



Short communication

Occurrence of *mcr-1* in *Escherichia coli* from rabbits of intensive farming

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ABSTRACT

The emergence of mobile colistin resistance genes (*mcr*) is yet another challenge in the fight against antimicrobial resistance, with reports proving the dissemination of these genes in different countries and different environments being of great concern. In the present study, we describe the recovery of three *E. coli* strains with *mcr-1* gene in InchI2 plasmids from intestinal content of necropsied meat rabbits reared in two intensive production systems in Portugal. Our findings are worrisome, given the high level of dependence on the usage of antibiotics in rabbit rearing and call for the development and implementation of an active surveillance system in this species.

1. Introduction

The continuous increase in antimicrobial resistance of Gram-negative bacteria combined with a lack of new antibiotics triggered the reintroduction of nephrotoxic and neurotoxic colistin into human medicine (Poirel and Nordmann, 2016). What was once a discontinued antibiotic, is now a last-resort antimicrobial classified by WHO as one of the “highest priority critically important for human medicine” (World Health Organization (WHO), 2017). However, the emergence of mobile colistin resistance genes (*mcr*) may undermine the usefulness of this antibiotic, with reports from nearly all over the world documenting the dissemination of *mcr* genes in isolates obtained from food-producing animals, food, humans and the environment (Sun et al., 2018).

Even though colistin was discontinued in human medicine in the 1980's, its use has been kept in veterinary medicine. In fact, some authors have linked the probable origin of *mcr-1* to pigs (Kieffer et al., 2017). Therefore, there is a need for ongoing animal surveillance to monitor colistin usage and for early identification of colistin resistance threats.

This communication describes the recovery of three MCR-1 producing *Escherichia coli* strains from rabbits reared in intensive production systems in Portugal. The isolates were recovered in 2015 and 2016.

2. Materials and methods

In the routine of the Microbiology and Food Technology Laboratory of ICBAS, University of Porto, three *E. coli* isolates displaying colistin resistance were recovered from intestinal content of necropsied meat rabbits collected in two commercial farms. All animals had a clinical history of enteric disease. The stockmanship was intensive, and the number of rabbits in each farm ranged from 7000 to about 25,000 commercial hybrids.

One isolate (1410/1) was recovered in January 2015 from an 18-day-old kit from a pen fed with a commercial diet medicated with 0.2 g/kg of neomycin, 0.25 g/kg of sulfadimethoxine and 0.025 g/kg of trimethoprim. Isolates designated as 2251/2 and 2252 were obtained in October 2016 from a 20-day-old kit fed with a diet containing colistin (0.08 g/kg), oxytetracycline (0.4 g/kg) and tilmicosin (100 g/kg), and a 42-day-old kit reared in the same farm and fed with a diet medicated with 0.1 g/kg of colistin, 0.5 g/kg of oxytetracycline and 0.03 g/kg of valnemulin, respectively.

All isolates were recovered from ileal content directly cultured on Tryptone Bile X-glucuronide agar (TBX; Biokar Diagnostics, Allonne, Beauvais, France).

Antimicrobial susceptibility testing was performed as recommended by the Clinical and Laboratory Standards Institute (Clinical and

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Laboratory Standards Institute (CLSI, 2017), using the Kirby-Bauer method on Mueller-Hinton agar (Biokar Diagnostics, Beauvais, France), and by determining minimal inhibitory concentrations (MIC) for colistin (Sigma-Aldrich, Saint Louis, Missouri, USA) using the broth microdilution method in cation-adjusted Mueller-Hinton broth (Sigma-Aldrich, Saint Louis, Missouri, USA).

Bacterial DNA extraction was carried out using the InstaGene™ matrix (Bio-Rad, Hercules, California, USA), according to the manufacturer's instructions. Phylo-groups were determined using the Clermont PCR-based method (Clermont et al., 2013) and sequence types (STs) were assigned using the multilocus sequence typing database for *E. coli* (https://enterobase.warwick.ac.uk/species/ecoli/allele_st_search). The isolates were screened for *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5* genes by PCR (Liu et al., 2016; Xavier et al., 2016; Borowiak et al., 2017; Carattoli et al., 2017; Yin et al., 2017). Positive results were confirmed by Sanger sequencing and compared with those in the NCBI database GenBank® using the Nucleotide BLAST® search tool (Altschul et al., 1990). The location of *mcr-1* was determined with S1-PFGE of total DNA followed by Southern blot hybridization using alkaline phosphatase-labelled probes targeting *mcr-1* and plasmid replicon type IncHI2 (Campos et al., 2016). Plasmid double locus sequence typing for IncHI2 plasmids was performed, and STs were assigned using the plasmid MLST database (<https://pubmlst.org/plasmid/>).

Conjugation assays were performed to assess the transmissibility of *mcr-1* gene to *E. coli* Hb101 recipient strain (resistant to sodium azide, streptomycin and kanamycin; colistin MIC 0.125 mg/L) as follows: donor and recipient strains were cultured overnight in Luria-Bertani broth (Sigma-Aldrich, Saint Louis, Missouri, USA) and were subcultured in fresh Luria-Bertani broth to reach logarithmic phase the following day. Donor and recipient strains were mixed at a ratio of 1:1 and 100 µL were transferred onto 0.45 µm nitrocellulose filters on Luria-Bertani agar plates, and incubated overnight at 37 °C. After incubation, the filters were resuspended in 0.85% NaCl, and 100 µL were plated on Luria-Bertani agar plates, Luria-Bertani plates supplemented with 100 mg/L sodium azide (Merck) and in Luria-Bertani plates supplemented with 100 mg/L sodium azide and 1 mg/L colistin.

3. Results

The three *E. coli* isolates from meat rabbits were resistant to colistin, with MIC ranging from 4 to 8 mg/L. Importantly, all three isolates were resistant to different clinically relevant antibiotic families (Table 1). Isolate 2251/2 was assigned to phylo-group A and ST206, while isolates 1410/1 and 2252 were assigned to phylo-group B1 and ST1589 and ST1431, respectively. Screening for colistin resistance genes revealed that all isolates were only positive for *mcr-1* gene. By means of S1-PFGE and Southern blot hybridization, *mcr-1* was located in IncHI2 plasmids with sizes ranging from 200 to 300 Kb and non-transferable by conjugation assays. Plasmid MLST for IncHI2 plasmids revealed ST2 in isolate 1410/1 and ST4 for the isolates 2252 and 2251/2 recovered

from the same farm (Table 1).

4. Discussion

Most of the *mcr-1* carrying *E. coli* have been isolated from pigs, poultry, food and clinical samples (Schwarz and Johnson, 2016). This is the first report of *mcr-1* carrying *E. coli* in meat rabbits in Portugal. To our knowledge, the only previous report of *mcr-1* gene in rabbits was from a study carried out in Italy (Agnoletti et al., 2018).

Isolates 2251/2 and 2252 were recovered from samples of kits fed with a commercial diet medicated with colistin, yet, isolate 1410/1 was retrieved from a rabbit not exposed to colistin. Nevertheless, it was an 18-day-old kit sharing its cage with a lactating doe fed with commercial diet medicated with 0.1 g/kg of colistin and 0.08 g/kg of tilmicosin. High stocking density is known to enable multidrug resistance spreading and this might partially explain our findings (da Costa et al., 2013).

Sequence typing showed that all three isolates belonged to different STs, even isolates 2251/2 and 2252, which were isolated from samples collected in the same farm. MCR-1-producing *E. coli* ST206 strains were first isolated in China in environmental water samples (Zhou et al., 2017), but have been isolated in human clinical samples as well (Luo et al., 2017; Zheng et al., 2018). ST1431 strains have already been identified in turkey meat samples originated from Germany, and this ST has reportedly been associated with the dissemination of extended-spectrum β-lactamase CTX-M-1 and carbapenemase OXA-48 in humans, companion animals and poultry (Zurfluh et al., 2016).

We were able to locate *mcr-1* in IncHI2 plasmids, an incompatibility type associated with the global spread of *mcr-1* (Matamoros et al., 2017). Plasmid MLST showed that isolate 1410/1 carries an IncHI2/ST2 plasmid, whereas isolates 2251/2 and 2252 carry an IncHI2/ST4. Both STs have been associated with *mcr-1*-harbouring IncHI2 plasmids (Veldman et al., 2016), including in Portugal, where IncHI2/ST4 plasmids have been described in *Salmonella* strains isolated from pork and humans (Campos et al., 2016).

None of the plasmids described herein were transferable in the conjugation assays performed. Even though IncHI2 plasmids are often transferable, non-transferable IncHI2 *mcr-1*-harbouring plasmids have been previously described in Portugal (Campos et al., 2016).

Since antibiotics were banned as growth promoters, an increase in the use of antimicrobials for therapeutic purposes has been reported (Hao et al., 2014). Rabbits have a digestive physiology that is very different from other livestock species and has not been able to adjust to a diet with high nutritional density nor to the stress inherent to intensive farming. This feature has driven their rearing towards a high level of dependence on the usage of antibiotics, including colistin, to manage their fragile intestinal microbiota, with intestinal health problems being the leading cause of impaired performance in growing rabbits (De Blas, 2013; Cunha et al., 2017). This is a practice that has to be revised and the need for antibiotics should be compensated with

Table 1

Characterization of *mcr-1*-carrying colistin-resistant *E. coli* isolates recovered from feces of rabbits in Portugal.

Strain	Farm	Phylo-group	ST (CC) ^a	Plasmid-mediated colistin resistance		Additional antibiotic resistance phenotype ^d
				Colistin MIC ^b , (mg/L)	Plasmid type carrying <i>mcr-1</i> gene (pMLST, Kb) ^c	
1410/1	I	B1	ST1589	8	HI2 (ST2, 298)	AMP, GEN, TOB, STR, TET, CIP, NAL, CHL
2251/2	II	A	ST206 (CC206)	4	HI2 (ST4, ND)	AMP, GEN, TOB, STR, TET, DOX, CIP, NAL, SXT, CHL
2252	II	B1	ST1431	8	HI2 (ST4, 200)	AMP, GEN, TOB, STR, TET, CIP, NAL, SXT, CHL, NIT

^a ST, sequence type; CC, clonal complex; as determined by Multilocus Sequence Typing.

^b MIC, minimal inhibitory concentration.

^c ND, non-determined; pMLST, plasmid multilocus sequence typing. In isolate 2251/2 *mcr-1*-carrying IncHI2 plasmid was only characterized by pMLST.

^d AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; DOX, doxycycline; GEN, gentamycin; NAL, nalidixic acid; NIT, nitrofurantoin; TET, tetracycline; TOB, tobramycin; STR, streptomycin; SXT, trimethoprim-sulfamethoxazole; other antibiotics tested included amikacin, amoxicillin-clavulanic acid, aztreonam, ceftazidime, cefotaxime, cefoxitin, ceftazidime, cephalotin, imipenem. All discs were provided by Oxoid (Basingstoke, Hampshire, UK).

alternative strategies such as post-weaning intake limitation, which has already been proven to reduce morbidity and mortality rates due to digestive disorders, reducing the need for antibiotics and, ultimately, improving the profitability of rabbit rearing (Gidenne et al., 2012). In fact, the usage of antibiotics constitutes a selective pressure for the acquisition of antimicrobial resistance genes (Garcia-Migura et al., 2014), with food-producing animals playing a pivotal role in their epidemiology (Agnoletti et al., 2018).

In Europe, in 2015, Portugal ranked seventh in the overall sales of antibiotics for veterinary medicine, fifth in the consumption of polymyxins, with sales in mg per population correction unit (PCU) being almost twice the European average (European Medicines Agency (EMA), 2017). However, rabbits represent the smallest population of food-producing species in Portugal (expressed in PCU) (European Medicines Agency (EMA), 2017), and there is no data available for their medication with colistin in national reports, since their sales are expressed in tonnes (Direcção Geral de Alimentação e Veterinária (DGAV), 2016). Nonetheless, their consumption of antibiotics is amongst the highest in food-producing animals, and the number of antimicrobials approved for usage in this species is limited, which calls for a better understanding of their role in the epidemiology of antimicrobial resistance (Agnoletti et al., 2018).

5. Conclusion

In Portugal, The National Plan for the Reduction of Antibiotics Usage in Animals was implemented in 2014, and colistin sales dropped 31% in 2015 (Direcção Geral de Alimentação e Veterinária (DGAV), 2013; European Medicines Agency (EMA), 2017). Nevertheless, sales of antimicrobials belonging to this class is an issue that requires further attention due to its relevance in human medicine.

The presence of three *mcr-1* carrying *E. coli* isolates belonging to three different clonal lineages in meat rabbits is worrisome and suggests that this might well be the tip of the iceberg. The findings described in this communication resulted of a routine diagnostic activity encompassing 13 samples collected from five commercial farms. Further studies are needed to fully determine *mcr-1* carrying *E. coli* prevalence in these and other farming systems. Notwithstanding, these call for the development and implementation of an active surveillance system in rabbits and for the banning of colistin from animal rearing.

Declaration of interest

None.

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