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Original article

The first record of *mcr-1* gene for colistin resistance in pigs from Serbia: should we be worried?

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Abstract

Colistin is being used as a last-resort drug to treat infections caused by multidrug-resistant (MDR) bacteria in humans. In veterinary medicine, colistin has been used for the treatment and prevention of infectious diseases. In the first study of *mcr* genes by multiplex PCR in healthy pigs from Serbia, we discovered *mcr-1* in 4.85% out of 350 fecal samples. The presence of *mcr-1* gene was detected on three farms located less than 100 km apart from each other, predominantly in piglet samples. The results point to the necessity of monitoring of colistin resistance and the *mcr* genes in food producing animals as well as restricting colistin usage on farms.

Key words: colistin resistance, pigs, mcr-1

Introduction

Colistin (polymyxin E) originally named "colimycin" was first isolated by Koyama et al. (1950), from the *Paenibacillus (Bacillus) polymyxa var. colistinus* in 1950 (Falagas et al. 2005, Andrae et al. 2020). Since its discovery, colistin has been used in human medicine to treat infections caused by Gram-negative bacteria. However, due to serious side effects such as nephrotoxicity, more potent and less toxic drugs such as aminoglycosides, quinolones and β-lactams replaced colistin in the 1970s. Over the last 20 years, colistin use was largely limited to topical and ophthalmic administration, while systemic use was limited to the treatment of secondary infections in patients with cystic fibrosis (Poirel et al. 2017).

Colistin is an old antimicrobial agent, but its clinical usefulness is being increasingly recognized now, because it is one of the few agents which are effective against multidrug-resistant Gram-negative

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bacteria (e.g. carbapenem-resistant enterobacteria, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*) (Andrade et al. 2020). The World Health Organization (WHO) has reclassified colistin into a category of very high importance for human medicine, under category B, which also refers to quinolones, 3rd- and 4th-generation cephalosporins, thus limiting the use in veterinary medicine in order to mitigate the risk to public health (WHO 2020). According to the European Medicines Agency (EMA), category B antibiotics should only be used in cases where category C and D antimicrobials are not clinically effective or there is no other alternative. In general, the use of antibiotics should be based on this rule, especially for the category B, where colistin belongs (EMA 2019).

Colistin usage in veterinary medicine, especially in farm animals such as chicken, swine and cattle, has continued up to date. It is used not only in clinical treatments of infections caused by enterobacteria, but also for prophylactic purposes and as a growth promoter (Katsunuma et al. 2007). In pig farming colistin is used to prevent infections with *Escherichia coli*, which can provoke severe diarrhoea, septicaemia and colibacillosis, mainly in young piglets, leading to huge economic losses (Kempft et al. 2013). Colistin is administered with food during or post weaning in order to reduce mortality and to enhance growth (Ahmed et al. 2021). Administration of antibiotics such as colistin has been suitable for growing farm industries because it allowed more successful parturition, higher population densities, and probably greater economic sustainability due to easier control of pathogens such as shigatoxin-producing *E. coli* strains (Rhouma et al. 2016).

The emergence and spread of colistin resistance among bacteria have diverse genetic background. Until 2015, resistance was considered to occur exclusively by chromosomal mutations and was not associated with horizontal gene transfer (El-Sayed et al. 2020). However, from 2011 through 2014, a plasmid-encoded colistin resistance gene, *mcr-1*, was identified in colistin-resistant *E. coli* isolated in China. Specifically, colistin resistance gene, *mcr-1,* was found in 21% of healthy swine at slaughter, 15% of pork and chicken meat from markets, and 1% of hospitalized human patients (Liu et al. 2016).

Untill now, the *mcr-1* gene has been found in various genera of the *Enterobacteriaceae* (*Escherichia, Klebsiella, Enterobacter, Cronobacter, Salmonella, Shigella*, and *Kluyvera*) isolated from the environment, vegetable and meat foods, animals and humans. The wide occurrence of colistin resistance in isolates recovered from farm animals strongly supports the presumption that livestock, especially pigs and cattle, might be the main source of *mcr-1* (Poirel et al. 2017).

There are no available data on the prevalence of transferable resistance to colistin in swine in South-Eastern Europe. Therefore, the objective of this study was to identify plasmid-borne *mcr* genes from pig feces using molecular methods.

Materials and Methods

We analyzed the total DNA extracts from pig fecal samples collected in a previous research during 2016 throughout Vojvodina region (the northern part of Serbia), which accounts for approximately 50% of swine production in Serbia. The minimum sample size in the population of 1.5 million pigs was calculated using Epitools epidemiological calculators (AusVet Animal Health Services and Australian Biosecurity Cooperative Research Centre for Emerging Infectious Disease, http://epitools.ausvet.com.au) and was determined as 288 samples. Finally, 403 individual fecal samples from healthy pigs in the period August--November 2016 were collected. The specimens were collected directly from the rectum of domestic pigs (*Sus scrofa domesticus*) on seven farms owned by the largest swine producing companies in the country which voluntarily consented to collaborate in this study. The samples were taken randomly and proportionally to each category (piglets, weaners, growers, finishers, gilts and sows). All pig samples were delivered to the laboratory within three hours after the sampling and processed immediately. From each fecal specimen, one aliquot of approximately 180-220 mg was frozen at -20°C until the further analysis. Genomic DNA extraction from fecal samples was performed using QIAamp DNA Stool Mini Kit (QIAGEN), following the manufacturer's instructions. Measurement of the extracted total DNA was done using De Novix Fluorometer, USA and a sufficient amount of genomic DNA was confirmed in all the 350 samples that were subjected to analysis.

For the multiplex PCR detection of five plasmid mediated colistin resistance genes (*mcr1, mcr2, mr3, mcr4 and mcr5*), we used the protocol of the European Union Reference Laboratory for Antimicrobial Resistance, previosly described by Rebelo et al. (2018). The composition of each PCR reaction was as follows: 12.5 µl of HotStart Taq2x MasterMix (BioLabs, USA), 5.5 µL of nuclease-free water, 0.5 µL of each of the 10 primers (10 µM), and 2 µL of DNA extract. Running conditions were: initial denaturation at 94°C for 15 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 90s and elongation at 72°C for 60 s, and a final elongation at 72°C for 10 min. PCR products were electrophoresed in 2% agarose gel. A molecular weight marker (GeneRuler 100bp Plus

DNA Ladder, ThermoScientific) was included in each run. The bands were visualized using UV gel documentation system SERVA BlueCube 300 (Germany) after the ethidium bromide staining. As a positive control strain for *mcr-1* gene *E. coli* NCTC 13846 was used.

Results

From the 350 samples of total fecal DNA, the *mcr-1* gene (product size 320bp) was detected in 17 (4.85%). The presence of *mcr-1* gene was detected on three farms located less than 100 km apart from each other. A total of 15 positive *mcr-1* gene samples came from piglets weighing up to 20 kg, one from weaning and one from gilts. None of the samples harbored other *mcr* genes, (*mcr-2*, *mcr-3*, *mcr-4* and *mcr-5*).

Discussion

The global use of antimicrobials in food animal production is projected to rise by 2030 (Poirel et al. 2017). However, as a result of restrictions in antibiotic usage on farms in the European Union, the consumption of polymyxins in 25 countries dramatically decreased in a period 2011-2020 by 76.5% (EMA 2021). To the contrary, in Serbia the trade of powdered colistin grew steadily from 8,4 tons in 2014 to 17,2 tons in 2018 (ALIMS 2018, ALIMS 2020). A significant level of antimicrobial usage in farrow-to-finish pig farms in Serbia is evident for prophylactic or therapeutic treatments. In sucklings, prophylactic treatment mostly included application of water-soluble colistin sulfate (13.51%) (Prodanov-Radulović et al. 2020).

So far, two molecular studies on colistin resistance in Serbia have been published, one in *Klebsiella pneumoniae* isolates from human population and one in *Salmonella* Infantis isolates from poultry. Twenty-seven colistin-resistant, carbapenemase-producing *Klebsiella pneumoniae* isolates were identified from hospitalized patients in Serbia between 2013 and 2016, with the majority of isolates originated from Vojvodina. None of the isolates possessed plasmid-borne *mcr-1* or *mcr-2* genes, whereas the overexpression of chromosomal *phoP* and *phoQ* genes was found (Novović et al. 2017). In a second study, genomic DNA of seven *Salmonella* Infantis isolates from poultry farms with reduced susceptibility to colistin were sequenced, and fluctuations in *phoPQ*, *pmrAB*, and *mgrB* mRNA levels, that are usually associated with colistin-resistance phenotype, were described (Jovčić et al. 2020).

Although ten plasmid mediated colistin resistance genes have been discovered so far, *mcr-1* remains the gene with the highest prevalence. Since the first detection of *mcr-1*, it has been identified in bacteria isolated from different animals and in over 30 countries. Shen and colleagues found that the origin of *mcr-1* dates back to the 1980s, when colistin resistant *E. coli* was isolated from chickens, at the time when colistin first started to be used in food-producing animals in China. The prevalence of *mcr-1* began to increase dramatically from 5.2% in 2009 to 30.0% in 2014 (Shen et al. 2020), but the ban of colistin usage in animal feed in 2016 led to a sharp decline of *mcr-1* prevalence in pig farms from 45% in 2016 to 19% in 2018 (Shen Chong et al. 2020).

In a long-term retrospective study of *E. coli* isolated from healthy cattle, swine and chickens in Japan during 1991-2014, out of 9,308 isolates, only 2 (0.02%) isolates positive for *mcr-1* were found (Kusumoto et al. 2016, Nakano et al. 2021). Another study from 2013-2015 revealed the prevalence of 20.43% *mcr*-1 among *E. coli* from healthy pigs (El-Sayed et al. 2020). Later, in 2018, the *mcr* gene was detected in 16 isolates: *mcr*-1 in 14 isolates of *E. coli* from 10 chicken samples (9.7%), and *mcr*-3 in two isolates of *Aeromonas sobria* from pork and chicken samples (1.0% each). The findings of this study highlight the necessity of surveillance of colistin resistance in bacteria that contaminate retail meats (Fukuda et al. 2018).

In Europe, in a 2004-2014 survey on 3018 *E. coli* isolates from pigs with various infections, only of 29 (0,96%) *mcr-1*-positive isolates were detected, with a yearly proportion varying from 0 to 2.20% (Garch et al. 2017). A total of 16 studies of plasmid mediated colistin resistance in swine were published from European countries in the period 2016-2021. The prevalence in healthy pigs was less than 1% of *mcr-1* positive findings (Valiakos and Kapna 2021).

In that perspective, the finding of 4.85% *mcr-1* positive DNA samples from healthy pigs in the largest swine producing region in Serbia raises concern. The main reason for such a discrepancy comparing to the rest of Europe is that the measures in the systemic monitoring of resistance and strict restriction in colistin consumption in Serbian farms are yet to be fully implemented, according to the National programme for the control of resistance of bacteria to antimicrobial drugs from 2019. The predominance of *mcr-1* positive samples from piglets comparing to the other age categories, strongly suggests that in Serbia the practice of colistin administration in the earliest stages of pig production as growth promoter has been used at the time of sampling. Finding only the most prevalent plasmid mediated gene *mcr-1* is in the concordance with many other studies (Valiakos and Kapna 2021). It could be expected that the prevalence would be even higher if we tested pigs with infections.

In our research, we used the samples of total fecal DNA from pigs rather than DNA isolated from individual bacterial colonies. By this screening approach, we raised the probability to detect plasmid-mediated colistin resistance determinants regardless of the host bacterium. However, this method lacks the capacity for the identification of bacterial species that are *mcr* gene carriers (Tong et al. 2018).

Conclusion

The results of this study demonstrated the presence of *mcr-1* gene in direct fecal samples of healthy pigs in Serbia. This is the first such study in South-Eastern Europe. The detection of high risk *mcr-1* resistance gene with the potential of horizontal dissemination in gut bacterial community of farm animals presents a threat in One Health perspective. In the context of withdrawal of colistin as growth promoter in Serbia, future surveliance of colistin resistance and the prevalence of *mcr* genes in food producing animals is of high importance.

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