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Research note

Stray dogs as carriers of *E. coli* resistant strains for the retracted and re-emerged antibiotic colistin, based on the *mcr-1* gene presence

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Abstract: Antimicrobial resistance (AMR) is becoming a major problem for animal and human health. Reports of resistance to colistin, an antibiotic that is considered a last resort drug against resistant Gram-negative bacteria, have been increasing over the last years. Among the different mechanisms that cause AMR to colistin, the mobilized colistin resistance (*mcr*) gene has been reported as responsible for the increased incidence in animals and humans since 2015. There are ten recognized distinct variants of this gene in bacteria isolated from animals, humans, food, and the environment. Companion animals could have a role in human infection by pathogenic and resistant *E. coli* strains as they share the same environment and are in close contact with humans. Considering this, our aim was to investigate antimicrobial resistance in companion domestic and stray dogs in Western Macedonia, Greece. Our results revealed that of the 43 individual fecal samples examined, 16% of them hosted the *mcr-1* gene, all of which were isolated from stray dogs. Our results suggested that companion dogs and stray dogs can serve as reservoirs for colistin-resistant *E. coli* strains.

Keywords: antibiotics; canine; colistin; mcr gene; companion animals

1. Introduction

Colistin is an antimicrobial agent of the polymyxin antibiotic class, discovered in 1947 in Japan. It is produced by strains of the Gram-positive bacterium *Paenibacillus polymyxa*, known also as *Bacillus polymyxa* [1]. There are five, chemically distinct, polymixins belonging to this class: A, B, C, D, and E. Among them, only polymyxin B and polymyxin E (colistin) are used in clinical procedures [2]. Colistin has been used since the 1950s to treat often life-threatening human infections caused by carbapenem-resistant bacteria, like *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli,* and other pathogens of the Enterobacteriaceae family, having an excellent bactericidal activity on various types of infections [3,4]. There are two forms available for clinical use, colistin methanesulfonate sodium (CMS), for parenteral administration and colistin sulfate (CS), administered orally or in topical formulations [5,6]. Moreover, colistin has been used extensively for years in veterinary medicine not only against Enterobacteriaceae infections but also as a growth promoter and as a protective agent in animal production [7,8].

Due to reported side effects, mostly nephrotoxicity and neurotoxicity, the clinical use of polymyxin antibiotics during the 1970s was significantly reduced and resulted in almost complete abandonment in the 1980s [9]. Over the last years, the incidence of resistant Gram-negative bacteria pathogens to available antimicrobial agents and the lack of new antibiotics to treat multidrug-resistant (MDR) infections have renewed interest in old antibiotics [10]. This emergence of MDR bacterial infections appeared as a result of excessive use of antibiotics in both human and animal medicine, posing a threat to healthcare systems worldwide [11]. Consequently, since the 1990s, polymyxin antibiotics, due to the restricted of possible alternatives and with an improved safety profile, have been considered as last-resort antibiotics, and although retracted in some countries, they have been re-emerged for the treatment of MDR Gram-negative infections [12,13]. Even though αantimicrobial resistance (AMR) to colistin was considered rare or incidental, extensive use of colistin has led to increased reports of colistin resistance [14,15]. AMR to colistin appears due to different mechanisms. The initial reports were about resistance due to chromosomal mutations. The fact that this form of chromosomal acquired resistance to colistin did not expand rapidly among bacterial populations, it has resulted in a restricted clinical impact and a minor public health concern [16]. However, in 2015, the first report in China of a plasmid-mediated colistin-resistance gene in *E. coli* that appeared to spread quickly among different bacterial populations was provided. The gene that was recognized as responsible for this resistance to colistin, was the mobilized colistin resistance (*mcr*) gene [17,18].

Ten distinct variants of the *mcr* gene (*mcr* 1-10) have been recognized in various bacteria isolated from humans, animals, food, and the environment [19]. Among *mcr* genes, *mcr-1*, which was the first one that was isolated, appears to be the most frequently detected in more than 60 countries worldwide [20]. Bacterial hosts of these genes, are mainly bacilli of the Enterobacteriaceae family, such as *E. coli* (*mcr* 1,2,3), *Salmonella enterica* (*mcr* 4,5,9), *K. pneumoniae* (*mcr* 7,8), *Enterobacter cloacae* complex (*mcr*-9), and *Enterobacter roggenkampii* (*mcr*-10), with the exception of the *mcr*-3 gene, which is frequently detected in *Aeromonas* spp., and *mcr*-6 gene, which is mainly detected in *Moraxella* spp. [21]. These genes code for transferases that alter the structure of the outer cell membrane of Gram-negative bacilli, a membrane that provides the microorganisms with additional protection, blocking antibiotics from accessing their target. This modification of the cell membrane is responsible for the development of colistin resistance [22,23].

E. coli, as the principal carrier of *mcr* genes, and, due to the ability to transfer easily between

various host species, is of great importance for the transferable colistin resistance [24]. *E. coli* is one of the most common Gram-negative bacteria worldwide, being a principal microorganism of the gut microbiota of many animal hosts and humans [25]. However, despite the fact that most of the bacterial strains are commensal, there are strains that can cause intestinal or extra intestinal infections in humans, like infections of the urinary tract and the nervous system [26]. Intestinal pathogenic *E. coli* strains are classified into six pathotypes: Enteropathogenic (EPEC), enterohaemorrhagic (EHEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAEC), and diffusely-adherent (DAEC) [27]. Extraintestinal pathogenic strains, on the other hand, are known under the name ExPEC [28]. Regarding phylogenetic classification of *E. coli* strains, there were initially four major groups: A, B1, B2, and D [29]. Additional groups have been added i.e., C, E, F, and G, along with one clade, I [30]. Among them, groups B2 and D are associated with the ExPEC strains and E, with the enterohemorrhagic Shiga toxin-producing *E. coli* O157: H7 being probably the most common *E. coli* pathogenic strain that causes illness in humans [31,32].

Humans may be infected by various pathogenic and resistant *E. coli* strains from farm animals [33–35]. A particular role in this infection may be played by pets, specifically stray dogs that have been abandoned and can act as bridge vectors [36–38]. In line with the general increase in the number of dogs occurring in urban and peri-urban areas, an increased number of unsuitable owners who lack the ability or even desire to care for their pets is also observed [39,40]. According to the 2023 annual report of the European pet food industry (FEDIAF), in Europe there are 340 million pet animals. Among them, the dog population is around 104 million. In Greece, there are 657.000 pet dogs, and 21% of households own at least one dog [41]. Close contact with companion animals and pet dogs, mostly due to poor or insufficient management of their waste, is considered a potential transmission route of resistant *E. coli* strains between them and humans [42,43].

For this reason, various actions take place for the control of stray dogs in different countries. One of the most common actions organized by the local authorities for the control of the stray dogs' populations is the Trapping-Neutering-Return (TNR) technique [44]. In Greece, there are few municipalities that implement programs for the management of stray dogs within their administrative boundaries (collection, electronic tagging, sterilization, efforts to adopt or return them to their natural environment), with expenses partially covered by the Ministry of Rural Development and Food (Official Government Gazette 5732/B/28-12-2020/KYA2654/356295). Yet, hundreds of thousands of stray dogs are reported in several parts of the country, mostly accounting for the abandonment of domestic dogs [45], representing a major public health problem due to pathogen transmission [46].

Hence, monitoring AMR in *E. coli* strains that come from companion animals is of great importance in the context of preventing further AMR development, an effort that has become a high priority worldwide [47]. However, to our best knowledge, there are no published data about resistant *E coli* strains in pet animals/dogs in Greece. With this in mind, we aim to investigate the antibiotic resistance in a rural area with a high number of farm animal facilities in Western Macedonia, Greece.

2. Materials and methods

2.1. Sample collection and bacterial cultures

Stray dogs (N = 24) were trapped in the Florina peri-urban area (Western Macedonia, Greece) in the frame of the TNR program organized by the Municipality of Florina and brought to the veterinary

clinic. Feces from 19 companion domestic dogs were also added in the analysis with a signed license by their owners. For stray dogs, folding dog traps and hunting snare traps were set and placed in the semi-mountainous peripheral region of Florina, in close proximity to sheep farms. Trapped animals were kept in captivity for no more than 24 h, as traps were checked on a daily basis. Trappings and collections were performed throughout the year, except for January, when the temperature is very low. Moreover, samples were collected with the help of owners, who kindly provided the feces in plastic bags. All animal manipulations were performed in compliance with the Directive 2010/63/EU on the protection of animals used for scientific purposes as well as with the Responsibilities of pet handlers (5-4039/2012) and approved by the Regular Meeting of the Municipal Council of Florina (Proceeding 30/24-10-2023).

All isolates originated from the animals' natural intestinal flora, and for stray dogs, apart from the stress, were in good health. No antibiotic treatment had been administered in any of the dogs investigated.

The ISO 16649-2:2001 method was applied for the culture of fecal samples in combination with indole and oxydase tests. Samples were embedded in Tryptone Bile X-glucuronide in agar (TBX; Oxoid, Basingstoke, UK), followed by incubation at 44 ℃ for 24 h or more; samples for *E. coli* were determined based on the blue or green coloration of the plate. Further, resistance to antibiotics applying the Kirby-Bauer agar Diffusion Method was carried out, and the minimal inhibitory concentration (MIC) of colistin was determined for all isolates.

2.2. Molecular analysis

DNA extraction was performed using the NucleoSpin Microbial DNA Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's recommended protocol. The presence of two major *mcr* genes (*mcr-1* and *mcr-2*) was investigated in the isolates of *E. coli* following the amplification procedures as described in Xexaki et al. [32]. Reactions were carried out using the FastGene Taq 2X Ready Mix (NIPPON Genetics, Europe), in total volumes of 20 μL, composed of 10 μL 2X Ready Mix, 0.6 pmol of each primer pair (mcr-1F - mcr-1R and mcr-2F - mcr-2R), and ultrapure water up to volume of 20 μL. The PCR conditions were 3 min at 95 ℃, followed by 36 cycles for 30 sec at 95 ℃, 40 sec at 55 ℃, and 45 sec at 72 ℃, with an additional final extension step for 5 min at 72 ℃. Positive samples, successfully amplified after depiction in agarose gel electrophoresis, were sequenced in both directions in an ABI-Prism 3730XL automatic sequencer, and after alignment in the software MEGA version 7 [48], the validity of the sequences was confirmed in the BLAST tool of NCBI website.

2.3. Statistics

A chi square test (χ^2) test was carried out to estimate the significant differences between the stray dogs and the companion ones concerning antibiotic resistance at the genetic level. The software version SPSS 22.0 was utilized for all performed tests.

3. Results and discussion

E. coli was identified in all fecal samples examined. No positive sample for either of the two genes was found in domestic companion dogs (Table 1). On the other hand, the *mcr-1* gene was

detected in seven stray dogs, supporting a statistically significant difference in stray dogs in contrast to domestic ones (Table 1). MIC test results were in total agreement with molecular analyses, inferring identical positivism rates, as shown in Table 1.

Table 1. Prevalence of *mcr* genes in the dogs investigated. The asterisk indicates statistical significance. Numerical values in parentheses indicate the % proportion of the gene.

gene	*mcr-1*	*mcr-2*
Stray dogs	7 (29.2%)*	0
Domestic dogs	0	0
Total	7 (16.2%)	0

On a global scale, AMR is becoming one of the most urgent public health concerns, and though it is a natural phenomenon of the bacterial evolution process, it is largely facilitated by excessive antimicrobial drug use in humans and animals. Around 700.000 people die every year, and it is estimated that by 2050, 10 million people will die annually due to AMR infections [49,50].

In human medicine, antimicrobials are used to treat various infections, which, in many cases, save lives. These drugs are also used in animals (farm and companion animals). In animal production, they are used not only to treat infections but also to promote productivity. It has been estimated that, on a global scale, 73% of traded antimicrobials are used in farm animals [51,52]. On the other hand, the treatment of companion animals with antimicrobial drugs, including those that are also licensed for use in human medicine, is often excessive and frequently occurs without proper bacterial identification and susceptibility testing **[**53–55].

Among drug-resistant pathogens, the World Health Organization (WHO) has recognized Gram-negative bacteria, including *E. coli* and other Enterobacteriaceae, as high priority AMR pathogens [56]. The WHO has also pointed out that *E. coli* constitutes a representative indicator of AMR since it harbors several resistance genes [50]. Furthermore, a major public health concern is the ability of *E. coli* to acquire and transfer resistant genes to other pathogens that share the same environment [57]. This raises the possibility of transmissions of resistant *E. coli* strains between animals and humans through various pathways, such as direct contact, contact with animal feces, or food consumption [57,58].

Until the end of 2015, the mechanisms related to the development of colistin resistance, without any proof for horizontal transfer, were the chromosomal mutations [59]. In 2016, the first report of a colistin resistance mechanism due to the plasmid-mediated mobilized colistin resistance (*mcr-1*) gene in *E. coli* strains in China was provided [8]. After its initial isolation in *E. coli* strains from food-producing animals, raw meat, and humans [17], the *mcr-1* gene spread to various countries worldwide and has been isolated since in different bacteria, mainly from in Enterobacteriaceae family, from people, wild and domestic animals, meat, vegetables, river water, and sewage. The *mcr-1* gene is known to provide adequate resistance against colistin and can spread rapidly by horizontal transfer, constituting a serious public health problem [60]. Companion animals (dogs and cats) have been gaining interest as a potential reservoir and a transmission pathway of resistance to humans, as the close contact between them and humans and the home environment favors transmission [61]. Moreover, these animals harbor bacteria resistant to most of the commonly used antibiotics in veterinary medicine and require the use of polymyxins for treatment, including hospital-associated pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *S. pseudintermedius*, vancomycin-resistant *Enterococci* (VRE), and extended-spectrum β-lactamase (ESBL)- or

carbapenemase-producing Enterobacteriaceae [61,62].

As for the *mcr-1* gene being more predominant in animals than humans, it shows a high potential for zoonotic transmission [59]. However, knowledge is limited concerning the extent this transmission occurs and the associated risk factors and transmission routes among companion animals and humans [63]. However, there are a few studies providing relative data on the epidemiology of the *mcr-1* gene in companion animals. Detection of *mcr-1* genes from these animals has been reported in China, where Zhang et al. [54] suggested that *mcr-1* positive *E. coli* strains can be transferred between companion animals and humans. In another study conducted in China, Lei et al. (2017) reported the presence of the *mcr-1* gene in *E. coli*, *K. pneumoniae*, and *Enterobacter aerogenes* strains isolated from companion animals and proposed that there was a possibility of transmission Enterobacteriaceae strains carrying this gene between companion animals and humans, posing a potential risk to public health [64]. In Ecuador, Loayza et al. [24] demonstrated the presence of the *mcr-1* gene in *E. coli* from companion animals. Moreover, these animals were sharing the same environment with a child diagnosed with a peritoneal infection by *mcr-1*- positive *E. coli* strains, a fact that led to the suggestion of a potential dissemination of the *mcr-1* gene in *E. coli* strains from different animals. In a study in Brazil, Kobs et al. [65] identified *mcr-1* positive isolates of *E. coli*, *Klebsiella* spp., and *Enterobacter* spp. from companion animals, proposing potential dissemination of resistance to polymyxins to the environment and humans.

In this study, we assessed the frequency of *mcr-1* positive *E. coli* strains in dogs from a rural area in Greece. These dogs were presented to a veterinary clinic in the city of Florina for the performance of a routine surgical procedure (ovariohysterectomy/castration) under a dog population management program that takes place annually. To the best of our knowledge, this is the first report of *mcr-1* gene positive *E. coli* strains in dogs in Greece. So far, the presence of the *mcr-1* gene in *E. coli* strains has been reported in farmed broilers and laying hens in Greece [32].

Our results revealed that of the 43 samples examined, 16% of them were hosting the *mcr-1* gene, all of which were isolated from stray dogs. Of the 43 dogs, 19 were owned and 24 were stray/feral dogs. Interestingly, all the *mcr-1* positive *E. coli* strains were found in the fecal samples collected from stray dogs. Stray dogs usually freely roam in a wide range of several kilometers searching for food, having a notable impact on public health, wild, and livestock animals [66]. The stray dogs mostly feed on wastes (human food and animal carcasses) and household scraps and they hunt wild and domestic animals, like rabbits and sheep [67]. It must be noted that more than 70% of all dog populations on a global scale are categorized as stray or free-ranging, and a considerable number of these dogs were previous owned dogs that were abandoned by their owners [67,68]. Apart from that, pet owned dogs in many cases consume commercial dog food, and as it has been shown in studies that dogs consuming commercial food have a lower prevalence of *mcr-1* positive *E. coli* strains compared to dogs that consume home-made food [69]. Moreover, most *mcr-1* positive *E. coli* strains are found in livestock animals' fecal samples, mainly pigs and broiler chickens [8,70]. In contrast with stray dogs, owned dogs potentially have minimal or no contact with these animals [64], and it has been suggested in previous research that farm animals are the source of *mcr* genes in sick pet dogs that had not received previous colistin treatment [21,59]. In addition, a fact that must been taken under consideration is that manure from these farm animals should be considered a possible source of antibiotic residues or *mcr-1* positive *E. coli* strains, contributing thus to their potential environmental dissemination [70]. In Florina, there are no reports of *mcr-1* positive *E. coli* isolates. Interestingly though, in a previous research study conducted in poultry farms from various areas of Greece, the *mcr-1* gene was found in

broilers' *E. coli* strains originating from the Regions of Epirus and Central Macedonia [32]. These geographical areas are adjacent to the Region of Western Macedonia, where Florina is located. In that way, stray dogs can potentially participate in the dissemination of various diseases to other animals or humans by fecal shedding of pathogens and environmental contamination (soil or water), acting as reservoirs for several zoonotic pathogens and for *mcr-1* positive *E. coli* strains [69]. Thus, stray dogs can be considered a potential reservoir and source of colistin-resistant bacteria, with the risk of their spreading to humans being facilitated through their feces [71]. In addition, as the *mcr-1* gene can spread quickly through horizontal transfer, and as *E. coli* has been classified by the WHO among the bacteria species that can be responsible for human health crises with an increasing antimicrobial resistance, the importance of colistin resistance in stray dogs is becoming a problem with serious public health implications [60,72].

It should be noted that our study was conducted in a geographical region exploring only dogs, which constitutes a limitation. Moreover, no phenotypic resistance tests for other antibiotics were performed on bacteria. Nevertheless, it can be inferred that living in the same environment and consuming similar food has an impact on both human and dog gut microbiome to a greater extent from cases when dogs feed on commercial food, suggesting that the presence of *mcr-1* positive bacteria has an increased possibility of dissemination among dogs and humans [73]. This fact combined with the roaming and feeding patterns of stray dogs could be a possible explanation of our results. In addition, the surgical procedure of ovariohysterectomy/castration in dogs can potentially have a cofounding effect, as hormonal and behavior changes due to this procedure can potentially influence colonization of *mcr-1* positive bacteria and have a general impact on the composition and diversity of dog's gut microbiota [73]. It is proposed that more extensive studies would be beneficial, with the addition of more geographical areas of the country and other companion animals (cats, rabbits etc.), as well as humans interacting in the same environment with these animals to more comprehensively explore this issue.

4. Conclusions

AMR is becoming a worldwide emerging problem for animal and public health, mainly due to the wrong and extensive use of antibiotics in both food and companion animals. This phenomenon has also resulted in the selection and transmission of resistant bacteria among animals or animals and humans. Resistance to colistin in the past was not considered a serious problem until reports of the plasmid-mediated colistin-resistance gene in *E. coli* strains emerged, a gene that shows that it can spread rapidly among bacterial populations. The existence in the same environment and the close contact of companion animals with humans could provide favorable conditions for such transmissions. Our results suggest that companion dogs and stray dogs can serve as reservoirs for colistin-resistant *E. coli* strains, possibly due to their roaming and feeding pattens, among others, causing the epidemiology of the mobilized colistin resistance (*mcr*) gene to be more complex. Providing this data, for the first time in Greece, we emphasize that additional research on this topic is necessary.

Author contributions

Conceptual idea: A. Parisi; E. Petridou; I. A. Giantsis; Sample collection: A. Parisi; M. V. Alvanou; Data analysis and interpretation: I. Tsakmakidis; D. K. Papadopoulos; K. Papageorgiou; Writing and editing: I. Tsakmakidis; M. V. Alvanou; K. Papageorgiou; E. Petridou, I. A. Giantsis.

Conflict of interest

The authors declare no conflict of interest.

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