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Determination of colistin resistance in *Escherichia coli* isolates from foods in Turkey, 2011-2015

Mustafa Güzel[1], M. Dilek Avşaroğlu[2], Yeşim Soyer[3]

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[1]Hitit University, Faculty of Engineering, Department of Food Engineering, Çorum, Turkey

[2] Ahi Evran University, Faculty of Agriculture, Department of Agricultural Biotechnology, Kırşehir, Turkey

[3]Middle East Technical University, Faculty of Engineering, Department of Food Engineering, Ankara, Turkey

ORCID IDs of the authors:

M.G. 0000-0002-4750-7488

M.D.A. 0000-0002-3106-9209

Y.S. 0000-0001-9687-9715

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Correspondence: Yeşim SOYER

E-mail: ysoyer@metu.edu.tr

ABSTRACT

Antimicrobial resistance of pathogenic microorganisms is an emerging public health concern. Intensive use of antibiotics in food animals might increase antimicrobial resistance in foodborne pathogens. Colistin is a last resort antibiotic for treatment of multidrug resistant (MDR) Gram negative pathogens. The recent antimicrobial resistance studies revealed a mobile antimicrobial resistance gene (*mcr*) that provides resistance to colistin. Furthermore, the gene has been found in different genera. Therefore, the aim of this study was to determine colistin resistance of *Escherichia coli* isolates (N=48) isolated in between 2011 and 2015 from food samples in Turkey. In addition, 5 *mcr* genes and their variants were screened by performing PCR on resistant isolates. 4 *E. coli* isolates were found resistant to colistin above the epidemiological cut-off value (Minimum inhibitory concentration (MIC) > 2mg/L). None of the resistant isolates had the *mcr* genes. Further studies with human and food isolates should be conducted to figure out which gene or genes are responsible for colistin resistance.

Keywords: Antimicrobial resistance, Colistin, *Escherichia coli*

Introduction

Antimicrobial resistance is one of the main public health concerns. Ineffective antibiotics might mean longer treatment, even death in some cases such as cancer treatment, organ transplant, surgeries, and dialysis (CDC, 2013). In the United States (US), more than two million of drug resistant case occurred, results in 23000 deaths, annually (CDC, 2013). It was estimated that the economic cost of drug resistant infections was around 35 billion dollars (CDC, 2013). One of the main reasons of antimicrobial resistance is to overuse of antibiotics in farm animals.

Colistin is a last resort antibiotic due to its nephrotoxic effect for humans. It is mainly used against MDR Gram negative infections (Ordooei et al., 2015). A pathogen is considered as MDR when it is resistant to 3 or more antibiotics (Tang et al., 2017). Colistin is also used in veterinary medicine to treat animal digestive disorders. Before the study of Liu et al. (2016), it was believed that colistin resistance occurred through chromosomal mutations, and thus, was only transferred vertically. However, Liu et al. (2016) discovered a transferrable, plasmid mediated gene (*mcr-1*) that causes colistin resistance in *E. coli*. The gene was dated back to mid 2000's and most likely spread from farm animals in China (Wang et al., 2018a).

After the identification of the gene, a number of reports showed that *mcr-1* has already spread globally, from South America (Arcilla et al., 2016; Fernandes et al., 2016), North America (McGann et al., 2016; Mulvey et al., 2016), Africa (Olaitan., 2016), to Japan (Suzuki et al., 2016) and South Asia (Tse and Yuen, 2016). Plasmid mediated colistin resistance has been found in almost all of the European countries (Campos et al., 2016; Doumith et al., 2016; Falgenhauer et al., 2016; Malhotra-Kumar et al., 2016; Prim et al., 2016; Zurfuh et al., 2016).

Although *mcr-1* gene has dominantly been found in *E. coli* (Arcilla et al., 2016), the gene was also identified in different species such as; *Enterobacter aerogenes* (Zeng et al., 2016), *Enterobacter cloacae* (Zeng et al., 2016), *Citrobacter freundii* (Li et al., 2017), *Citrobacter braakii* (Sennati et al., 2017), *Klebsiella pneumonia* (Liu et al., 2016), and *Salmonella enterica* (Webb et al., 2016). In addition, resistant isolates found in poultry, cattle (Hu et al., 2016), fowl (Yang et al., 2018), turkey, and pigs (Perrin-Guyomard et al., 2016) as well as environmental samples (Zurfuh et al., 2016). What is more, *mcr-1* was found in a number of different plasmid replicon types, which further contributes the dissemination of resistance (Wang et al., 2018b; Ye et al., 2016).

Recently, several variants of *mcr-1*, which were also provide resistance to colistin, were discovered. One of the genes, *mcr-2*, was found in Belgium (Xavier et al., 2016). The gene was on a 35k base plasmid, and was more prevalent than *mcr-1* in colistin resistant bacteria. It was also reported that the plasmid harboring *mcr-2* has a higher conjugation rate than original *mcr-1* harboring plasmid (Xavier et al., 2016). Yin et al. (2017) reported the discovery of *mcr-3*, another plasmid encoded colistin resistance variant of *mcr-1*. After the first report, *mcr-3* gene was found in *Salmonella* isolates from human patients in Denmark (Litrup et al., 2017; Roer et al., 2017), and *E. coli* isolates from cattle in Spain (Hernandez et al., 2017). In both studies, there were isolates carried both *mcr-1* and *mcr-3* gene. Soon after the discovery of *mcr-3*, Carattoli et al. (2017) were discovered the existence of *mcr-4* in *Salmonella* and *E. coli* isolates. The gene was found in pathogens that were isolated in Italy, in Spain, and in Belgium in a time frame between 2013 to 2016 (Carattoli et al., 2017). Borowiak et al. (2017) defined a transposon associated resistance gene, *mcr-5*, in *Salmonella* Paratyphi B. Yang et al. (2016) identified *mcr-7* in a IncI2 type plasmid in a *K. pneumoniae* isolate, in China. Wang et al. (2018) discovered mcr-8 in NDM producing *K. pneumoniae*. Lastly, *mcr-9* was identified in a *Salmonella* Typhimurium, in the US (Carroll et al., 2019). To date, more than 40 *mcr* genes and variants has been described. Additionally, co-occurrence of different *mcr* genes has been reported in several studies (Garcia et al., 2018; Hernandez et al., 2017; Yang et al., 2016).

Studies showed the animal to human transmission of *mcr-1* (Hasman et al., 2016; Liu et al., 2016; Ye et al., 2016). Considering the rapid dissemination of the gene and the importance of colistin in medicine, public health implications are severe. Therefore, the purpose of this study was to determine the colistin resistance, and to investigate the existence of *mcr-1* to *mcr-5* genes and their variants by screening the *E. coli* strains previously isolated from food samples collected in Van and Ankara, Turkey.

Materials and Methods

In this study, 48 *E. coli* strains that were isolated in between 2011-2015 from foods in previous studies, were screened (Table 1). Detailed description of Van samples was given by Kyere et al. (2015).

Isolation and Conformation of E. coli

A total of 28 samples were isolated in Van, 2011, and the rest were isolated in Ankara (n:20), 2015. *E. coli* isolates had been held in -80˚C prior the experiments, and was a part of METU Food Safety Lab collection. In both experiments, subsampling and isolation were performed following the *E. coli* isolation method of the Food and Drug Administration (FDA) (Feng et al., 2011).

Table 1. Van *E. coli* isolates, their sources, isolation locations, and phenotypic resistances

METU IDs	Specific food source	City Collected	Antibiotics resistant to
MET-K1-001	Raw milk	Van	NR
MET-K1-002	Herby Cheese	Van	NR
MET-K1-003	Raw milk	Van	Gentamycin
MET-K1-004*	Raw milk	Van	NR
MET-K1-005	Raw milk	Van	NR
MET-K1-006	Raw patty meat	Van	NR
MET-K1-007	Chicken wings	Van	AMP, FOX, NA
MET-K1-008	Salted cheese	Van	NR
MET-K1-009	Chicken drumstick	Van	AMP, AMC, SF, SXT, TE, CN, S
MET-K1-010	Chicken drumstick	Van	AMP, AMC, SF, SXT, TE, CN, S
MET-K1-011	Chicken drumstick	Van	AMP, SF, SXT, NA, TE, CN, S
MET-K1-012	Turkey wings	Van	AMP, TE
MET-K1-013	Chicken drumstick	Van	NR
MET-K1-014*	Chicken drumstick	Van	AMC, SF, SXT, NA, TE, S, K
MET-K1-015*	Chicken drumstick	Van	AMP, AMC, SF, SXT, NA, TE, S
MET-K1-016	Chicken drumstick	Van	AMP, AMC, SF, SXT, NA, TE, S
MET-K1-017	Chicken drumstick	Van	AMP, AMC, SF, SXT, NA, TE
MET-K1-018	Chicken drumstick	Van	AMP, SF, SXT, NA, TE, S, K, C
MET-K1-019	Chicken drumstick	Van	AMP, KF, SF, SXT, NA, TE, CN, C
MET-K1-020	Chicken drumstick	Van	NR
MET-K1-021	Chicken drumstick	Van	AMP, SF, SXT, TE, S
MET-K1-022	Chicken drumstick	Van	AMP, KF, SF, SXT, NA, TE, S, K, C
MET-K1-023	Raw milk	Van	NR
MET-K1-024*	Herby cheese	Van	AMP, AMC, SF, NA, TE, S, C
MET-K1-025	Chicken drumstick	Van	AMP
MET-K1-026	Herby cheese	Van	NR
MET-K1-027	Raw milk	Van	NR
MET-K1-028	Chicken drumstick	Van	AMP, SF, S, C

* Strains that exhibited colistin resistance.

AMP: Ampicillin, AMC: Amoxycillin/Clavulanic Acid, SF: Sulfafurazole, SXT: Sulphamethoxazole/Trimethoprim, TE: Tetracycline, S: Streptomycin, K: Kanamycin, C: Chloramphenicol Cro: Ceftriaxone, Eft: Ceftiofur, , Imp: Imipenem, Ak: Amikacin, Cn: Gentamicin, , Cip: Ciprofloxacin, N: Nalidixic acid, Fox: Cefoxitin, Kf: Cephalothin, Etp: Ertapenem, NR: Not resistant

Briefly, food samples were collected from the markets in city center of Van and Ankara, for *E. coli* isolation (Table 1). The samples then were transferred to the laboratory at the Food Engineering Department of Yuzuncu Yil University in Van and Food Engineering Department of METU. 25 g of each food sample was weighted aseptically, and was transferred to 225 mL of buffered peptone water for enrichment of *E. coli*, followed by homogenization using a stomacher. Homogenates were incubated at 36˚C for 18 h for cell enrichment, then 20 µL of each homogenate was sub-cultured on Endo Agar. After 18-24 h of incubation at 36˚C in an incubator, suspected colonies were isolated for further confirmation. In case of Van samples, suspected *E. coli* isolates were stored at −20˚C in brain heart infusion (BHI) broth with 15% (v/v) glycerol prior to transport to the Food Engineering Department at Middle East Technical University (METU), Ankara, Turkey.

Determination of Antimicrobial Resistance

The existence of *mcr-1* to *mcr-5* in the isolates were carried out in two steps. Initially, phenotypic colistin resistance of *E. coli* isolates was determined by MIC. Then borderline susceptible and resistance isolates was screened by PCR for the

existence of *mcr-1, mcr-2, mcr-3, mcr-4, mcr-5* genes (Rebelo et al., 2018).

Phenotypic Characterization of Antimicrobial Resistance

MIC testing was conducted with broth dilution method based on CLSI standards and EUCAST criteria (CLSI, 2014; EUCAST, 2016). For this, series of concentrations of colistin were prepared from commercial lyophilized colistin sulphate (Sigma-Aldrich), and added to the cation adjusted Muller Hilton Broth (CAMHB) in the test tubes, respectively. To standardize the inoculum density, 0.5 McFarland standard was used as turbidity standard. For every test, negative control (without antibiotic) was prepared.

PCR Screening for mcr Genes

After the MIC testing, isolates with 2 mg/L or higher colistin resistance was checked for *mcr-1* to *mcr-5* genes. PCR analysis was done described by Rebelo et al. (2018) by using the primers *mcr-1*-F (5′-GGCACCAGTATTGGCCTGCT-3′), *mcr-1*-R (5′- CATATGCGCCACAATGTGTTG -3′), *mcr-2-F* (CAAGTGTGTTGGTCGCAGTT), *mcr-2-R* (TCTAGCCCGACAAGCATACC), mcr-3-F (AAATAAAAATTGTTCCGCTTATG), *mcr-3-R* (AATGGAGATCCCCGTTTTT), mcr-4-F (TCACTTTCATCACTGCGTTG), *mcr-4-R* (TTGGTCCATGACTACCAATG) *mcr-5-F* (ATGCGGTTGTCTGCATTTATC), *mcr-5-R* (TCATTGTGGTTGTCCTTTTCTG). *E. coli* NTCC 13846 strain was used as positive control for *mcr-1* gene.

Conditions of PCR was as follows; 94 °C for 15 minutes for initial denaturation, 94 °C for 30 seconds, 58 °C for 90 seconds, 72 °C for 60 seconds for 25 cycle, and a final elongation at 72 °C for 10 minutes. Samples were taken from Eppendorf tubes, placed into gel, and ran in electrophoresis for 45 minutes. The gel was placed into staining-bath contains Ethidium Bromide solution for 30 minutes, rinsed in water, and visualized in BioRad Molecular Imager Gel-Dox XR.

Results and Discussion

In total, 48 *E. coli* strains isolated from various sources in two different cities were tested. 20 samples were isolated from poultry in Ankara, 2015. Rest of the samples were isolated from various sources (17 poultry, 6 raw milk, 4 cheese, and 1 meat) in Van, 2011 (Table 1) (Kyere et al., 2015). 11 samples were susceptible to antibiotics. Two samples were resistant to one antibiotic while the rest of the samples showed multi-resistance of two or more antibiotics. Furthermore, three of the samples were resistant to eight antibiotics. All resistant isolates were collected from food samples and were non-pathogenic. Van isolates were discussed in detail by Kyere et al. (2015). Unlike Van isolates, only three of the Ankara isolates were susceptible to antibiotics, phenotypically (Table 2). The rest were resistant to two or more antibiotics. Furthermore, 10 of the isolates were resistant to five or more antibiotics. Antibiotic resistance of Ankara isolates further characterized with PCR screening of antibiotic resistance genes. Majority of the isolates carried more than one antibiotic resistance gene (Table 3). Nine of the Ankara isolates carried Extended Spectrum β-Lactamase (bla_{TEM1}) gene. Although three isolates showed phenotypic resistance, resistance genes could not been determined.

MIC results revealed that 4 isolates were resistant to colistin (MIC ≥ 2mg/L) (Table 4). 3 of these isolates were resistant to at least two more antibiotics. All of the colistin resistant samples were isolated in Van, 2011. Among them two of the samples were isolated from dairy product (one cheese and one raw milk), the other two were isolated from poultry products. However, PCR screening of isolates for *mcr* genes were negative. The results implied that resistant isolates had a different resistance mechanism (e.g. vertical gene transfer) than plasmid mediated resistance. Another possible explanation might be the existence of other *mcr* genes. In this study, we screened samples only for *mcr-1* to *mcr-5*. However, there are 4 more *mcr* genes *mcr-6*, *mcr-7*, *mcr-8*, *mcr-9*, and their variants confer colistin resistance. Previous studies showed that currently ratio of plasmid mediated colistin resistance, due to *mcr-1*, is relatively low. For example, El Garch et al. (2017), reported that only 45 of 292 colistin resistant isolates had the *mcr-1* gene. Negative results might be associated with this low ratio.

Although *mcr* genes and their variants has already spread to the ecosystem, prevalence of *mcr-1* in food samples relatively low compared to wastewater and animal feces. Chen et al. (2017) reported the prevalence of *mcr-1* gene in colistin resistant bacteria from food samples as 36%, while the prevalence of *mcr-1* was 51% and 71% in animal feces and water, respectively. This difference might be due to the variety of gene acquisition mechanisms. For example, while plasmids that carry *mcr-1* in *Salmonella* food isolates were conjugative, plasmids in *Salmonella* animal isolates were not. It was suggested that *mcr-1* acquisition in food *Salmonella* isolates probably due to a cross species conjugation from *E. coli*, instead of *Salmonella* animal samples (Cui et al., 2017).

Table 2. Ankara *E. coli* isolates, their sources, isolation locations, and phenotypic resistances

METU IDs	Specific food source	City Collected	Antibiotics resistant to
MET A1-001	Chicken Breast	Ankara	CRO, EFT, AMP, AMC, FOX, KF
MET A1-002	Chicken Thigh	Ankara	AMP, AMC, FOX, KF
MET A1-003	Chicken Wing	Ankara	SF, SXT, C, CN, K, S, CIP, N, AMP, T
MET A1-004	Chicken Thigh	Ankara	CIP, N
MET A1-005	Chicken Thigh	Ankara	SF, SXT CN, K, CIP, N, AMP, T
MET A1-007	Chicken Wing	Ankara	S, AMP, KF
MET A1-008	Chicken Breast	Ankara	SF, SXT, C, K, S, CIP, N, AMP, T, KF
MET A1-009	Chicken Rib	Ankara	KF
MET A1-010	Chicken Wing	Ankara	SF, SXT, C, CN, S, CIP, N, AMP, T
MET A1-011	Chicken Wing	Ankara	SF, S, CIP, N, T
MET A1-012	Chicken Wing	Ankara	SF, K, CIP, N, T
MET A1-014	Chicken Wing	Ankara	NR
MET A1-015	Chicken Thigh	Ankara	SF, SXT, C, S, CIP, N, AMP, T
MET A1-016	Chicken Wing	Ankara	SF, SXT, C, CN, S, CIP, N, AMP, T, KF
MET A1-017	Chicken Rib	Ankara	NR
MET A1-018	Chicken Thigh	Ankara	NR
MET A1-019	Chicken Wing	Ankara	SF, SXT, C, K, S, CIP, N, AMP. T, KF
MET A1-020	Chicken Thigh	Ankara	SF, T
MET A1-021	Chicken Thigh	Ankara	CIP, N, ETP

AMP: Ampicillin, AMC: Amoxycillin/Clavulanic Acid, SF: Sulfafurazole, SXT: Sulphamethoxazole/Trimethoprim, TE: Tetracycline, S: Streptomycin, K: Kanamycin, C: Chloramphenicol Cro: Ceftriaxone, Eft: Ceftiofur, Imp: Imipenem, Ak: Amikacin, Cn: Gentamicin, , Cip: Ciprofloxacin, N: Nalidixic acid, Fox: Cefoxitin, Kf: Cephalothin, Etp: Ertapenem, NR: Not resistant

Table 3. Specific source and resistance profiles of *E. coli*, isolated in Ankara, 2015

Isolate ID	Specific Source	Phenotypic Resistance	Resistance genes
MET A1-001	Chicken Breast	CroEftAmpAmcFoxKf	*bla*$_{CMY-2}$
MET A1-002	Chicken Thigh	AmpAmcFoxKf	ND
MET A1-003	Chicken Wing	SfSxtCCnKSCipNAmpT	*bla*$_{TEM-1}$, *flo, aadA1, aadA2, aphA1-Iab, dhfrI, tetA, sul1*
MET A1-004	Chicken Thigh	CipN	ND
MET A1-005	Chicken Thigh	SfSxtCnKCipNAmpT	*bla*$_{TEM-1}$, *aadA1, aadA2, aphA1-Iab, tetA*
MET A1-007	Chicken Wing	SAmpKf	*bla*$_{TEM-1}$, *aadA1*
MET A1-008	Chicken Breast	SfSxtCKSCipNAmpTKf	*bla*$_{TEM-1}$, *cat1, aphA1-Iab*
MET A1-009	Chicken Rib	Kf	ND
MET A1-010	Chicken Wing	SfSxtCCnSCipNAmpT	*bla*$_{TEM-1}$, *flo, aadA1, dhfrI, tetA, sul1*
MET A1-011	Chicken Wing	SfSCipNT	*tetA*
MET A1-012	Chicken Wing	SfKCipNT	*aadA1, aadA2, tetA*
MET A1-014	Chicken Wing	Susceptible	*aadA2*
MET A1-015	Chicken Thigh	SfSxtCSCipNAmpT	*bla*$_{TEM-1}$, *flo, aadA1, aphA1-Iab, tetA, aadA2, sul1*
MET A1-016	Chicken Wing	SfSxtCCnSCipNAmpTKf	*flo, aadA1, aphA1-Iab, dhfrI, tetA, sul1*
MET A1-017	Chicken Rib	Susceptible	*bla*$_{TEM-1}$
MET A1-018	Chicken Thigh	Susceptible	*bla*$_{TEM-1}$, *aphA1-lab*
MET A1-019	Chicken Wing	SfSxtCKSCipNAmpTKf	*bla*$_{CMY-2}$, *cat1*
MET A1-020	Chicken Thigh	SfT	*tetA*
MET A1-021	Chicken Thigh	CipNEtp	*bla*$_{TEM-1}$, *aadA2*

Cro: Ceftriaxone, Eft: Ceftiofur, Sf: Sulfafurazole, Sxt: Sulphamethaxazole/trimethoprim, C: Chloramphenicol, Imp: Imipenem, Ak: Amikacin, Cn: Gentamicin, K: Kanamycin, S: Streptomycin, Cip: Ciprofloxacin, N: Nalidixic acid, Amp: Ampicillin, Amc: Amoxicillin-clavulanic acid, T: Tetracycline, Fox: Cefoxitin, Kf: Cephalothin, Etp: Ertapenem, ND: Not determined

Table 4. Colistin resistant *E. coli* isolates, their sources, other resistances, and MIC values

Isolate ID	Specific Source	Antibiotics resistant to	MIC
MET-K1-004	Raw milk	Susceptible	≥2mg/L
MET-K1-014	Chicken drumstick	AMC, SF, SXT, NA, TE, S, and K.	≥2mg/L
MET-K1-015	Chicken drumstick	AMP, AMC, SF, SXT, NA, TE, and S.	≥2mg/L
MET-K1-024	Herby cheese	AMP, AMC, SF, NA, TE, S, and C.	≥2mg/L

AMP: Ampicillin, AMC: Amoxycillin/Clavulanic Acid, SF: Sulfafurazole, SXT: Sulphamethoxazole/Trimethoprim, TE: Tetracycline, S: Streptomycin, NA: Nalidixic Acid, K: Kanamycin, C: Chloramphenicol

Nearly all plasmid mediated colistin studies showed that resistant isolates had multidrug resistance (MDR) including Extended Spectrum β-Lactamase (*bla*$_{ESBL}$, *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-55}$, *bla*$_{TEM1}$) (Li et al., 2016; Zeng et al., 2016; Zurfuh et al., 2016), Carbapanemases (Sun et al., 2016), and New Delhi Metallo β-Lactamase (*bla*$_{NDM-5}$, *bla*$_{NDM-9}$) (Borowiak et al., 2017; Yao et al., 2016). While our multidrug resistant isolates did not have *mcr* genes, the reports highlight the possible significant challenge in treatment of MDR infections.

Conclusion

MDR pathogens can be associated with increased treatment durations to life threatening conditions. There are currently a few antibiotics deployed against MDR infections. Colistin is a last line of defense against MDR Gram negative pathogens. Plasmid mediated colistin resistance disseminated rapidly between the several sources, bacterial species and continents. The absence of colistin treatment might have severe consequences. Previous studies showed the importance of farm animals and foods as gene transfer media. As a result, studies should focus on reducing the unnecessary antibiotic usage, and alternative treatments, especially in veterinary medicine.

Foodborne pathogen infections are still a serious threat for human health. In this regard, antibiotic resistance of foodborne pathogens is a critical concern. Colistin has been used for treatment of farm animals across all European countries. Considering the fact that plasmid mediated colistin resistance has spread globally, our results do not show or imply that *mcr* genes was not present in Van and Ankara, Turkey. Further studies with more isolates should be conducted. More sources such as hospitals, wastewater, and poultry farms should be monitored.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived the conflict of interests.

Ethics committee approval: Author declare that this study does not include any experiments with human or animal subjects.

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