

Original Research Article

INSIDIOUS INCREASE IN ITS COLISTIN RESISTANCE TOGETHER EXTENDED-SPECTRUM BETA-LACTAMASE-PRODUCING *ESCHERICHIA COLI* IN DAIRY COWS IN TÜRKİYE

ABSTRACT

Background: In recent years, the reporting of extended-spectrum beta-lactamase producing and colistin-resistant *Escherichia coli* (*E. coli*) in food-producing livestock animals is of great importance as a potential vector of multi-drug resistant (MDR) *E. coli* for the farm environment, farm workers, and veterinarians who are in close contact with these animals.

Materials and Methods: In this study, it was aimed to determine antibiotic resistance profiles of *E. coli* from diarrhoeic dairy cows, and observe the resistance against beta-lactam group antibiotics and colistin in 3 years' time. Fifty-four *E. coli* isolated from the fecal samples of diarrhoeic dairy cows were investigated for the multi-drug resistance. Forty-four *E. coli* showed MDR resistance, including colistin; nine *E. coli* isolates showed MDR resistance profiles involving at least six to ten antibiotics, but not for colistin. It was discovered that MDR *E. coli* isolates were also resistant to colistin. Resistance to beta-lactams were observed 100% in 2021 and 2022, but not for 2020. Colistin resistance was found to have increased progressively year over year, reaching 0% in 2020 and 37.03% and 46.29% in the next two years, respectively.

Conclusion: As a conclusion, circulation of beta-lactamase producing *E. coli* accompanied with colistin resistant *E. coli* in live-stock animals reared for the purpose of milk production should be concerned as a potential public health problem in terms of one health concept and more detailed investigations should be planned to question the ground origins of gradual increase.

Keywords: beta-lactamase, colistin, resistance, *E. coli*, bovine feces

1. INTRODUCTION

Escherichia coli is a common causative agent of enteric foodborne illness, urinary tract infections, blood stream infections, and gastroenteritis in animals and humans worldwide [1]. The emergence of multidrug resistant (MDR) Enterobacteriaceae including *E. coli* has become a major concern throughout the world and limited availability of novel antibiotics, colistin usage has been resumed as a last resort antibiotics in human and veterinary medicine [2].

Since the 1950s, colistin has been used for decades in veterinary medicine to treat and prevent infectious diseases, especially gastrointestinal infections brought on by Gram-negative bacteria in systems of intensive husbandry [3]. The gradual increase of colistin-resistant *E. coli* prevalence in foods and food-producing animals have great importance for human health since colistin, a last-resort antibiotics used for the human potentially fatal infection treatment by multiresistant-enterobacteria [4]. Since resistance to antibiotics like extended-spectrum cephalosporins (ESCs), carbapenems, and colistin has spread widely in humans, animals, and the environment, antimicrobial resistance

has grown to be a serious public health concern [1]. The extended-spectrum cephalosporins are among the beta-lactam antibiotics to which *Enterobacteriaceae* seek resistance through a variety of molecular strategies. The production of extended spectrum beta-lactamases (ESBLs) is the primary mechanism by which resistance to these antibiotics is induced [5]. Colistin resistance in gram negative bacteria is generally divided into two mechanisms: plasmid mediated and chromosomal mediated [1]. Recent years have seen a focus on plasmid-mediated resistance to ESCs and colistin in animals that are reared as food-production [6]. Given need to retain the efficacy of antimicrobials used to treat MDR infections in humans, the use of colistin in veterinary medicine is re-evaluated.

Numerous studies on chicken and chicken meat have also been carried out, and reports of a significant worldwide prevalence of ESC-resistant and colistin resistant *E. coli* have been made [7, 8]. Contrarily, there is a lack of research conducted on ruminants. In France and the Netherlands, the range of rate between 20.4-39.0% ESC-R *E. coli* harboring *mcr-1* gene was reported [9, 10].

In recent years, the coexistence of colistin resistance with ESBL-resistant *E. coli* of both human and veterinary origin, and the circulation of common resistance patterns between these two populations, has raised questions about the use of colistin, considered an antibiotic of last resort, in the veterinary field as well. The aims of this study were to determine antimicrobial resistance profiles of *E. coli* from diarrhoeic dairy cows, screen and evaluate for resistance to beta-lactam antibiotics and colistin resistance between 2020-2022.

2.MATERIAL AND METHODS

2.1 Fecal Samples

Between 2020 and 2022, 54 fecal samples were collected from the same dairy cow farm in Bandırma, Türkiye. To get fresh stools, the faeces were collected with the assistance of a veterinarian. A steril wooden medical spatula were used to collect 10 gr fecal sample per animal. The veterinarian reported that animals in the farm were under standart rules of hygiene, food consumption and restricted antibiotic use. Samples were collected using sterile tubes and sterile spatula. The samples were transferred to laboratory in two hours, immediately under cold chain.

2.2 Isolation and identification of *E. coli*:

Detection of *E. coli* was carried out according to the protocol of Quin et al. [11]. Approximately 1g of sample was suspended into 9 mL of modified Tryptone Soya Broth (Oxoid, CM0129) and incubated overnight at 37 °C. After the incubation, the broth was inoculated on MacConkey Agar (Oxoid, CM0115.) and incubated aerobically at 37 °C for 24h. The suspected colonies with round shapes, smooth surfaces and pink color on MacConkey Agar were inoculated on Eosin-Methylene Blue (EMB) Agar (Oxoid, CM0069). At the end of incubation, the suspected colonies of *E. coli* for the characteristic metallic sheen on Agar were examined with Gram's stain. After the examination, one *E. coli* colony was inoculated into Brain Heat Infusion Broth (BHIB) (Oxoid, CM1135) Pure culture of *E. coli* were identified on biochemical tests, oxidase test, coagulase test, catalase test, indole test, methyl red test, Voges-Proskauer test, citrate (Simmons) test [12]. Afterwards, the cultured samples were stored at -20 °C for antibiotic susceptibility testing.

2.3 Antimicrobial susceptibility testing:

For antimicrobial susceptibility testing, a total of 11 antibiotic discs (Oxoid) belonging to six different antibiotic classes- enrofloxacin (ENR 5µg), gentamicin (GEN 10µg), amoxicillin/cluvalanic acid (AMC 20/10µg), trimethoprim-sulfamethoxazole (SXT 25µg), oxytetracycline (OT 30µg), cephaperazone (CFP 75µg), neomycin (N 30µg), cefquinom (CEQ 30µg), cephalixin/kanamycin (CFXK 15 µg), tetracyclin (TE 30 µg), colistin (CT 1 µg)- were used in Kirby-Bauer Disc Diffusion Method. The zone diameters were interpreted according to European Committee on Antimicrobial Susceptibility Testing Standarts (EUCAST) [13], and evaluated as sensitive, intermediate and resistant.

3.RESULTS

In our study, all the samples were collected from the cow farm in Bandirma-Türkiye. Between 2000- 2022, 41 out of 54 *E. coli* isolated from dairy cows which suffered diare were found to be haemolytic, the remaining 13 were non-haemolytic. In order to cure enteritis the antibiotics emphasized in Table 1 were used.

Between 2020 and 2022, phenotypically nine and 44 out of 54 were determined to be moderate sensitive and resistant to colistin, respectively. Three out of 13 non-haemolytic *E. coli* were determined to be colistin resistant, the remains were moderate sensitive while all haemolytic *E. coli* exhibited colistin resistance. When the rates of colistin resistance were evaluated over the three years, the rate, which was found to be 0% in the year 2020, increased to 37.03% and 46.29% in the subsequent two years,

respectively. Except for the year 2020, in the other two years, 100% resistance to beta-lactam group antibiotics was exhibited, and parallel to this, it was observed that resistance was demonstrated in all 20 *E. coli* isolates except for five isolates in 2021, and in all 25 isolates in 2022 (Table 1).

E. coli isolates from dairy cows with diarrhea displayed multi-drug resistance to fluoroquinolone (ENR), aminoglycoside (GEN and N), beta-lactam (AMC, CFP, CEQ, CFXX), tetracycline (TE and OT), sulfonamide (SXT) group antibiotics. MDR profiles *E. coli* were emphasized in detail in Table 2.

Nine *E. coli* isolates exhibited MDR resistance profiles at least six to ten antibiotics, but not for colistin, 44 *E. coli* exhibited MDR resistance including colistin. Colistin resistant *E. coli* isolates were found to be MDR, as well (Table 2).

When we evaluated resistance to beta lactam group antibiotics; in 2020 none of the *E. coli* isolates exhibited resistance against colistin while AMC/CFP/CEQ/CFXX, CFP/CFXX, CFXX and CEQ resistance profiles were observed in each one among four *E. coli* isolates, respectively. In 2021, AMC/CFP/CEQ/CFXX resistance profiles were observed in five *E. coli* isolates however those were sensitive to colistin. In the same year and following year, AMC/CFP/CEQ/CFXX/CT resistant profiles were determined in 20 and 25 isolates, respectively. An increase in resistance to colistin has been observed in parallel with the year-by-year increase in resistance to beta-lactam group antibiotics (Table 3).

4. DISCUSSION AND CONCLUSIONS

There is a dearth of information on the colistin resistance of animal microorganisms and foodstuffs. The European surveillance of bacteria of animal origin did not include colistin in its mandatory antimicrobial panel for *Enterobacteriaceae* until 2014 [14]. Similarly, polypeptides were not relatively considered in the antimicrobial resistance monitoring schemes of many other countries. This condition can be explained by several factors. First, the exclusion of polypeptides from epidemiological studies and animal-origin bacterial monitoring systems in many nations likely stemmed from the lack of colistin usage or its restricted application in both humans and animals in these regions. Subsequently, scientists and veterinarians likely assumed that colistin resistance was rare due to co-selection of colistin resistance by other antibiotics or clonal expansion of a colistin-resistant (CST-R) isolate because colistin resistance was thought to be limited in *Enterobacteriaceae* (which are the main indicators of resistance in Gram-negative bacteria). It was thought to solely been caused by chromosomal mutations until the end of 2015 [15]. Ultimately, they neglected colistin resistance surveillance. However, the use of colistin as a last-resort antibiotic in people has increased, making it necessary to monitor the development of resistance to this polypeptide in humans, companion animals, and food animals with more precision and thoroughness.

In veterinary field, among farm animals such as pigs, ruminants, poultry and companion animals such as rabbits, horses, dogs, cats colistin usage has been widely-spreaded in all continents for decades [16]. Isolated CST-R *E. coli* in pigs, hens, broilers, ducks, rabbits from France [17], in companion animals such as horses, dogs, cats from Sweden [18], Norway [19], Germany [20], Türkiye [21], in piglets from Thailand [22], in yaks from

India [23], in diarrhoeic ruminants from Spain [24] were reported among the animals including companion animals and live-stock animals.

In Türkiye, the studies conducted on colistin resistance was limited and studied group comprised animals and poultry [25-27]. Erzaim [25] declared that fifteen out of 200 *E. coli* isolated from the intestine of broiler chickens was phenotypically resistant to Polimiksin E and none of colistin resistance were found to be plasmid-mediated. Sezener et al [28] reported 22.66% colistin resistance among 75 *E. coli* isolated from cats and dogs, and none of the isolates were found to harbor mcr 1-3 gen. When Tok [27] investigated *E. coli* and *Salmonella* isolates the author declared that *E. coli* and *Salmonella* isolates collected from chicken meat samples between 2018-2019 showed evidence of CST-R with the rate of 25% and 53%, respectively while historical *E. coli* and *Salmonella* isolates exhibited 0% and 8%, respectively. According to the results of Tok [27], colistin resistance was seen to be increased over time among both *Salmonella* and *E. coli* isolates. Parallel to the previous study the rate of colistin resistance in the current study was found to be gradually increased over the years. When the rates of colistin resistance were evaluated over the three years, the rate, which was found to be 0% in the year 2000, increased to 37.03% and 46.29% in the subsequent two years, respectively. The inappropriate use of colistin in agriculture as a growth promoter in livestock has contributed to the selection of resistant strains. Inadequate infection control practices, poor sanitation, mobility of people and animals have also contributed to the spread of CST-R *E. coli* in the dairy farm [28].

High MDR resistance occurred in *E. coli* isolates in the current study to fluoroquinolone (ENR), aminoglycoside (CN and N), tetracycline (TE and OT), sulfonamide (SXT)

group, polymyxine (colistine sulphate) antibiotics were thought not to be astonishing. In alignment with our study, MDR *E. coli* from the feces of bovine animals in Europa was declared [29]. Sezener et al. [26] also reported multi-drug resistance to similar antibiotics in *E. coli* isolates from cats and dogs in Türkiye. Recently, global increase in human *E. coli* isolates exhibiting resistance to beta lactams, cephalosporins possessing *bla*_{CTX}, *bla*_{NDM}, *bla*_{TEM} were reported to be resistant to colistin harboring plasmid mediated *mcr* genes, as well food-producing farm animals in Latin America [30], in Europea, and Asia [16, 31]. In our study, the same situation was observed in a dairy cow farm. Except for the year 2020, along with 100% resistance to beta-lactam group antibiotics in the other two years, colistin resistance was demonstrated in all 20 *E. coli* isolates except for five isolates in 2021, and in all 25 isolates in 2022 (Table 1). *E. coli* can be found in the intestines of animals and humans, and food. CST-R *E. coli* in livestock can contaminate meat and milk; should they are not handled or cooked properly, great risks to human health can occur [32]. Recent studies showed that ESBLs and carbapenem resistance accompanied emerging CST resistance in the same microbes have great significance in terms of health associated MDR infections [1]. In our study, beta lactam group-AMC, CFXK and ESBL group- CFP, CEQ- resistant *E. coli* isolated from the feces of diarrhoeic dairy were also found to be resistant to colistin. Supporting our results, it has been demonstrated that ESBL-producing *E. coli* can acquire colistin resistance in vitro experiments by Nakayama et al [33]. In parallel to our results, in a study conducted in Tunisia, Saidani et al. [34] declared where they detected 14 ESBL-producing *E. coli* in 219 fecal samples, they found that 11 of those also exhibited colistin resistance. Similiarly, in France of 106 out of 517 ESBL-

producing *E. coli* isolates carrying *mcr-1* gene were also found to be resistant to colistin [35]. Moreover, *Mcr-1* gene carried on IncHI2 plasmid in CST-R *E. coli* from calf fecal samples in Tunisia between 2016-2017 and bovine digestive samples in France between 2004-2014 [36] were reported. Those were co-localized with *bla*_{CTX-M-14}, *bla*_{CTX-M-55} and *bla*_{CTX-M-14}, *bla*_{CTX-M-1} in isolated ESBL producing *E. coli* [34]. Although we did not examine the presence of both *mcr* and *bla*_{CTX} genes in our isolates, colistin resistant *E. coli* exhibiting resistance to beta lactam group antibiotic AMC; CFXK (2. Generation cephalosporin); and ESBL group antibiotics, CFP (3. Generation cephalosporin); CEQ (4. Generation cephalosporin) were suspected to harbor those genes. In further studies, the presence of those will be investigated in detail in our isolates. The study's findings all point to the necessity of a thorough, one health strategy that prioritizes cleanliness, prudent use of antibiotics in agriculture, and antibiotic stewardship in clinical settings [37].

Consequently, the increasing colistin resistance exhibited by ESBL-resistant *E. coli* in food-producing farm animals from year to year, along with the similar resistance patterns observed in human *E. coli* isolates globally poses a significant public health threat. Although our study focused on phenotypic resistance and did not examine the transfer of common resistance genes, future research should specifically investigate similar resistance genes in live-stock animals, as well as the spread of epidemic plasmids in both humans and animals. As a result, concerted efforts are required in both human and veterinary medicine.

ETHICAL APPROVAL

According to the subparagraph k of the 8th article of the "Regulation on the Principles and Procedures of Animal Experiment Ethics Committees" published in the Official Gazette dated 15.02.2014 and numbered 28914, the collection of fecal or bedding samples and sample collection by swabbing are not subject to the approval of the Local Ethics Committee for Animal Experiments (HAYDEK).

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UNDER PEER REVIEW

Table 1. Resistant profiles of *E. coli* isolates between 2020-2022

Year	Number of bacteria	ANTIBIOTICS										
		ENR	GEN	AMC	SXT	OT	CFP	N	CEQ	CFXK	TE	CT
2020	1	R	I	S	R	R	I	R	I	R	R	I
	1	R	R	R	R	R	R	R	R	R	R	I
	1	R	R	S	R	R	S	S	R	S	R	I
	1	R	R	I	R	R	R	R	S	R	R	I
	1	R	R	R	R	R	R	R	R	R	R	I

	1	S	R	R	R	R	R	R	R	R	R	R	I
	1	R	R	R	R	R	R	R	R	R	R	R	I
2021	1	R	I	R	R	R	R	R	R	R	R	R	R
	17*	R	R	R	R	R	R	R	R	R	R	R	R
	1	R	I	R	R	R	R	R	R	R	R	R	I
	2	R	I	R	R	R	R	R	R	R	R	R	R
	1	R	I	R	R	R	R	R	R	R	R	R	I
2022	24*	R	R	R	R	R	R	R	R	R	R	R	R
	1	S	R	R	I	R	R	R	R	R	R	R	R
TOTAL	54												

*, Haemolytic *E. coli*; ENR, enrofloxacin; GEN, gentamicin; AMC, amoxicillin/cluvalanic acid; SXT, trimethoprim-sulfamethoxazole; OT; oxytetracycline; CFP, cephaperazone; N, neomycin; CEQ, cefquinom; CFXXK, cephalixin/kanamycin, TE, tetracyclin; CT, colistin; R, resistance; I, Intermediate; S, Sensitive.

Table 2. MDR profiles of *E. coli* isolates

Number of <i>E. coli</i>	MDR profiles
41	ENR/GEN/AMC/SXT/OT/CFP/N/CEQ/CFXK/TE/CT
1	GEN/AMC/OT/CFP/N/CEQ/CFXK/TE/CT
1	ENR/AMC/SXT/OT/CFP/N/CEQ/CFXK/TE
2	ENR/AMC/SXT/OT/CFP/N/CEQ/CFXK/TE/CT
1	ENR/AMC/SXT/OT/CFP/N/CEQ/CFXK/TE
1	ENR/AMC/SXT/OT/CFP/N/CEQ/CFXK/TE/CT
1	ENR/GEN/AMC/SXT/OT/CFP/N/CEQ/CFXK/TE
1	GEN/AMC/SXT/OT/CFP/N/CEQ/CFXK/TE
1	ENR/GEN/AMC/SXT/OT/CFP/N/CEQ/CFXK/TE
1	ENR/GEN/SXT/OT/CFP/N/CFXK/TE
1	ENR/GEN/SXT/OT/CEQ/TE

1	ENR/GEN/AMC/SXT/OT/CFP/N/CEQ/CFXK/TE
1	ENR/SXT/OT/N/CFXK/TE

ENR, enrofloxacin; GEN, gentamicin; AMC, amoxicillin/clavulanic acid; SXT, trimethoprim-sulfamethoxazole; OT; oxytetracycline; CFP, cephaperazone; N, neomycin; CEQ, cefquinom; CFXK, cephalixin/kanamycin, TE, tetracyclin; CT, colistin.

Table 3. The increased beta-lactam and colistin resistance over the years

Year	Number of <i>E. coli</i>	Resistance profiles of beta-lactams and colistin
	1	CFCK
2000	1	AMC/CFP/CEQ/CFXK
	1	CEQ
	1	CFP/CFXK
2001	5	AMC/CFP/CEQ/CFXK
	20	AMC/CFP/CEQ/CFXK/CT
2022	25	AMC/CFP/CEQ/CFXK/CT

AMC, amoxicillin/clavulanic acid; CFP, cephaperazone; CEQ, cefquinom; CFXK, cephalixin/kanamycin; CT, colistin.

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