2009, and 77 isolates from 76 patients in 2013 and 2014. Three isolates that were collected in 2014 were not available for whole genome sequencing. In the SoM study, a total number of 711 clinical ESBL-E isolates were obtained from 654 ESBL-E-positive patients.

Outbreaks in healthcare settings

Since 2009, several outbreaks with antimicrobial-resistant bacteria in Dutch hospitals and nursing homes have been documented. Six outbreaks, comprising 215 isolates, for which whole genome sequence data were available, were included in this analysis: (i) an outbreak of CTX-M-15-producing *K. pneumoniae* in several wards of a hospital and an associated rehabilitation centre in 2012–2015 (29 isolates) [19]; (ii) an outbreak of *Enterobacter cloacae* in a surgical ward in 2014 (14 isolates); (iii) an outbreak of colistin-resistant *E. cloacae* in an intensive care unit between 2009 and 2014 (86 isolates); (iv) an outbreak of colistin-resistant KPC-producing *K. pneumoniae* in a nursing home in 2012 (10 isolates) [20]; (v) an outbreak of colistin-resistant *K. pneumoniae* in patients after endoscopic retrograde cholangio-pancreaticography (ERCP) procedures in

Table 3

Antimicrobial susceptibility of *mcr-1*-positive *Escherichia coli* isolates from retail chicken meat, the Netherlands, 2009–2015

Antimicrobial agent	Isolate					
	213		14M009386		14M009387	
	MIC (mg/L)	S/I/R	MIC (mg/L)	S/I/R	MIC (mg/L)	S/I/R[a]
Polymyxins						
Colistin	3[b]	R	≥16	R	≥16	R
Penicillins						
Ampicillin	≥32	R	≥32	R	≥32	R
Amoxicillin/clavulanic acid	8	S	≤2	S	4	S
Piperacillin/tazobactam	≤4	S	≤4	S	≤4	S
Cephalosporins						
Cefuroxime	≥64	R	16	R	16	R
Cefotaxime	8	R	4	R	4	R
Ceftazidime	≤1	S	16	R	16	R
Cefepime	2	I	≤1	S	≤1	S
Cefoxitin	≤4	S[c]	≤4	S[c]	≤4	S[c]
Carbapenems						
Meropenem	≤0.25	S	≤0.25	S	≤0.25	S
Imipenem	≤0.25	S	≤0.25	S	≤0.25	S
Aminoglycosides						
Gentamicin	≤1.0	S	≤1	S	≤1	S
Tobramycin	≤1.0	S	≤1	S	≤1	S
Fluoroquinolones						
Ciprofloxacin	0.5	S	≤0.25	S	≤0.25	S
Norfloxacin	2	R	≤0.5	S	≤0.5	S
Folate pathway inhibitors						
Trimethoprim/sulfamethoxazol	≥16/304	R	≤1/19	S	≤1/19	S

I: intermediate; MIC: minimum inhibitory concentration; R: resistant; S: susceptible.

[a] According to the European Committee on Antimicrobial Susceptibility (EUCAST) clinical breakpoints [26].

[b] Etest: MIC = 3 mg/L; Vitek2: MIC = 2 mg/L.

[c] No clinical breakpoint available; S refers to the screening breakpoint for AmpC Enterobacteriaceae.

2014–2015 (50 isolates); and (vi) an outbreak of (intrinsic) colistin-resistant *Serratia marcescens* in a neonatal intensive care unit in 2014–2015 (26 isolates).

Whole genome sequencing and analysis of sequence data

Whole genome sequencing (WGS) was performed, on either a MiSeq, HiSeq 2500 or NextSeq sequencer (Illumina). De novo assembly was performed using CLC genomics Workbench 7.0.4 (Qiagen) or the open source SPAdes 3.5.0 software (http://bioinf.spbau.ru/spades) [21]. Sequence data were screened for the presence of the *mrc-1* gene by running the assembled sequences against a task template containing the *mcr*-1 gene sequence in Ridom SeqSphere+version 3.0.1 (Ridom, Germany) or by uploading the assembled sequences to the open access bioinformatic webtool ResFinder (updated version 2.1, including the *mcr-1* sequence) of the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/) [22]. For isolates from two outbreaks (colistin-resistant *E. cloacae* and ERCP-related colistin-resistant *K. pneumonia*), the thresholds for sequence identity and coverage length were set to 98% and 60%, respectively, while for all other isolates both thresholds were set to 80%. The sequence data of the *mcr-1*-positive isolates were further analysed by using a genotyping plugin that allowed serotyping of the isolates and detection of acquired antibiotic resistance genes and plasmids with a 80% threshold for both sequence identity and coverage length (BioNumerics v7.6 beta software, Applied Maths). Reference data for acquired antimicrobial resistance genes and plasmid replicons were retrieved from the ResFinder and PlasmidFinder databases (version 9 November 2015) of the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/) [22,23]. Whole genome multilocus sequence typing (wgMLST) analysis was performed using a pan-genome MLST scheme comprising 9,347 genes, based on 19 well-annotated reference genomes of *E. coli* and *Shigella* spp. (BioNumerics v7.6 beta, Applied Maths). Additionally, single nucleotide polymorphism (SNP) calling was performed by mapping the paired-end reads of isolate 14M009387 and isolate 213 to the de novo assembled genome of isolate 14M009386, using Bowtie 2.5.5 [24] and SAMtools [25]. Resulting Binary Alignment Maps (BAM) files were

Table 4

Whole genome multilocus sequence typing analysis and whole genome single nucleotide polymorphism analysis of *mcr-1*-positive isolates from retail chicken meat, the Netherlands, 2009–2015[a]

Isolate	wgMLST			wgSNP
	Loci shared	Different alleles within shared loci		SNP positions
	n	n	%	n
14M009387	4,243	3	0.07%	8
213	3,791	3,606	95.1%	100,215

MLST: multilocus sequence typing; SNP: single nucleotide polymorphism; wg: whole genome.

[a] Isolate 14M0009386 was used as reference.

used to perform whole genome SNP (wgSNP) analysis (BioNumerics v7.6 beta, Applied Maths).

Antimicrobial susceptibility testing

Isolates for which antimicrobial susceptibility data were available were screened for the presence of colistin resistance. Susceptibility testing of the three *mcr*-1-positive *E. coli* isolates was performed using Vitek2 (bioMérieux, France) and Etest (bioMérieux, France). The breakpoint tables of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were used for the interpretation of minimum inhibitory concentrations (MICs) [26]. Isolates with a colistin MIC›2 mg/L were considered colistin-resistant.

Results

An overview of the 2,471 *Enterobacteriaceae* isolates from retail chicken meat, rectal samples, clinical cultures and outbreaks is presented in Table 1. Colistin resistance was found in two (1.6%) of 122 chicken meat-derived ESBL-E isolates, in 14 (1.1%) of 1,247 isolates from ESBL-E rectal carriers, and in 15 (1.8%) of 813 ESBL-E isolates from clinical cultures. The *mcr*-1 gene was detected in three (1.5%) of 196 chicken meat-derived ESBL-producing *E. coli* isolates, one cultured in 2009 and two in 2014. For all three isolates, the *mcr-1* sequence showed 100% similarity to the gene reported in China [2]. None of the 2,275 human isolates harboured the *mcr-1* gene.

Table 2 shows the general and molecular characteristics of the three *mcr-1*-positive *E. coli* isolates. The isolate that was cultured in 2009 had sequence type ST2079, was CTX-M-1-positive and harboured 17 acquired resistance genes. Both isolates from 2014 had sequence type ST117, were SHV-12-positive and harboured five acquired resistance genes. Although these two isolates were cultured from different meat samples of non-Dutch origin, the meat samples had the same lot number and were bought in the same supermarket on the same day. Plasmid replicons were identified in all three isolates, eight in the isolate from 2009 and two in both isolates from 2014. However, none of the plasmid replicons could be linked to the *mcr*-1 gene.

Antimicrobial susceptibilities for the three *mcr*-1-positive *E. coli* isolates are shown in Table 3. All three isolates were colistin-resistant (MIC›2 mg/L). The isolate from 2009 tested colistin-susceptible by Vitek2 (MIC = 2 mg/L), but resistant by Etest (MIC = 3 mg/L). wgMLST analysis showed that the two isolates from 2014 differed by only three (0.07%) of 4,243 shared loci, whereas the isolate from 2009 differed by 3,606 (95.1%) of 3,791 shared loci (Table 4). The two isolates from 2014 differed by only eight SNPs in wgSNP analysis.

Discussion

In our study, covering the period 2009 to 2015, we detected the recently described plasmid-mediated colistin resistance gene, *mcr-1*, in three ESBL-producing *E. coli* isolates from retail chicken meat samples obtained from Dutch supermarkets in 2009 and 2014. All three *mcr*-1-positive isolates were colistin-resistant, and two of them were genetically closely related. No *mcr-1*-positive isolates were detected in a large collection of *Enterobacteriaceae* isolates of human origin that were collected during the same time period and included isolates of four outbreaks with colistin-resistant *Enterobacteriaceae*.

In addition to the recent reports on the global occurrence of the *mcr-1* gene in *Enterobacteriaceae* cultured from humans, food animals and food [2-13], our findings confirm the presence of the *mcr-1* gene in the European setting already since 2009.

The observed 1.5% prevalence of *mcr*-1-positive isolates is comparable with the reported 2% (5/255) prevalence in imported chicken meat in Denmark, and is lower than the 15% (78/523) prevalence in retail meat in China [2,3]. This lower prevalence may be related to the relatively low rates of polymyxin use in livestock in Europe. In 2014, polymyxins constituted only 0.4% (0.34 defined daily dose animal (DDDA)/animal year) of all antibiotics used in broilers in the Netherlands, with a decreasing trend over the last few years [27].

It is noteworthy that the observed 1.5% prevalence of *mcr-1*-positive isolates in ESBL-E isolates from retail chicken meat in this study is similar to the 1.5% phenotypic colistin resistance that was found in *E. coli* isolates cultured from Dutch retail chicken meat in 2014 [27]. Unfortunately, no data are currently available on the resistance mechanisms involved in this phenotypic colistin resistance.

The genetic identity between the two *mcr-1*-positive isolates that were obtained from the same batch of meat samples most likely represents batch contamination from a common source.

The *mcr*-1-positive isolates in this study belong to different sequence types as compared with those that were found to be related to the *mcr*-1 gene in the Chinese and Danish study [2,3]. *E. coli* ST2097 is uncommon in

humans, but has been reported once before in a study on ESBL-producing bacteria in flies from broiler farms in the Netherlands [28]. *E. coli* ST117, on the other hand, is common in both poultry and humans [16,29]. The detection of the *mcr*-1 gene in isolates that belong to different sequence types illustrates the potential for horizontal transfer of this resistance gene.

Although all chicken meat samples were bought in Dutch supermarkets, the labelling of the samples did not provide any clue with respect to the country where animals were raised. Available data on the origin of the chicken meat were limited to the producing country for the samples from 2014 (non-Dutch, European), for the 2009 isolate this information was not available. A non-European origin of the *mcr-1*-positive meat samples can, therefore, neither be confirmed, nor excluded.

The absence of the *mcr*-1 gene in human isolates of various origins is in accordance with observations in previous studies that the presence of the *mcr-1* gene in clinical isolates is still rare. In China, 1.4% (13/902) of clinical *E. coli* isolates and 0.7% (3/420) of clinical *K. pneumoniae* isolates were *mcr-1*-positive, and in Denmark, only 0.2% (1/417) of ESBL- and AmpC-producing *E. coli* isolates from bloodstream infections [2,3]. This absence of the *mcr-1* gene in current Dutch collections of human *Enterobacteriaceae* may in part be due to the low use of colistin and its analogues, the polymyxins, in humans in the Netherlands. In 2014, polymyxins constituted less than 0.1% (0.01 defined daily dose (DDD)/1,000 inhabitant-days) of all systemic antimicrobials used in primary care and ca 0.3% (0.2 DDD/100 patient-days) of systemic antimicrobials used in the hospital setting [30].

Short-read sequence data are not optimal for the assembly of plasmid sequences, which are known to contain multiple repetitive elements. This may explain why the analysis of our sequence data did not reveal a link between the *mcr-1* gene and the plasmid replicons identified.

Although the prevalence of *mcr-1*-positive isolates in meat samples was low, the presence of this colistin resistance gene in food represents a potential public health threat, as it is located on mobile genetic elements that have the potential to spread horizontally to other bacteria. With the increase in carbapenem resistance, the use of colistin is increasing and, herewith, the selective pressure for the spread of *mcr*-1 gene-containing plasmids. As colistin has become one of the last resort antibiotic options to treat severe infections with Gram-negative bacteria, the continued monitoring of colistin resistance and its underlying resistance mechanisms is important, not only in humans, but also in food production animals and food. The emergence of plasmid-mediated colistin resistance underpins the recent proposal of veterinary experts to reconsider the use of colistin and its analogues in food production animals [31].

In conclusion, the plasmid-mediated colistin resistance gene *mcr*-1 was detected in three ESBL-producing *E. coli* isolates that had been cultured from retail chicken meat from Dutch supermarkets in 2009 and 2014. Two isolates were obtained from the same batch of meat samples, which most likely represents contamination from a common source. The *mcr*-1 gene was not present in a large collection of human isolates collected between 2009 and 2015 in the Netherlands. These findings indicate that *mcr*-1-based colistin resistance currently poses no threat to healthcare in the Netherlands, but requires continued monitoring of colistin resistance and its underlying mechanisms in humans, livestock and food.

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Conflict of interest

Katrien De Bruyne is an employee of Applied Maths, a company that develops and sells software for microbiological typing methods. All other authors have no competing interest to declare.

Authors' contributions

MK, MJMB, JR, PH collected the data, MK, MB, JR and KDB performed the molecular analysis, MK, PH, MJMB, MB, KDB, JR, AF, PS and JK participated in drafting the manuscript, MK coordinated and edited the manuscript.

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Letter to the editor: *Escherichia coli* harbouring *mcr-1* gene isolated from poultry not exposed to polymyxins in Brazil

SA Lentz [1,2], D de Lima-Morales [2,3], VM Cuppertino [1], LdS Nunes [3], AS da Motta [1], AP Zavascki [4], AL Barth [3], AF Martins [1,3]

1. 1. ICBS – Instituto de Ciências Básicas da Saúde, UFRGS - Univ. Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
2. These authors contributed equally to this work.
3. LABRESIS – Lab. de Pesquisa em Resistência Bacteriana, HCPA - Hosp. de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil
4. Infectious Diseases Service, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil

Correspondence: Afonso Luis Barth (albarth@hcpa.edu.br)

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To the editor: The recent paper by Fernandes et al. [1] described the presence of the *mcr-1* gene in *Escherichia coli* from pigs and poultry in Brazil. The authors stated that microbiology laboratories worldwide should be aware of *mcr*-1 isolates resistant to polymyxins in patients living in or returning from Latin American countries and highlighted that the *mcr*-1 gene dissemination results from polymyxins' misuse as growth promoter in food animals. In view of the concerning spread of antibiotic resistance, we screened *E. coli* isolates obtained from a poultry slaughterhouse in southern Brazil with official reports on antibiotic use.

Poultry rectal swabs were collected between August and October 2015. A total of 340 chickens farmed in Brazil and belonging to 17 batches were included in the study. All poultry had received bacitracin, narasin and nicarbazin during a first period of life (between the 2nd and the 18th day) and avilamycin and salinomycin during a second period (between the 20th and 35th day); the chickens of batches 10 and 11 had also received doxycycline during at total of 3 days, in the second period of life. Poultry included in this study were not exposed to polymyxin during their entire life (around 40 days).

A total of 343 isolates were evaluated by polymerase chain reaction (PCR) for the *mcr*-1 gene [2] and 10 (3%) were positive. The *mcr*-1 gene was confirmed by sequencing the PCR amplicon. The *mcr*-1 positive isolates were obtained from 10 different chickens belonging to three batches from three different breeders. The polymyxin B minimum inhibitory concentrations (MIC) of the 10 *mcr*-1 positive isolates were 2 mg/L (8 isolates), 1 mg/L and 0.25 mg/L and they could be classified as susceptible to polymyxin B, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (resistance›2 mg/L). In contrast, most reports indicate that the *mcr*-1 gene is usually found in isolates presenting resistance to polymyxins [2-6].

The mcr-1 positive isolates were submitted to DNA macrorestriction typing by pulsed-field gel electrophoresis (PFGE) and five isolates, from the same batch, proved to be clonally related while the other five isolates were unrelated. Conjugation experiments with the *E. coli* J53 were successful for two *mcr*-1 positive isolates which confirmed that the *mcr*-1 gene was located in a plasmid. The transconjugants presented positive results by PCR for the *mcr*-1 gene and had a polymyxin B MIC of 2 mg/L.

According to Brazilian law, all slaughterhouses must submit in advance to the Federal Inspection Service of the Ministry of Agriculture, the bulletin of health of each batch of animals to be slaughtered. It is of note that the chickens evaluated in this study have received antibiotics as growth promoters, but polymyxins were not included among these compounds. This goes against the hypothesis that the emergence of the *mcr*-1 gene is linked to the use of polymyxins in animal feed in Brazilian livestock [1] and suggests that others compounds or factors may also be involved in the selection of this gene.

Finally, the fact that the *mcr*-1 was originally described in China and thereafter in several other countries including Europe indicates that this gene is already widespread in the world. Therefore, isolates with *mcr*-1 should be considered in any patient, regardless of whether they were living in or returning from Latin America or not.

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Conflict of interest

None declared.

Authors' contributions

Conceived the project: AFM; Managed sample collection: SAML, ASM, AFM; Performed laboratory investigations: SAML, DLM, LSN, VMLC; Drafted the article: DLM; Revised the article: APZ, ALB, AFM.

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Authors' reply: *Escherichia coli* harbouring *mcr-1* gene isolated from poultry not exposed to polymyxins in Brazil

MR Fernandes [1], Q Moura [2], F Esposito [1], N Lincopan [1,2], on behalf of the authors of the original article [3]
1. Department of Clinical Analysis, School of Pharmacy, Universidade de São Paulo, São Paulo, Brazil
2. Department of Microbiology, Institute of Biomedical Sciences, Universidade de São Paulo, São Paulo, Brazil
3. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=22458

Correspondence: Nilton Lincopan (lincopan@usp.br)

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To the editor: The foregoing letter by Lentz et al. examined the occurrence of the *mcr-1* gene in *Escherichia coli* isolates recovered from chicken cloacal swabs collected between August and October of 2015, from a poultry slaughterhouse in southern Brazil [1], providing valuable additional data on the epidemiology of this novel gene. Of 343 animals screened, 10 (3%) different chickens belonging to three flocks from three different breeders were found with *mcr-1* positive *E. coli* isolates [1]. None of these chickens had been reportedly exposed to polymyxins (as growth promoter) [1]. The authors therefore considered their findings as contradicting the plausible hypothesis that the emergence of the *mcr-1* gene is linked to the use of polymyxins in animal feed in Brazilian livestock [2], suggesting that others compounds or factors may also be involved in the selection of this gene.

With regard to this interpretation however, we put forward several points that might be taken into account. Indeed, the investigation by Lentz et al. was conducted as a prospective and short three-month period study, whereby the use of antibiotics other than polymyxins to promote growth might have been part of a transitory change in local agricultural practices, which is not reflected in the whole country. Furthermore, the chickens were only tested for *mcr-1*-harbouring bacteria after 35 days of life, so it is not known if they already had acquired *E. coli* with this gene at a younger stage. For example, if the *mcr-1* gene had been detected already at one day of age, this could have suggested vertical transmission from breeder flocks, as well as the capacity of *mcr-1* positive strains to survive the hatchery process[3]. In addition, retrospective use of colistin, in the studied breeder flocks, was not raised. So, although the authors state that polymyxins were not employed as a growth promoters throughout the study period, the possible use of colistin in the past years, along the poultry production chain, cannot be ruled out. This could explain the polymyxin susceptibility (i.e. polymyxin B minimum inhibitory concentration: MIC ≤ 2 mg/L) exhibited by *mcr-1* positive *E. coli* strains found in the study [1]. In fact, the persistence of a resistance gene may be related to the stability of the plasmid in its host, where the expression of resistance is normally silent until it is induced by antibiotic pressure [4,5]. Moreover, antibiotic-resistant bacteria may also be acquired from external sources, and potentially transferred to current animals, from animals kept at the same location during the previous farming cycle ('carry-over') [3]. In brief, we believe that studies conducted to evaluate the presence of *E. coli* harbouring *mcr-1* gene in poultry not exposed to polymyxins should be preferably addressed in experimental farm settings where antimicrobial exposure is well controlled.

Regardless, valuable information in the letter by Lentz et al. was the identification of more *mcr-1* positive *E. coli* in southern Brazil, which is worrisome, since strains of *E. coli* carrying *mcr-1* have been previously identified in food-producing animals from Minas Gerais, São Paulo, Paraná and Santa Catarina states [2]. Recently, we have also identified the first colistin-resistant *mcr-1* positive *E. coli* isolate from a human infection in Rio Grande do Norte State, north-eastern Brazil (data not shown; GenBank accession number: CP015977). Thus, although data on MCR-1 are currently few, there is supportive evidence that *E. coli* strains carrying *mcr-1* genes are widespread in Brazil in both humans and animals. Currently, to optimise the performance of farming, use of colistin sulfate is allowed within the levels recommended by the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) in diets of food-producing animals [6].

In summary, these results should encourage greater restrictions of colistin in farming systems. Furthermore, the emergence of *mcr-1* positive *E. coli* isolates and their potential spread require very close monitoring and surveillance.

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Conflict of interest

None declared.

Authors' contributions

MRF, QM, FE and NL wrote the letter.

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Hungary

Epinfo (az Országos Epidemiológiai Központ epidemiológiai információs hetilapja)
National Center For Epidemiology, Budapest
Weekly, online. In Hungarian.
http://www.oek.hu/oek.web?to=839&nid=41&pid=7&lang=hun

Iceland

EPI-ICE
Landlæknisembættið
Directorate Of Health, Seltjarnarnes
Monthly, online. In Icelandic and English.
http://www.landlaeknir.is

Ireland

EPI-INSIGHT
Health Protection Surveillance Centre, Dublin
Monthly, print and online. In English.
http://www.hpsc.ie/hpsc/EPI-Insight

Italy

Notiziario dell'Istituto Superiore di Sanità
Istituto Superiore di Sanità, Reparto di Malattie Infettive, Rome
Monthly, online. In Italian.
http://www.iss.it/publ/noti/index.php?lang=1&tipo=4

Bolletino Epidemiologico Nazionale (BEN)
Istituto Superiore di Sanità, Reparto di Malattie Infettive, Rome
Monthly, online. In Italian.
http://www.epicentro.iss.it/ben

Latvia

Epidemiologijas Bileteni
Sabiedribas veselibas agentura
Public Health Agency, Riga
Online. In Latvian.
http://www.sva.lv/epidemiologija/bileteni

Lithuania

Epidemiologijos žinios
Užkreciamuju ligu profilaktikos ir kontroles centras
Center for Communicable Disease Prevention and Control, Vilnius
Online. In Lithuanian.
http://www.ulac.lt/index.php?pl=26

Netherlands

Infectieziekten Bulletin
Rijksinstituut voor Volksgezondheid en Milieu
National Institute of Public Health and the Environment, Bilthoven
Monthly, print and online. In Dutch.
http://www.rivm.nl/infectieziektenbulletin

Norway

MSIS-rapport
Folkehelseinstituttet, Oslo
Weekly, print and online. In Norwegian.
http://www.folkehelsa.no/nyhetsbrev/msis

Poland

Meldunki o zachorowaniach na choroby zakazne i zatruciach w Polsce
Panstwowy Zaklad Higieny,
National Institute of Hygiene, Warsaw
Fortnightly, online. In Polish and English.
http://www.pzh.gov.pl

Portugal

Saúde em Números
Ministério da Saúde,
Direcção-Geral da Saúde, Lisbon
Sporadic, print only. In Portuguese.
http://www.dgs.pt

Romania

Info Epidemiologia
Centrul pentru Prevenirea si Controlul Bolilor Transmisibile, National Centre of Communicable Diseases Prevention and Control, Institute of Public Health, Bucharest
Sporadic, print only. In Romanian.
Sporadic, print only. In Romanian.
http://www.insp.gov.ro/cnscbt/index.php?option=com_docman&Itemid=12

Slovenia

CNB Novice
Inštitut za varovanje zdravja, Center za nalezljive bolezni, Institute of Public Health, Center for Infectious Diseases, Ljubljana
Monthly, online. In Slovene.
http://www.ivz.si

Spain

Boletín Epidemiológico Semanal
Centro Nacional de Epidemiología, Instituto de Salud Carlos III, Madrid
Fortnightly, print and online. In Spanish.
http://revista.isciii.es

Sweden

Folkhälsomyndighetens nyhetsbrev
Folkhälsomyndigheten, Stockholm
Weekly, online. In Swedish.
http://www.folkhalsomyndigheten.se/

United Kingdom

England and Wales

Health Protection Report
Public Health England, London
Weekly, online only. In English.
https://www.gov.uk/government/collections/health-protection-report-latest-infection-reports

Northern Ireland

Communicable Diseases Monthly Report
Communicable Disease Surveillance Centre, Northern Ireland, Belfast
Monthly, print and online. In English.
http://www.cdscni.org.uk/publications

Scotland

Health Protection Scotland Weekly Report
Health Protection Scotland, Glasgow
Weekly, print and online. In English.
http://www.hps.scot.nhs.uk/ewr/

European Union

"Europa" is the official portal of the European Union. It provides up-to-date coverage of main events and information on activities and institutions of the European Union.
http://europa.eu

European Commission - Public Health

The website of European Commission Directorate General for Health and Consumer Protection (DG SANCO).
http://ec.europa.eu/health/

Health-EU Portal

The Health-EU Portal (the official public health portal of the European Union) includes a wide range of information and data on health-related issues and activities at both European and international level.
http://ec.europa.eu/health-eu/

European Centre for Disease Prevention and Control

European Centre for Disease Prevention and Control (ECDC)
The European Centre for Disease Prevention and Control (ECDC) was established in 2005. It is an EU agency with aim to strengthen Europe's defences against infectious diseases. It is seated in Stockholm, Sweden.
http://www.ecdc.europa.eu

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