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ORIGINAL ARTICLE

Phenotypic and genotypic resistance to colistin in *E. coli* isolated from wild boar (*Sus scrofa*) hunted in Italy

Giovanni Cilia[1] · Barbara Turchi[1] · Filippo Fratini[1] · Valentina Virginia Ebani[1] · Luca Turini[1] · Domenico Cerri[1] · Fabrizio Bertelloni[1]

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Abstract
The One Health approach is not only focused on diseases and zoonosis control but also on antimicrobial resistance. As concern this important issue, the problem of plasmid-mediated colistin resistance recently emerged. Few studies reported data about colistin resistance and *mcr* genes in bacteria from wildlife. In this manuscript, 168 *Escherichia coli* isolated from hunted wild boar were tested; colistin resistance was evaluated by MIC microdilution method, and the presence of *mcr-1* and *mcr-2* genes was evaluated by PCR. Overall, 27.9% of isolates resulted resistant to colistin, and most of them showed a MIC value > 256 µg/mL. A percentage of 44.6% of tested *E. coli* scored positive for one or both genes. In details, 13.6% of isolated harbored *mcr-1* and *mcr-2* in combination; most of them exhibiting the highest MIC values. Interestingly, 19.6% of *mcr*-positive *E. coli* resulted phenotypically susceptible to colistin. Wild boar could be considered a potential reservoir of colistin-resistant bacteria. In the light of the possible contacts with domestic animals and humans, this wild species could play an important role in the diffusion of colistin resistance. Thus, the monitoring programs on wildlife should include this aspect.

Keywords Colistin resistance · *E. coli* · *mcr-1* · *mcr-2* · Wild boar

Introduction

Colistin, also known as polymyxin E, is a cationic polypeptide antibiotic belonging to the class of polymyxins. It was firstly isolated from *Paenibacillus polymyxa* subsp. *colistinus* in 1947 (Benedict and Langlykke 1947) and recently regained attention as one of the last resort antibiotics against some multidrug-resistant bacteria belonging to the Enterobacteriaceae family, including *Escherichia coli*, *Enterobacter* spp., *Citrobacter* spp., *Klebsiella* spp., and *Salmonella* spp. (Falagas and Kasiakou 2005). The main colistin mechanism of action relies on the cell membrane destabilization and increased permeability of the lipopolysaccharide due to the interaction between the α,γ-diaminobutyric acid (Dab) residue of the positively charged polymyxin and the phosphate groups of the negatively charged lipid A membrane, displacing divalent cations (Ca^{2+} and Mg^{2+}) from the phosphate groups of membrane lipids (Dixon and Chopra 1986). Due to its high toxicity, from the Seventies, colistin employment in human medicine was limited (Koch-Weser et al. 1970), while it was frequent in veterinary practices. Colistin has been used for animal treatment for decades, especially in Italy, and it is still widely employed. However, in Europe, the use of colistin in veterinary medicine drastically decreased in recent years (European Medicines Agency 2020). Colistin is generally used for the treatment of gastrointestinal infections caused by *E. coli* or other Enterobacteriaceae in poultry and pigs, especially in intensive settings (Rhouma et al. 2016; Kempf et al. 2016). Furthermore, it is employed for the prevention of infectious disease and as a growth promoter in certain countries; indeed, oral administration of colistin to pigs and chickens via feed significantly stimulates the growth of young animals, enhances feed conversion, and increases economic returns for the farmer (Shen et al. 2020). This antimicrobial can be administered topically, by injection, via intramammary route, and orally. In intensive farms, the last option allows the concurrent administration of many subjects.

In 2015, the first identification of a transferable plasmid-located colistin resistance determinant, called *mobile colistin resistance 1 gene* (*mcr-1*), in *E. coli* from animals, food, and human samples in China (Liu et al. 2016) opened the way to

✉ Barbara Turchi
barbara.turchi@unipi.it

[1] Department of Veterinary Science, University of Pisa, 56126 Pisa, Italy

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many studies. These were mainly focused on the molecular epidemiology of *mcr*-mediated colistin resistance and lead to the identification of nine variants of the *mcr* gene (Luo et al. 2020).

Despite the extensive use of colistin in animal farming, in many European countries, the resistance against colistin in *E. coli* from healthy animals seems to be low (< 1%) (Kempf et al. 2013). This situation is also observed in other countries, such as Brazil and the USA, which importantly contribute to food animal farming (Meinersmann et al. 2017; Palmeira et al. 2018). However, in the last years, the observed resistance rates in Europe and China suggested a rapid increase among indicator *E. coli* from food animals (0.9–76.9%) (Liu and Liu 2018).

If on the one hand information on colistin resistance, especially the *mcr1*-mediated resistance, in bacteria from farm animals and animal-derived food products is available (Liu and Liu 2018), those on the prevalence of colistin resistance in wildlife are scarce (Shen et al. 2020). Recently, Wasyl et al. reported a low prevalence of colistin resistance among *E. coli* from wild boars (n = 278) and wild ruminants (n = 264) hunted in Poland, with only two resistant isolates from roe deer, which were *mcr*-negative (Wasyl et al. 2018). This was in accordance with data by Navarro-Gonzalez et al. who did not detect any colistin-resistant *E. coli* among isolates from wild boar (*Sus scrofa*) and Iberian ibex (*Capra pyrenaica*) in a National Game Reserve in northeastern Spain (Navarro-Gonzalez et al. 2013). As for Italy, few surveys on antibiotic resistance including colistin in Enterobacteriaceae from wildlife were performed. They were carried out employing the disk-diffusion method (Foti et al. 2009; Zottola et al. 2013; Botti et al. 2013), which is not reliable (Tan and Ng 2006).

The present study aimed to investigate the phenotypic resistance against colistin in *E. coli* from hunted wild boar and to evaluate the presence of *mcr-1* and *mcr-2* genes in resistant and susceptible isolates. Moreover, the potential role of wild boar as a *reservoir* and carrier of colistin-resistant bacteria and *mobile colistin resistance* genes has been evaluated.

Materials and methods

Sample collection and *Escherichia coli* isolation

Two hundred rectal swabs were collected from wild boar during hunting seasons 2018–2019 in 4 provinces (Pisa, Livorno, Grosseto, Siena) of Tuscany, Italy. All animals were killed during the hunting season following the regional hunting law (Regolamento di attuazione della legge regionale 12 gennaio 1994, n. 3 D.P.G.R. 48/R/2017), and no animals were specifically sacrificed for this study purpose.

Swabs were collected before slaughtering, and at the same time, information about sex and age (Sáez-Royuela et al. 1989) of animals was recorded. *Escherichia coli* isolation was performed on Tryptone Bile X-glucoronide (TBX) Agar (Oxoid, Milan, Italy), after enrichment in buffered peptone water (Oxoid) at 37 °C for 24 h. Plates were incubated at 42 °C for 24 h, and one single isolated blue colony from each sample was selected as presumptive *E. coli* isolate, purified on tryptone soy agar (TSA) (Oxoid), and subsequently confirmed as *E. coli* using conventional biochemical tests. Isolates were sub-cultured in brain and heart infusion broth (BHI) (Oxoid) and frozen at − 80 °C, after the addition of glycerol as cryoprotectant.

Phenotypic colistin resistance determination

Minimum inhibitory concentrations (MIC) for colistin were determined by the broth microdilution method, following CLSI guidelines (CLSI 2015). MIC test was performed in cation adjusted Mueller Hinton (MH) broth (Oxoid) employing colistin sulfate (CARLO ERBA Reagents, Cornaredo, Italy). Two-fold dilutions were performed from 256 to 0.5 µg/mL. According to CLSI and EUCAST recommendations, isolates with MIC values ≤ 2 µg/mL were considered susceptible, whereas those with MIC values > 2 µg/mL were recorded as resistant (CLSI 2018; EUCAST 2020).

Presence of colistin resistance genes

The occurrence of plasmid-borne colistin resistance genes *mcr-1* and *mcr-2* was evaluated by PCR, employing primers and protocols previously reported (Table 1) (Xavier et al. 2016; Barbieri et al. 2017). DNA extraction was performed by Quick-DNA Plus Kits (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. PCRs were performed in 25 µl of reaction mixtures containing 200 µM of deoxynucleotide triphosphates, 0.5 µM of each primer, 1.25 U of Taq polymerase (Lucigen Corporation, Middleton, Wisconsin, USA), and 2 µl of extracted DNA. All amplifications were carried out in the automated thermal cycler Gene-Amp PCR System 2700 (Perkin Elmer, Norwalk, Connecticut, USA). PCR products were analyzed by electrophoresis at 100 V for 45 min on 1.5% agarose gel stained with ethidium bromide, and PCR Sizer 100-bp DNA ladder (Norgen Biotek, Thorold, Canada) was used as a DNA marker.

Results

Escherichia coli isolation

One hundred and sixty-eight *E. coli* were isolated from wild boar. In particular, 74 isolates were from males and 94 from females. Sixty-three, 25, and 80 isolates were from

Table 1 Employed primers and related information

Gene	Primer	Sequence	Annealing temperature	Expected product (bp)	Reference
mcr-1	mcr1-F	CGGTCAGTCCGTTTGTTC	58 °C	309	Barbieri et al. (2017)
	mcr1-R	CTTGGTCGGTCTGTAGGG			
mcr-2	mcr2-IF	TGTTGCTTGTGCCGATTGGA	65 °C	566	Xavier et al. (2016)
	mcr2-IR	AGATGGTATTGTTGGTTGCTG			

young, sub-adult, and adult wild boar, respectively. As for geographical distribution, 60 *E. coli* were isolated from samples collected in Grosseto province, 53 in Pisa, 45 in Siena, and 10 in Livorno (Table S1).

Phenotypic and genotypic colistin resistance

Overall, 47/168 (27.9%) *E. coli* scored resistant to colistin, with MIC values ranging between 4 and >256 µg/mL (Table 2). Thirty out of 168 isolates (17.8%) showed a MIC higher than 256 µg/mL. Among susceptible isolates (121/168–72.0%), 95/168 (56.5%) showed a MIC lower or equal to 0.5 µg/mL, which was the lowest tested concentration.

Genes responsible for colistin resistance, *mcr-1* and *mcr-2*, were equally distributed in the studied population. Particularly, 23/168 (13.6%) *E. coli* harbored both genes, 26/168 (15.4%) scored positive only for *mcr-1*, and 26/168 (15.4%) for *mcr-2*. The presence of both genes was mainly associated with higher MIC values: 21 *mcr*-positive isolates had a MIC value ≥ 256 µg/mL. Whereas the presence of *mcr-1* or *mcr-2* alone was also detected in susceptible *E. coli* (Table 1), in particular, 33/168 (19.6%) isolates were PCR-positive, but they showed a MIC value ≤ 2 µg/mL Ninety-three out of 168 isolates (53.3%) did not show resistance genes; most of them exhibited a low MIC value and were, consequently, categorized as colistin susceptible (Table 2).

Figure 1 shows the geographic distribution of *mcr* genotypic profiles. No statistical differences were observed among sex, age, and provinces considering both phenotypic colistin resistance and *mcr* gene distribution (Figs. S1 and S2).

Discussion

Antimicrobial resistance is one of the main issues for human and veterinary medicine with some authors forecasting the return in few years to a situation similar to that of the pre-antibiotic era (de Kraker et al. 2016; Martens and Demain 2017). The rise of multidrug resistance and in particular the resistance to β-lactams lead to the urgent need for new antibiotics or to the reintroduction of those molecules which were no longer in use due to several reasons (Livermore et al. 2011). Polymyxins, in particular colistin, were recently reintroduced for humans treatment, despite their toxic effects (Li et al. 2006). Contrariwise, the large employment of colistin in veterinary medicine was constant (European Medicines Agency 2019). Recently, the discovery of a plasmid-mediate resistance gene (*mcr-1*) encoding for colistin resistance was a matter of great concern. This gene, or its variants, was subsequently detected all over the world, especially among *E. coli* or other Enterobacteriaceae (Nang et al. 2019). In Italy, some studies reported the presence of *mcr*-positive colistin-resistant bacteria isolated from humans (Cannatelli et al. 2016; Simoni et al. 2018). At the same time, some authors reported the spreading of colistin-resistant/*mcr*-positive *E. coli* among breeding animals (Curcio et al. 2017; Alba et al. 2018; Magistrali et al. 2018). However, no information is available about wild animals, excluding some studies which provided evidence of phenotypic colistin resistance using the disc diffusion method (Foti et al. 2009; Zottola et al. 2013; Botti et al. 2013).

Table 2 Distribution of MIC values in relation to the observed genotype

Genes	MIC µg/mL											
	≤0.5	1	2*	4	8	16	32	64	128	256	>256	Total
mcr-1 + *mcr-2*	1	1	0	0	0	0	0	0	0	2	19	23
mcr-1	11	2	0	0	0	0	0	2	5	0	6	26
mcr-2	15	2	1	0	0	0	0	2	0	1	5	26
mcr-negative	68	13	7	2	2	0	0	1	0	0	0	93
Total	95	18	8	2	2	0	0	5	5	3	30	168

*Isolates with MIC values > 2 µg/mL were considered resistant according to CLSI and EUCAST guidelines

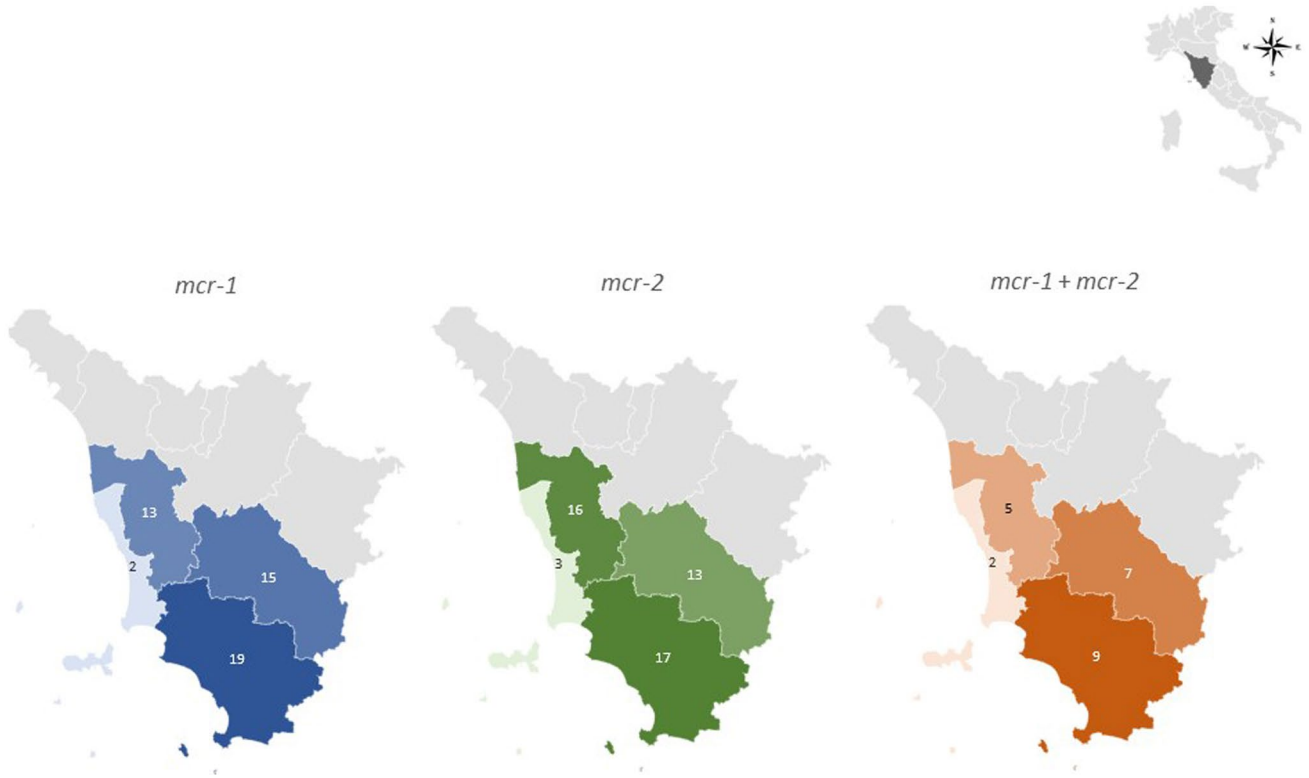

Fig. 1 Geographical distribution of the *mcr*-positive *E. coli* isolated form wild boar in relation to investigated provinces (Tuscany region, Italy)

In the present work, colistin resistance in 168 *E. coli* isolated from hunted wild boar was evaluated, using the recommended MIC microdilution method (CLSI 2015; Tan and Ng 2006). A percentage of 28.0% of tested bacteria resulted phenotypically resistant to colistin. Obtained results showed a higher percentage of colistin-resistant *E. coli* compared to that obtained in Italy for isolates from farm swine. In particular, Alba et al. (2018) detected resistance rates ranging from 0.6 to 6.5% in *E. coli* from fattening pigs. The last EFSA report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals, and food showed a percentage of colistin-resistant indicator *E. coli* in fattening pigs of 0.6% in Italy (EFSA and ECDC 2019). The higher percentage of colistin-resistant *E. coli* detected in wild boar could be related to the environmental pollution and the behavior of this animal species. Although wild animals are not directly exposed to antimicrobials for treatment, they could easily come in contact with antimicrobial residues or antimicrobial-resistant bacteria. Indeed, many human activities (ex. hospitals, livestock facilities, sewage system, wastewater treatment facilities, agricultural fertilization) could contribute to the release in the environment of antimicrobials or antimicrobic resistant bacteria (Dolejska and Papagiannitsis 2018). Some studies highlighted that antimicrobial-resistant bacteria are more often detected in omnivorous wild animals (Vittecoq et al. 2016), and this could be linked to a possible "accumulation" process. Wild boars are omnivorous, scavengers, and semi-synanthropic animals; furthermore, they could easily come in contact with livestock and breeding environments; for these reasons, these animals seem to be perfect candidates to acquire and cumulate antimicrobial-resistant bacteria. To support this hypothesis, it should be noted that isolates examined in this investigation were previously characterized for their antibiotic susceptibility profile and resulted resistant to many other antimicrobials, (Bertelloni et al. 2020a). To the best of author knowledge, no data on colistin resistance in *E. coli*, or other bacteria, from wild boar in Italy, are available. Furthermore, considering other European countries, few studies were performed. One of them was carried out in Poland and one in Spain, and in both cases, authors did not detect colistin-resistant *E. coli* (Navarro-Gonzalez et al. 2013; Wasyl et al. 2018).

As regard resistance genes, 44.64% of test *E. coli* resulted positive for one or both tested *mcr* genes. The two genes resulted equally distributed in the studied bacterial population. This is in contrast with other studies carried out in Italy on animal isolates, where *mcr-1* resulted the predominant gene, while *mcr-2* was rarely detected. However, these investigations were conducted on *E. coli* isolated from livestock

and in different geographic areas (Curcio et al. 2017; Alba et al. 2018; Magistrali et al. 2018). Furthermore, *mcr-2* was detected in migratory birds which could be a possible source of diffusion in the ecosystem (Ahmed et al. 2019). Most *mcr*-positive *E. coli* resulted phenotypically resistant, in particular those harboring both genes. The high prevalence of the resistance genes is consistent with the high percentage of resistant isolates detected by the phenotypic test. Nevertheless, 19.6% of isolates were *mcr*-positive but phenotypically susceptible to colistin. This is an unexpected result since the available information is scant. Indeed, most of the studies evaluated the presence of *mcr* genes in resistant isolates, and only a few authors reported the occurrence of susceptible phenotype in *mcr*-positive strains (Liassine et al. 2016; Quan et al. 2017; Magistrali et al. 2018). However, some hypotheses could be done. The lack of the expression of resistance genes is well documented, recently also for colistin, and it could be related to many different causes (Hughes and Andersson 2017; Nang et al. 2019). Furthermore, many variants exist for *mcr* genes (Partridge et al. 2018), and this may be suggestive of their high variability. So, it is plausible to speculate that some mutations occurred, impairing the gene functions. Furthermore, these defective genes can rapidly spread in bacteria populations, considering that they are plasmid-associated. In accordance with our study, some recent works showed a poor concordance between the *mcr* genes presence and the phenotypic resistance detected (Aguirre et al. 2020; Vidal et al. 2020). Finally, some isolates (3.0%) scored negative for both *mcr-1* and *mcr-2,* but resulted phenotypically resistant. This could be due to the presence of other mobile colistin resistance elements; some of them reported in Italy like *mcr-3* and *mcr-4* (Alba et al. 2018), or to chromosomal resistance genes (Moffatt et al. 2019).

Conclusions

Wild boar could share habitats with domestic animals, especially where extensive farming is adopted. This could lead to the transmission of pathogens to breeding animals. Furthermore, wild boar is one of the most abundant hunted species in some countries, like Italy (Bertelloni et al. 2020b), and, recently, these animals gain access to peri-urban and urban environments; these circumstances could, directly or indirectly, expose humans to infections by zoonotic or antimicrobial-resistant microorganisms carried by wild boar (Torres et al. 2019; Bertelloni et al. 2020b; Cilia et al. 2020). Polymyxins are considered last-line treatments for multidrug-resistant Gram-negative bacterial infections. Considering the rising problem of colistin resistance linked to plasmid-mediated genes, it is important to monitor all possible reservoir niches. This work showed the high circulation of phenotypic resistant and *mcr-1*- and *mcr-2*-positive *E. coli* isolates among the wild boar population of Central Italy. These results highlight by one side the possible impact of antimicrobial pollution on wild boar and, more in general, on ecosystems, on the other side the possible role these animals could play as carriers of colistin-resistant bacteria and genes. Wild boar could reintroduce these bacteria in food animals producing system or transmit them directly to humans, representing a serious hazard for animals and human health. In the One Health approach to antimicrobial resistance, it remains important the constant monitoring of wildlife populations.

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Declarations

Conflict of interest The authors declare no competing interests.

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