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Colistin Resistance Profiles and Genotypes of *Escherichia coli* Isolates from Dogs and Cats*

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ABSTRACT

***Background*:** Nowadays, antibiotic resistance has become an important problem, posing a serious threat to both human and animal medicine. Colistin is one of the last-resort drugs for the treatment of particularly caused by multidrug resistant bacteria. The aim of this study was to investigate the resistance of *Escherichia coli* strains against colistin and the presence of colistin resistance genes (*mcr1*, *mcr2* and *mcr3*) in them. Antibiotyping and genotyping of all strains was also aimed.
***Materials, Methods & Results*:** A total of 75 isolates of *Escherichia coli* from healthy animals (38 dogs and 37 cats) were screened for colistin resistance by cultivation in a screening agar and then microbroth dilution method was performed. Antibiotic susceptibilities of the isolates were determined by KBDDM. The presences of *mcr1*, *mcr2* and *mcr3* genes were investigated by PCR. The colistin resistant strains were genotyped by using RAPD-PCR, and antibiotyped based on resistance profiles. In the screening test, 1 strain in cats and 2 strains in dogs were colistin-resistant. However, 18.6% of strains (from 14 cats and 3 dogs) were found as colistin-resistant in the microdilution test. MDR status was 76.31% and 97.29% in dog and cat strains, respectively. The colistin-resistant strains showed 78-100% and 65-90% similarities with respect to their antibiotypes and genotypes, respectively. *mcr1*, *mcr2* and *mcr3* genes were not found in any of the strains.
***Discussion*:** There is an increase in infections brought on by Gram negative bacteria with various antibiotic resistances in addition to infections brought on by bacteria that are antibiotic-resistant. In order to cure illnesses caused by resistant bacteria, the repurposing of outdated antibiotics may be on the table. Colistin is a crucial antibiotic in veterinary medicine, according to a number of published perspectives, although it should only be administered with caution. However, the discovery of the plasmid-derived *mcr1* gene and subsequent reports that this gene has propagated around the world. *Escherichia coli* strains isolated from companion animals have been found to carry the *mcr1* (colistin resistance gene), and possible human-animal cross-contamination has been looked into. The findings demonstrated that *mcr1*-carrying *E. coli* might inhabit pets and spread between people and animals. The cat and dog strains used in this investigation had variable colistin resistance rates, which varied between trials. Although no isolates were found to be positive for the *mcr1-3* genes in this study, it is believed that colistin resistance, which is determined phenotypically, should not be ignored in terms of spreading both in cat and dog populations as well as in terms of risk to human health, given the possibility that resistance could occur with other different mechanisms. Epidemiological research still uses *in vitro* antibacterial susceptibility patterns. Our antibiotyping method, which was based on an analysis of several antibiotic resistances, provided quantitative data. Commercial software was utilized to conduct the evaluation. There are no reports or publications that provide quantitative antibiotyping data for *E. coli* strains in the literature. A popular technique for genotyping different bacterial species is RAPD-PCR. By determining if certain specific genotypes are similar to those of other resistance strains, RAPD-PCR and other genotyping data can be compared with antibiotic resistance profiles to determine the specific risk of treatment resistance in infectious diseases. All organisms that were colistin resistant exhibited multiple antibiotic resistance, and these findings were also related to RAPD genotypes. The findings indicated that colistin-resistant *E. coli* bacteria could potentially represent a risk to human health and were thought to be transmitted from cats and dogs to humans and vice versa.

Keywords: antibiotyping, cats, colistin resistance, dogs, *Escherichia coli*, genotyping.

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INTRODUCTION

Colistin (Polymyxin E) is a bactericidal effective polycationic lipopeptide, which was introduced in 1950 for human medicine but have been systematically restricted due to its toxicity. Various infections caused by Gram-negative bacteria cause high morbidity and mortality and colistin is considered to be a "last resort drug" in the treatment of infections with high mortality in human medicine showing multiple antibiotic resistance (MDR). Colistin plays a key role in public health, despite all the limitations of its reliability profile. Colistin has been used in veterinary medicine for decades regularly both for treatment and prophylaxis. Considering the growing need for antimicrobials for the treatment of infections caused by multiple antibiotic resistant agents in humans, the use of this drug in veterinary medicine is questioned [2,5].

Colistin resistance has been shown to be due to a phosphoethanolamine transferase encoded by the plasmid-derived *mcr1* gene at the end of 2015 [2,5,10]. After the initial reporting of the mobile colistin resistance gene *mcr1*, it was reported in Enterobacteriacea isolated from animals, animal products, humans and environment in more than 30 countries on 5 continents. In the meantime, 6 variants of the *mcr* gene (*mcr1.2-mcr1.7*) have been reported.

The objectives of the study were: i) to investigate the resistance profiles of *Escherichia coli* strains kept in Culture Collection of Veterinary Microbiology Department of Veterinary Faculty against various antibiotics including colistin; ii) to determine the presence of colistin resistance genes in the strains, iii) to antibiotype and genotype of the colistin resistant strains.

MATERIALS AND METHODS

Bacterial strains

A total of 75 *Escherichia coli* isolates including 38 dog- and 37 cat-originated isolates in a collection of Veterinary Microbiology Department of the University of Ondokuz Mayıs, Faculty of Veterinary Medicine were investigated in a study. All isolates have been isolated from faeces of healthy animals in a period of 2010-2012. *E. coli* ATCC 25922 strain and *Proteus vulgaris* ATCC 8427 strain were used as reference strains in antibiotic susceptibility tests.

The isolates kept in culture collection were cultured on %7 sheep blood agar[1] plates at 37°C for 24 h. Then, colonies were identified by conventional methods [12] and isolates identified as *E. coli* were used for further analyzes.

Antimicrobial susceptibility testing and antibiotyping

Total of 13 antibiotic discs[2] belonging to 7 different antibiotic classes [colistin (10 µg), gentamycin (10 µg), ampicilin (10 µg), amoxycilin-clavulonic acid (20/10 µg), trimetophrim-sulphamethaxazole (1.25/23.75 µg), oxytetracycline (30 µg), neomycin (30 µg), lincomycin (15 µg), enrofloxacin (5 µg), imipenem (10 µg), cefazolin (30 µg), cefquinom (30 µg) and cefaperazone (75 µg)] were used in Kirby-Bauer Disc Diffusion Method (KBDDM). The zone diameters were interpreted according to EUCAST [4] breakpoints, and evaluated as sensitive, intermediate and resistant.

The antibiotyping was performed according to the susceptibility profiles of the strains by means of the Unweighted Pair Group Method using arithmetic averages (UPGMA) cluster analysis[3], and the dendrogram was created for evaluation of relatedness between the strains.

Determination of colistin resistance

All isolates were screened at a single screening concentration of 2 mg/L of colistin sulfate[1]. Isolates growth in screening medium (MacConkey Agar[1] containing 2 mg/L colistin) were tested for susceptibility to colistin by microbroth dilution test. Briefly, serial of two-fold dilutions of colistin sulphate powder in Mueller Hinton Broth[1] ranging from 0.25 to 128 µg/mL. Bacterial suspensions adjusted as 0.5 MacFarland were added to each well. After incubation at 35°C for 18-20 h, obtained MIC values were interpreted according to EUCAST criteria (susceptible ≤ 2 mg/L, resistant > 2 mg/L).

Determination of mcr genes

Escherichia coli strains were screened for *mcr1*, *mcr2* and *mcr3* genes using oligonucleotid primers described in Table 1. *mcr1*, *mcr2* and *mcr3* genes specific PCR analysis were performed in PCR conditions as described in the literature [10,20,21].

Genotyping

ERIC-2 oligonucleotide primer[4] was used to determine RAPD-PCR patterns of colistin positive isolates. The amplification was performed by modifying the method reported previously [18]. A total of 25

µl RAPD master mix containing DEPC-treated water, 1XPCR Buffer, 2.5 mM $MgCl_2$, 200 µM each dNTP[2], 2.5 U Taq DNA polymerase[2], 25 pmol primer and 5 µL template DNA was prepared. Amplification was carried out as follows: initial denaturation at 94°C for 5 min, 40 cycles of 94°C for 1, 40°C for 1 min, 72° C for 3 min and final extension at 72°C for 7 min. Amplification products loaded onto 1.5% agarose gel containing ethidium bromide were visualized after electrophoresis.

Dendrograms were generated from RAPD-PCR of isolates using UPGMA (Unweighted Pair Group Method with Arithmetic Averages).

Table 1. Oligonucleotide primers for screening of *mcr1*, *mcr2* and *mcr3*.

Target gene	Primer	Sequence
mcr1	CLR F	CGGTCAGTCCGTTTGTTC
	CLR R	CTTGGTCGGTCTGTAGGG
mcr2	MCR2 IF	TGTTGCTTGTGCCGATTGGA
	MCR2 IR	AGATGGTATTGTTGGTTGCTG
mcr3	MCR3 F	TTGGCACTGTATTTTGCATTT
	MCR3 R	TTAACGAAATTGGCTGGAACA
ERIC2	ERIC2	AAGTAAGTGACTGGGGTGAGCG

RESULTS

Bacterial strains

All 75 isolates selected from culture collection were identified conventionally as *Escherichia coli*.

Agar disc diffusion tests and antibiotyping

The resistance of dog and cat strains to all antibiotics used in the study is given in Figure 1. The rates of resistance to at least 3 and more antibiotics from different antibiotic classes (multi-drug resistance) were found to be 76.31% and 97.29% in dog and cat strains, respectively. Multi-drug resistances in cat and dog isolates were presented in Figure 2. All isolates were found as multi-drug resistant. Most of the isolates (14 and 16 isolates from cats and dogs, respectively) were resistant against 3 antibiotic class. However only three cat isolates were resistant against all 7 antibiotic class. It was determined that more cat isolates (91.89%) showed multiple antibiotic resistance than dog isolates (73.68%).

Colistin resistant strains showed similarity between 78-100% in terms of their antibiotic susceptibilities. The dendrograms showing the result of the antibiotyping is given in Figure 3.

Determination of colistin resistance

After screening of *Escherichia coli* strains in terms of their colistin resistance performed using MacConkey Agar containing colistin sulphate, 1 strain from cat and 2 from dogs were found as colistin resistant. In microbroth dilution test of these strains, they were also colistin resistant. However, a total of 17 strains (14 cats and 3 dog strains) were found to be colistin resistant in the microbroth dilution test (Table 2).

Determination of mcr genes

None of the strains were found to be positive for *mcr1*, *mcr2* and *mcr3* colistin resistance genes in PCR analysis.

RAPD-PCR

As a result of RAPD-PCR analysis of colistin positive cat and dog strains, it was determined that cat strains showed genotypic similarity between 59-96% and dog strains showed 63-67%. When examined all the strains phenotypically resistant to colistin, it was determined that the strains were divided into 3 clusters (DA, DB and DC) based on 70% similarity threshold. The strains showed genotypic similarity between 65-96%. Dendrogram obtained from RAPD profile analysis of all colistin resistant *Escherichia coli* strains is given in Figure 4.

Table 2. Microbroth dilution test results of dog and cat strains.

Strain No	Origin	MIC(µg/mL)
1	Dog	128
2	Dog	128
3	Dog	64
4	Cat	32
5	Cat	8
6	Cat	16
7	Cat	64
8	Cat	32
9	Cat	8
10	Cat	4
11	Cat	16
12	Cat	4
13	Cat	8
14	Cat	64
15	Cat	16
16	Cat	4
17	Cat	2

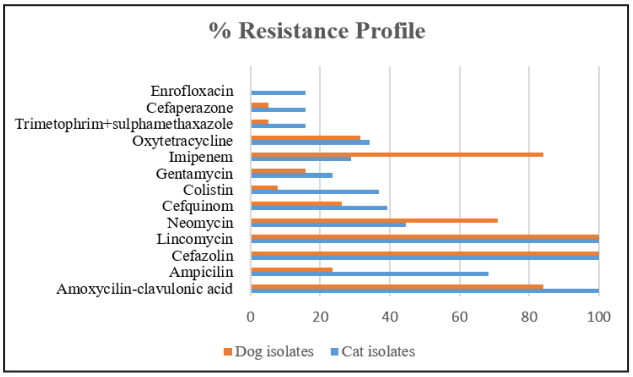

Figure 1. The resistance rates against all antibiotics in dog and cat isolates.

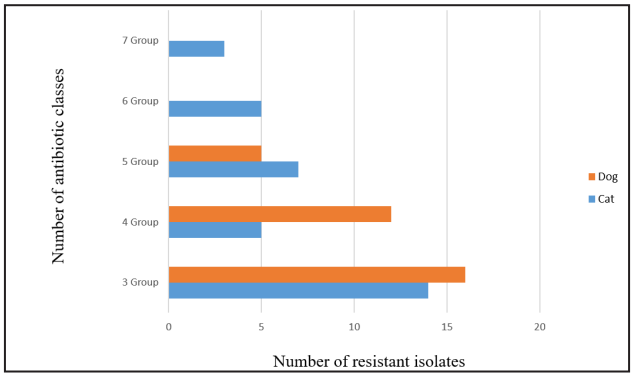

Figure 2. Multiple antibiotic resistance in dog and cat isolates.

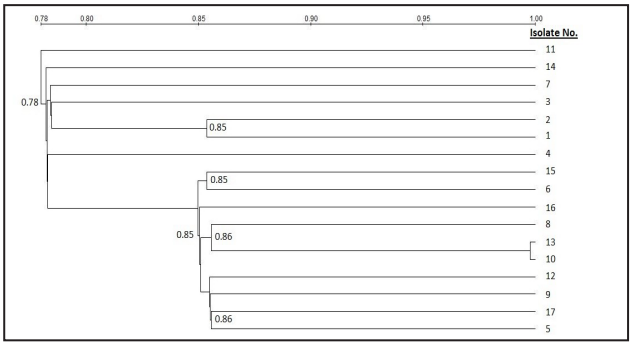

Figure 3. Dendrogram from antibiotyping of colistin resistant isolates.

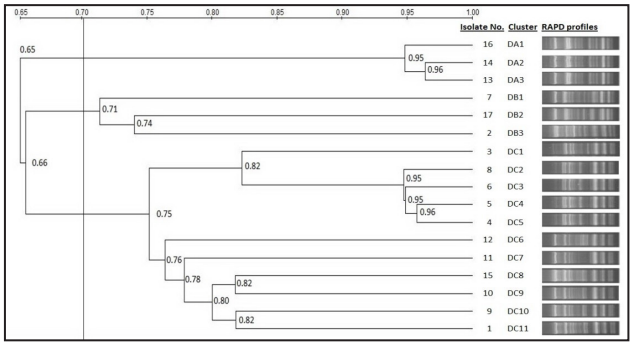

Figure 4. Dendrogram obtained from RAPD-PCR analysis of all colistin resistant strains.

DISCUSSION

In addition to infections caused by antibiotic resistant bacteria, there is an increase in infections caused by Gram negative bacteria with multiple antibiotic resistances. However, the development of new antibiotics is not sufficient to cope with this resistance problem. Therefore, different alternatives are being investigated for the treatment of infections with resistant bacteria and re-use of old antibiotics may be on the agenda. Colistin is one of the most important of such antibiotics that was introduced in the 1950s and was abandoned in the 1970s with less toxic and easier to use antibiotics. Colistin can be said to be one of the oldest, but increasingly important, specific agents used in the treatment of infections caused by resistant microorganisms [1]. Various opinions have been published that suggest that colistin is an important antibiotic in veterinary medicine, and that it should only be used with caution [6]. However, the role of colistin in the veterinary therapeutic arsenal has changed with the discovery of the plasmid-derived *mcr1* gene and then with many reports that this gene has spread worldwide, especially in animals, and also in humans and in the environment. These views were reviewed in 2016, according to these new findings. Since then, *mcr2* to *mcr5* genes have been identified. It is also important to note that other chromosomal mechanisms also cause colistin resistance. EMA updated recommendations on the use of drugs containing colistin in animals and recommended that the sale of such drugs should be minimized throughout the European Union. In addition, it has been suggested to use colistin-containing drugs as second-line therapy only in animals [7].

The plasmid-mediated colistin resistance gene, *mcr1*, has first been identified in 2015 in *Escherichia coli* strains isolated from food, food-producing animals and human patients [10]. The presence of *mcr1* in Enterobacteriaceae strains isolated from human and food-producing animals has subsequently been reported in about 12 countries [8,11,13,16]. In addition, *mcr1* colistin resistance gene has also been detected in *E. coli* strains isolated from companion animals

and probable contamination between these animals and humans has been investigated. The results have showed that *E. coli* carrying *mcr1* could be colonized in companion animals and transferred between humans and animals. In addition, it has been shown that, in addition to food animals and humans, accompanying animals may also play a role as a colistin-resistant *E. coli* reservoir, adding another complexity to the rapidly developing epidemiology of plasmid-mediated colistin resistance [22]. In China, *E. coli* which contained both the *mcr1* and *blaNDM*-5 gene mobile IncX3-X4 hybrid plasmid [15] has been identified. It has been also reported that mcr1 carrying *E. coli* strains were transported between companion animals and humans [9]. *E. coli* strains isolated from stool samples of the animals were *mcr1* positive by 7.4% in Real-time polymerase chain reaction [3]. In this study, colistin resistance was detected in 37.8% and 7.8% of *E. coli* strains isolated from healthy dog and cat stool samples, respectively. However, none of the strains found to have *mcr1* gene. The new plasmid-derived gene, *mcr2*, was found only in Belgium, leading to colistin resistance [20]. Thus, the hypothesis that *mcr2* induced resistance developing mechanism is different from that of the *mcr1* gene has emerged. In this study, no *mcr2* gene was detected in any of the strains with phenotypic colistin resistance. In 2017, another mcr variant, the *mcr3* gene, in porcine *E. coli* strains along with 18 other resistance genes on a conjugative plasmid has identified [21]. In this study, no *mcr3* gene was found in any of the *E. coli* strains.

In a Swiss study [14], colistin resistance has been determined in 2% of the isolates isolated from clinical samples of cats and dogs (2 of 4 isolates, of all dogs). Only one of these strains was *E. coli* (MIC 2 mg / L) isolated from urine. The colistin resistance rates in this study are different in cat and dog strains and differ with other studies. Although no isolates were found to be positive for *mcr1*, *mcr2* and *mcr3* genes in this study, considering the possibility of resistance to occur with other different mechanisms, it is thought that this colistin resistance, which is determined phenotypically, should not be ignored in terms of spreading both in cat and dog populations and in terms of risk to human health. However, reports for use of colistin in companion animals in Turkey should be followed and to prevent conditions that could support the emergence of plasmid-mediated colistin resistance, the polymyxins should be considered to use in a rational way in these animals.

MDR bacteria are one of the most important current threats both to public and animal health. Although, MDR bacteria are typically cause nosocomial infections, some MDR bacteria have found to be associated quite prevalently with community-acquired infections. The spread of MDR bacteria into the community is a crucial treat causing increased morbidity, mortality, healthcare costs and antibiotic use [17]. There is limited novel alternative antimicrobial for the treatment of infections due to MDR pathogens in the near future. The uses of colistin and polymyxin B have also been registered for topical administration to individual veterinary patients. In companion animals, prescription eye and eardrops are available with colistin alone, or in combination with other antimicrobials [7]. We found that multi-drug resistance in E. coli strains from healthy dogs and cats as 76.31% and 97.29%, respectively. EMA [7] has reported the percentage of MDR isolates in *E. coli* from poultry populations and meat thereof, reported as resistant to colistin in a table. This table includes the number (%) of isolates resistant to colistin only and the numbers (%) of isolates resistant to colistin being also resistant to one antimicrobial class /to none of the 9 additional antimicrobial classes. We found that all isolates showed MDR. It was detected that 3 colistin resistant cat isolates were also resistant 4 and more antibiotic classes and from 14 colistin resistant dog isolates, 5, 4 and 5 were also resistant against 6, 5 and 4 different antibiotic classes. In vitro antibacterial susceptibility patterns are still used in epidemiological investigations. The antibiotyping method used gives us quantitative results and based on the evaluation of multiple antibiotic resistances. The evaluation was performed using commercial software. No report/publication has been found where quantitative antibiotyping results of *E. coli* strains were given in a literature. The antibiotyping results in this study showed that all cat and dog isolates were more than 78% similar. Moreover, most of the cat isolates (71.4%) were more than 85% similar.

RAPD-PCR is a widely used method for genotyping various bacterial species. Comparison of RAPD-PCR and other genotyping results with antibiotic resistance profiles may be important in terms of showing the specific risk of resistance to treatment in infectious diseases, by showing whether some specific genotypes are similar to those of multiple resistance strains. The researchers have found a relationship between RAPD patterns and antibiotic resistance profiles of

Klebsiella pneumoniae strains isolated from clinical samples [19]. In this study, the RAPD patterns of *E. coli* strains isolated from cats and dogs were evaluated both in and between themselves. RAPD-PCR resulted in 14 and 3 different genotypes in cats and dogs, respectively. Based on 70% similarity threshold, it was observed that cat strains were grouped under 3 clusters (CA, CB and CC) among themselves, while 3 dog isolates were observed as "unique types". The genotypic similarities of the strains within the CC cluster, which contained a large proportion (64.28%) of the cat strains, ranged from 73% to 96%, while all the strains within the CB cluster showed similarity over 90%. The RAPD analysis of the dog isolates revealed that the genotypic similarity between the 3 strains found to be resistant to colistin was below 70%. Although the genotypic similarity of the 3 colistin-resistant dog isolates examined in the study was low, in the dendrogram in which the RAPD profiles were evaluated collectively, it was found that more than 80% similarity between the cat and dog isolates was occurred. When examined all the strains phenotypically resistant to colistin, it was determined that the strains were divided into 3 clusters (DA, DB and DC) based on 70% similarity threshold. The strains showed genotypic similarity between 65-96%. All colistin resistant strains also showed multiple antibiotic resistance and these results were also associated with RAPD genotypes. The results show that genotypically similar colistin-resistant strains can pose a risk of infection in cats and dogs and may also pose a potential risk to human health.

CONCLUSIONS

In conclusion, 18.6% of *Escherichia coli* strains isolated from cats and dogs were phenotypically determined colistin resistance, but this phenotype was not associated with *mcr1*, *mcr2* and *mcr3* genes investigated in the study. However, considering the presence of other resistance genes and other mechanisms that could lead to colistin resistance, resistant strains should be examined for other resistance genes. However, the results of genotypic typing performed in the study showed that cat and dog colistin resistant strains were genetically similar to each other. In the study, there is a risk that the colistin strains which are conventionally colistin resistant can spread between cats and dogs and also can be transferred to humans and these strains are considered to be a potential risk for human health.

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