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Revealing *mcr-1*-positive ESBL-producing *Escherichia coli* strains among *Enterobacteriaceae* from food-producing animals (bovine, swine and poultry) and meat (bovine and swine), Portugal, 2010–2015

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ABSTRACT

We screened 1840 *Enterobacteriaceae* isolates from food-producing animals, meat, meat products and animal feed, for the detection of plasmid-mediated colistin resistance, during 2010–2015. The *mcr-1* gene was detected in 8.0% (97/1206) *Escherichia coli* and in 0.47% (3/634) *Salmonella enterica* isolates, with a high number of *mcr-1* positive *E. coli* isolates (45.7%) being extended-spectrum β-lactamase or plasmid-mediated AmpC β-lactamase co-producers. No *mcr-2* gene was detected. Our findings highlight the spread of *mcr-1* genes within a wide-ranging sample of food-producing animals and meat, in Portugal.

1. Introduction

The increasing prevalence of human infections caused by extended-spectrum β-lactamases (ESBLs) and carbapenemases among multidrug-resistant (MDR) Gram negative bacteria, including *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, has resulted in a renewed interest in colistin, considered to be the last resort therapeutic action (Catry et al., 2015). On the other hand, colistin has been widely used for decades in the veterinary practice, for prevention and treatment of gastrointestinal infections caused by *Enterobacteriaceae* in food-producing animals (Patersen and Harris, 2015).

Following the original report of plasmid-mediated colistin resistance (PMCR) in China by the end of 2015 (Liu et al., 2016), several studies in different countries reported a worldwide distribution of the *mcr-1* gene in *Enterobacteriaceae* isolates from humans, food and companion animals, meat and environment (Poirel et al., 2017). Since then, seven novel variants were detected. The *mcr-2* gene was found in colistin resistant *Escherichia coli* isolates from sick calves and piglets in Belgium (Xavier et al., 2016); *mcr-3* was identified in *E. coli* isolates from cattle in Spain, swine in China and a human bloodstream infection in Denmark (Hernández et al., 2017; Roer et al., 2017; Yin et al., 2017).The *mcr-4* was detected in *Salmonella enterica* serotype [4,5,12:i:-] from a healthy swine in Italy (Carattoli et al., 2017); and *mcr-5* in *Salmonella* Paratyphi B dTa + originating from food-producing animals and food products, in Germany (Borowiak et al., 2017). More recently, in China, *mcr-7* was described in *Klebsiella pneumoniae* strains recovered from chickens (Yang et al., 2018); and *mcr-8* was noted in NDM-producing *K. pneumoniae* from both food-producing animals and human clinical samples (Wang et al., 2018). The *mcr-6.1* gene was recently annotated and deposited into GenBank (NG_055781). Meanwhile, several gene variants of the phosphoethanolamine transferase enzyme MCR-1 have been described (MCR-1.2 to MCR-1.12), differing from MCR-1 at a single amino acid (Di Pilato et al., 2016; Lu et al., 2017; Tijet et al., 2017; Yang et al., 2017).

More worrisome is the presence of *mcr* genes in *Enterobacteriaceae* carrying other resistance determinants namely, ESBL and/or carbapenemase genes. The first report of co-localization of *mcr-1* and ESBL genes dates to 2006 (Haenni et al., 2016a). Since then, an increase on the proportion of *mcr-1* genes among ESBL-producing *E. coli* in animals, has been noticed and clearly differing from the low prevalence of *mcr-1* in non-ESBL-producing *E. coli* strains, suggesting that the use of extended-spectrum cephalosporins may have simultaneously favoured the

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spread of *mcr-1* (Haenni et al., 2016a; Perrin-Guyomard et al., 2016).

Currently, carbapenemases are still rare in animals. However, the finding of two swine in Italy housed at the same farm, revealed the detection of $bla_{OXA-181}$ associated to *mcr-1*, suggesting that livestock may become a relevant reservoir of strains conferring resistance to last-line antibiotics, including polymyxins, β-lactams and carbapenems (Pulss et al., 2017).

The purpose of the present study was to investigate the presence of PMCR-encoding genes, *mcr-1* and *mcr-2*, in a collection of *Enterobacteriaceae* isolates from food-producing animals, meat, meat products and animal feed within the period 2010–2015.

2. Material and methods

2.1. Bacterial isolates

This study was conducted in Portugal and included 1206 *E. coli* isolates selected and recovered from cecum samples [bovine (n = 350), swine (n = 398) and poultry (n = 387)] collected at slaughter, 51 meat samples [bovine (n = 12) and swine (n = 39)] collected at retail stores, and 20 clinical samples collected from food-producing animals, either stool specimens from sick animals or, intestinal contents from deceased animals.

Additionally, 634 *Salmonella enterica* isolates recovered from animal faeces and faecal environmental samples (n = 388) collected at poultry houses, bovine faeces (n = 5) collected at slaughter, animal feed (n = 13) collected at feed mills and, meat samples and meat derived products (n = 228) from bovine, swine and poultry collected at retail stores, were analysed. All isolates were kept frozen at −80 °C, revived on Tryptose Soya Agar plates (Oxoid, UK), before being submitted to antimicrobial susceptibility testing.

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of 1206 *E. coli* and 634 *S. enterica* isolates from food-producing animals, meat and animal feed (Tables 1 and 2) was studied. Minimum Inhibitory Concentrations (MIC) of colistin, cefotaxime, ceftazidime and meropenem (Glentham Life Sciences, UK) were determined using the agar dilution method (CLSI, 2015). At the time of the study, this method was one of the standard methods for susceptibility testing towards colistin (CLSI, 2015; Jerke et al., 2016), with results comparable to the currently recommended broth dilution in terms of reproducibility and robustness (Turlej-Rogacka et al., 2018). Results were interpreted according to the epidemiological cut-off values of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, http://mic.eucast.org/Eucast2/). Non-wild type isolates towards cefotaxime and ceftazidime, were tested for the phenotypic detection of extended-spectrum β-lactamases (ESBL) and plasmid-mediated AmpC β-lactamases (PMAβ), using the microdilution technique in commercial standardized microplates (EUVSEC2, TREK, USA), accordingly to manufacturer's instructions.

2.3. Detection of PMCR-encoding genes

For genotypic testing, all DNAs were extracted using a boiling method. Colistin-resistant *E. coli* and *S. enterica* isolates were screened for the presence of PMCR-encoding genes (*mcr-1* and *mcr-2*), using a multiplex PCR with primers and conditions previously described (Cavaco et al., 2016) from EU Reference Laboratory for Antimicrobial Resistance (EURL-AR, http://eurl-ar.eu/), followed by sequencing of the amplicons on both strands using automatic sequencer ABI3100 (Applied Biosystems, Warrington, UK). Template DNAs from National Reference Laboratory for Antibiotic Resistance collection were used as positive control in all PCR reactions.

2.4. Identification of β-lactamase-encoding genes

The isolates evidencing non-susceptible phenotype to cefotaxime, ceftazidime and/or cefoxitin were screened by PCR for the presence of *bla* (bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{CTX-M}, bla_{CMY}, bla_{MOX}, bla_{FOX}, bla_{LAT}, bla_{ACT}, bla_{MIR}, bla_{DHA}, bla_{MOR}, bla_{ACC}) genes, as previously described (Jones-Dias et al., 2016a).

E. coli chromosomal *ampC* gene, including its promoter region, was also analysed by PCR by using Int-B2 and Int-H1 primers for the five strains with no ESBL- and/or PMAβ-encoding gene detected (Mammeri et al., 2006). Chromosomal AmpC from *E. coli* ATCC 25922 was used as standard. Positive and negative controls were used in all PCR reactions. PCR products were purified, and all amplicons, except those obtained for TEM-family, were further sequenced directly on both strands using automatic sequencer ABI3100 (Applied Biosystems, Warrington, UK).

2.5. Nucleotide sequence accession number

The new *mcr-1.9* nucleotide sequence was submitted with accession number KY780959.

2.6. Statistical analysis

OpenEpi software, version 3.01 (Dean et al., 2013) was used for statistical analysis. Fisher exact test was used to assess differences in antibiotic nonsusceptibility between different groups. One-tailed P values of < 0.05 were considered statistically significant. Associations were determined by calculation of odds ratios with 95% confidence intervals. This analysis identified variables significantly and independently associated with colistin nonsusceptibility and/or production of MCR-1-type.

3. Results and discussion

In this study, from 1206 *E. coli* and 634 *S.* enterica isolates, reduced susceptibility to colistin was observed in 8.6% (103/1206) and 5.5% (35/634), respectively (Tables 1 and 2). Indeed, in our study, *E. coli* constituted a risk factor (OR = 1.598, 95% CI: 1.064–2.449), for the occurrence of colistin resistance (P = .011) when compared to *Salmonella* spp.

Overall, we detected *mcr-1*-like genes in 100 colistin-resistant *Enterobacteriaceae* isolates (*E. coli*, n = 97 and *S. enterica*, n = 3) (Tables 1 and 2). All amplicons except one, exhibited a sequence with 100% homology to the previously described *mcr-1* (Liu et al., 2016); one amplicon (of the commensal CTX-M-8- and TEM-type-producing LV23529 isolate collected from a swine), hereafter named *mcr-1.9* (KY780959), differed from *mcr-1* by one-point mutation (T1238C), leading to Val413Ala substitution.

None of our isolates were positive for *mcr-2* gene, which is in accordance with other studies (Kieffer et al., 2017; Roschanski et al., 2017). On the contrary, Xavier et al. (2016) in Belgium detected a higher frequency of *E. coli mcr-2*-harboring isolates from diseased piglets, compared to *mcr-1* and more recently, in China, in both swine and poultry (Zhang et al., 2018). In our study, although not statistically significant, 94.1% (1135/1206; P = .423) of the *E. coli* isolates investigated were from healthy animals (i.e. excluding clinical cases and meat samples).

To our knowledge, this was the most wide-ranging study conducted in Portugal, reporting the occurrence of *mcr* genes in *E. coli* isolates from turkeys (27.0%, 50/185; $P \leq .001$), swine (10.1%, 40/398; P = .052), and swine meat (5.1%, 2/39; P = .569), as well as, from broilers (2%, 4/202; P ≤ .001), never reported before in the country.

In Portugal, PMCR had been detected in different *Enterobacteriaceae* species isolated from humans, food-producing animals and meat, and in the environment (Campos et al., 2016; Figueiredo et al., 2016; Jones-Dias et al., 2016b; Kieffer et al., 2017; Manageiro et al., 2017; Mendes

Table 1
Escherichia coli (n = 1206), from food-producing animals, meat and meat products.

Isolate origin/Animal species	Year	Colistin R/Total isolates tested (%)	MIC[a] range (μg/mL)	*mcr* positive isolates/total colistin R (%)	MIC range (μg/mL)	Frequency of *mcr* positive isolates (%)	Frequency of ESBL and/or PMAβ/*mcr* positive isolates (%)	Genetic profile of *bla* and *ampC* genes
Clinical cases								
Food animals	2010–2013	2/20 (10%)	8–16	2/2 (100%)	8–16	2/20 (10%)	0/2	
Cecum samples								
Broilers	2014	6/202 (3%)	8–16	4/6 (66.7%)	8–16	4/202 (2%)	0/4	
Turkeys	2014	50/185 (27%)	8– > 16	50/50 (100%)	8– > 16	50/185 (27%)	2/50 (4%)	bla_{TEM}, bla_{SHV-12} (n = 1) $ampC$ (mutation in promotor) (n = 1)
Bovine	2015	0/350						
Swine	2015	42/398 (10.6%)	8– > 16	40/42 (95.2%)	8– > 16	40/398 (10.1)	38/40 (95%)	bla_{TEM}, $bla_{CTX-M-1}$ (n = 11) $bla_{CTX-M-1}$ (n = 1) bla_{TEM}, $bla_{CTX-M-8}$ (n = 1)[b] bla_{TEM}, $bla_{CTX-M-14}$ (n = 5) bla_{TEM}, $bla_{CTX-M-27}$ (n = 1) bla_{TEM}, $bla_{CTX-M-32}$ (n = 13) bla_{TEM}, bla_{SHV-12} (n = 2) $ampC$ (mutation in promotor) (n = 1) $ampC$ (n = 3)
Meat samples								
Bovine	2015	0/12						
Swine	2015	3/39 (7.7%)	8–16	2/3 (66.7%)	16	2/39 (5.1%)	2/2 (100%)	bla_{TEM}; $bla_{CTX-M-1}$ (n = 2)
Total		103/1206 (8.5%)		98/103 (95.1%)		98/1206 (8.1%)	42/98(45.7%)	

[a] Minimum Inhibitory Concentration.
[b] *mcr-1.9* gene positive isolate.

Table 2
Salmonella spp. (n = 634), from food-producing animals, meat, meat products and animal feed.

Isolate origin/Animal species	Serotypes	Year	Colistin R/Total isolates tested (%)	MIC[a] range (μg/mL)	*mcr* positive isolates/total colistin R (%)	MIC range (μg/mL)	Frequency of *mcr* positive isolates (%)
Faeces/Environment							
Poultry	Enteritidis	2013–2015	11/47 (23.4%)	4–8	0/11		
	Typhimurium	2014–2015	1/11 (9.1%)	8	0/1		
	4,5,12:i:-	2014–2015	0/5				
	Other	2011–2015	1[b]/325 (0.3%)	> 16	0/1		
Bovine	Typhimurium	2014–2015	0/1	4–16			
	4,5,12:i:-	2014–2015	0/4				
Animal feed	Enteritidis	2014–2015	6/6 (100%)	4–16	0/6		
	Other	2014–2015	0/7				
Meat and meat products	Enteritidis	2014–2015	6/20 (30%)	4–8	0/6		
	Typhimurium	2014–2015	0/37				
	4,5,12:i:-	2014–2015	6/54 (11.1%)	8–16	2/6 (33.3%)[c,e]	8–16	2/54 (3.7%)
	Other	2014–2015	4/117 (3.4%)	4– > 16	1/4 (25%)[d,e]	8	1/117 (0.9%)
Total			35/634 (5.5%)		3/35 (8.6%)		3/634 (0.47%)

[a] Minimum Inhibitory Concentration.
[b] *S.*Havana.
[c] Bovine and swine meat.
[d] *S.* Reading (swine meat).
[e] Non-ESBL or non-PMAβ producers.

et al., 2018; Tacão et al., 2017).

In our study, fattening turkeys showed a higher frequency of colistin resistant and *mcr*-positive *E. coli* isolates (P ≤ .001). Regarding fattening swine and broilers, colistin resistance occurred at relatively lower levels, but remaining relevant (particularly in swine) as reported in other studies (EFSA, 2017; Alba et al., 2018). Therefore, fattening turkeys constituted a risk factor for the existence of isolates resistant to colistin (OR = 6.75, 95% CI: 4.31–10.58); in contrast, fattening broilers were negatively associated with colistin resistance (OR = 0.286, 95% CI: 0.101–0.660).

Additionally, 2/20 (10.0%; P = .493) MCR-1-producing *E. coli* isolates were detected among clinical cases of food-producing animals (Table 1). Of note is the first detection of an *E. coli* isolate from a diseased swine already in 2010, suggesting a long-term and silent dissemination of this resistance gene in animals in the country.

In this respect, the selection pressure exerted by the overall high use of colistin in the meat production, mainly in swine and poultry sectors, might have played a major role. Portugal is the fourth European country following Spain, Italy and Croatia, with a higher level of sales of colistin (a peak in 2013), and the third country regarding consumption of colistin in food-producing animals (ECDC/EFSA/EMA, 2017; EMA/ESVAC, 2017).

No colistin-resistant *E. coli* isolates were detected in bovine animals and bovine meat (P ≤ .01); these findings agree with some studies (EFSA, 2017), though contradicting others, reporting a higher frequency of *mcr-1*-positive isolates from veal calves, particularly ESBL-producing isolates (Haenni et al., 2016a; Haenni et al., 2016b; Xavier et al., 2016).

Worryingly, we observed that forty-two (45.7%) MCR-1-producing *E. coli* isolates from swine (n = 38), turkeys (n = 2) and swine meat (n = 2), evidenced a non-susceptible phenotype to cefotaxime, ceftazidime and/or cefoxitin and were ESBL or PMAβ co-producers: $bla_{\text{CTX-M-1}}$, n = 14; $bla_{\text{CTX-M-32}}$, n = 13; $bla_{\text{CTX-M-14}}$, n = 5; $bla_{\text{CTX-M-27}}$, n = 1; $bla_{\text{SHV-12}}$, n = 3; $bla_{\text{CMY-2}}$, n = 3 (Table 1). Among ESBL isolates, $bla_{\text{CTX-M-1}}$ gene, was the most prevalent, which is in line with other European studies (Schill et al., 2017), followed by $bla_{\text{CTX-M-32}}$, very common in piggeries in Portugal (Ramos et al., 2013; Rodrigues et al., 2017). Haenni et al., 2016a verified that the proportion of *mcr-1*-positive ESBL-producing *E. coli* strains has been increasing, clearly differing from the low *mcr-1* prevalence in non-ESBL-producing *E. coli* strains (Haenni et al., 2016a). Indeed, selection pressure exerted by broad-spectrum cephalosporins and other antimicrobials may enhance the rapid dissemination of PMCR and vice-versa (Haenni et al., 2016a; Haenni et al., 2016b).

Regarding *S. enterica*, *mcr-1* gene was detected in 0.47% (3/634) of *S. enterica* isolates: in two *S.* 4,5,12:i:- from bovine and swine meat (P = .020) and one *S.* Reading from swine meat (P = .458) (Table 2). This is, at our knowledge, the first description of a MCR-1-producing *S.* Reading isolate, which highlights the facility of dissemination of this antibiotic resistance mechanism intra- and inter-species. In Portugal, PMCR had been detected in *S.* Typhimurium, *S.* Rissen and *S.* 1,4,[5],12:i:- isolates collected from humans and food-producing animals (Campos et al., 2016; Figueiredo et al., 2016).

Indeed, the presence of colistin resistance gene in food represents a potential public health threat, as it is located in mobile genetic elements that have the potential to spread horizontally. All isolates studied remained wild type to meropenem, corroborating that carbapenem-resistant *Enterobacteriaceae* are not commonly found in bacteria from non-human sources, despite its globally occurrence in livestock, pets, wildlife, and seafood (Köck et al., 2018).

Results obtained in this study support that meat-producing animals intensively farmed (turkeys, swine, broilers, and veal calves) are at risk of being largely exposed to colistin and promptly spread the plasmid-mediated *mcr* gene. Worryingly, animal feed constituted a risk factor for the existence of colistin-resistant *Salmonella* isolates (OR = 17.26, 95% CI: 4.491–64.41, P ≤ .001). As polymyxins are becoming a last resort antimicrobial for use in humans, National Competent Authorities and the farming industry are now taking measures to reduce the use of colistin in food-producing animals, mainly in turkeys and swine, following the recommendations of the European Medicines Agency (EMA/CVMP/CHMP, 2016). In Portugal, since 2013, there has been a decrease in sales (mg/PCU) (population correction unit) of polymyxins, dropping to 12.13 mg/PCU (−31% compared to the previous year) in 2015 (ESVAC, 2017), which is still above the maximum level of 5 mg colistin/PCU (EMA/CVMP/CHMP, 2016). In parallel, a five-year National Action Plan for the Reduction of Use of Antibiotics in Animals was initiated on 1st January 2014 to promote the prudent use of antimicrobials and to raise awareness about antimicrobial resistance.

4. Conclusions

In this study, we described a new MCR-1 variant (MCR-1.9) in an *E. coli* from a swine, among *Enterobacteriaceae* isolates from food-producing animals, meat, meat products and animal feed, screened for the

detection of plasmid-mediated colistin resistance. Our findings highlight the spread of *mcr-1* genes within food-producing animals and meat in Portugal (detected in 8% of *E. coli* and in 0.5% of *S. enterica* isolates), and a high number of *mcr-1* positive *E. coli* isolates (45.7%) that were ESBL or PMAβ co-producers.

These findings add new concerns to the rapid emergence of PMCR in different countries.

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Conflict of interest

None declared.

Ethical approval

None required.

Authors' contributions

LC performed microbiological and molecular experiments, analysed the data and wrote the manuscript. VM performed molecular experiments and bioinformatics analysis, interpreted other data and wrote the manuscript. EF performed microbiological and molecular experiments. IC, AA, PT and TA acquired laboratory data and performed microbiological experiments. MC designed the study, wrote, reviewed and edited the manuscript. All authors read and approved the final manuscript.

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