Investigation of Antibiotic Resistance, Phylogenetic Groups and Clonal Relationships of Colistin Resistant *Escherichia coli* Isolates Obtained from Broilers

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ABSTRACT

The emergence of resistance mechanisms in bacteria that can be carried and spread through plasmids can lead to problems, particularly in densely populated poultry environments such as broilers, where the spread of antibiotic resistance and the reduction of treatment options can occur. The aims of this study were to investigate the antibiotic resistance profiles of *Escherichia coli* isolates obtained from broilers with colibacillosis, the presence of the most prevalent plasmid-mediated mobile colistin resistance gene-1 (*mcr*-1) and the phylogenetic groups and clonal relationships of colistin resistant isolates. The material for the study consisted of 184 *E. coli* isolates obtained from commercial broilers. Bacterial identification was performed using conventional methods. The susceptibility of the isolates to 20 antibiotics belonging to nine antimicrobial families was examined using the BD Phoenix™ M100 automated system and NMIC/ID 433 panels. Isolates resistant to at least three or more antibiotic classes were considered as multidrug-resistant (MDR). The presence of the *mcr*-1 in the isolates was investigated by polymerase chain reaction (PCR), while their phylogenetic groups were determined by quadruplex PCR and clonal relationships were analyzed using enterobacterial repetitive intergenic consensus sequences (ERIC) PCR. According to the results of the antibiotic susceptibility testing, the highest resistance was observed against tigecycline (100.0%), ampicillin (78.8%), and ciprofloxacin (53.8%), while the highest susceptibility was observed against amikacin, imipenem, and meropenem (100.0%). Of the isolates, colistin resistance was detected phenotypically in 4.4% (8/184) and genotypically in 5.4% (9/184). All phenotypically resistant isolates carried the *mcr*-1 gene. Of all the isolates, 72.8% were MDR, while 100.0% of the colistin-resistant isolates were MDR. Among the nine colistin-resistant *E. coli* isolates, eight (88.9%) were classified into four phylogenetic groups (B1, C, F, E), while one isolate (11.1%) could not be classified. Using ERIC typing, a total of seven ERIC types were identified, including six single and one multiple genotypes from seven farms. Two isolates obtained from the same farm had the same genetic background and one isolate obtained from a different farm was closely related. This indicates that there is clonal dissemination among isolates carrying the *mcr*-1 gene, and it suggests that this resistance gene can spread within the same farm. This study is the first research in Türkiye demonstrating the coexistence of low colistin resistance and high tigecycline resistance in APEC isolates obtained from broilers in the food chain.

Keywords: Broiler; Clonality; Colistin Resistance; *Escherichia coli*; Phylogenotyping.

INTRODUCTION

In addition to commensal *Escherichia coli* in humans and animals, many pathogenic *E. coli* strains cause intestinal and/or extraintestinal infections. One of these strains, avian pathogenic *E. coli* (APEC), easily spreads among poultry through horizontal and vertical transmission routes, leading to extraintestinal infections with a septicemic course and fatal outcomes in poultry (1).

Antimicrobial resistance is a significant problem in APEC strains. It has been reported that APEC isolates show resistance to various antibiotics commonly used in the poultry industry, such as tetracyclines, sulfonamides, and aminoglycosides (1). Resistant forms of APEC strains to antibiotics are known to activate antibiotic resistance genes in other pathogenic *E. coli* strains and these resistance genes can be easily transmitted between animals and humans (2).

Infections caused by multidrug-resistant bacteria are often treated with carbapenems in humans, and in cases where the bacteria are resistant to carbapenems, colistin is used as a last alternative (3). Colistin is a polypeptide antibacterial agent belonging to the polymyxin group, which exhibits activity against Gram-negative bacteria. Colistin interacts with the negatively charged lipopolysaccharide (LPS) layer of bacterial cell membranes. This interaction involves the addition of a compound called phosphatidyl ethanolamine to the lipid A region of LPS, altering the structure of the targeted LPS layer and reducing the effectiveness of colistin (4).

Colistin is used in the treatment of *E. coli* induced gastrointestinal infections in cattle, sheep, pigs, and poultry (5). Initially, colistin resistance was believed to be solely caused by chromosomal mutations. However, the emergence of plasmid-mediated resistance mediated by mobile colistin resistance (*mcr*) genes has increased the significance of this problem (6). Since these genes are located on plasmids, they can be easily transferred to other bacteria (7). To date, ten different variants of the *mcr*-1 gene have been identified in various bacteria isolated from animals, food, farms, humans, and the environment (8).

Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences are repetitive palindromic sequences of 127 base pairs in length that are present in multiple copies in bacterial genomes. ERIC-PCR is useful for identifying clonal groups formed by isolates of the same origin (9). ERIC-PCR is an easily applicable and rapid method that involves comparing the generated clusters. If isolates have similar ERIC-PCR profiles, it may be inferred that they have the same origin and are part of the same clonal group (10).

The *E. coli* phylotyping method contributes to our understanding of the genetic diversity and relationships of *E. coli* strains, aiding in disease control and outbreak prevention strategies. A quadruplex PCR method has been developed to characterize the phylogroups of *E. coli* based on the presence of different marker genes (*arp*A, *chu*A, *yja*A, and *TspE*4. C2) (11). Taxonomically, eight phylogenetic groups of *E. coli* have been recognized (A, B1, B2, C, D, E, F, clade I) (11). Recently, it has been reported that certain strains belong to an intermediate group called phylogroup G, situated between the F and B2 phylogroups (12). According to the Clermont classification, phylogroup A primarily consists of low-virulence isolates and represents commensal strains, while phylogroup B1, commonly found in the intestinal flora, represents environmentally derived strains (13). The more pathogenic isolates are found in phylogroups B2 and D (14). Phylogroup B2 is highly virulent and poses a greater risk to human health. Phylogroup F, often containing enterohemorrhagic isolates, is the sister group of phylogroup B2 and exhibits lower virulence. Phylogroup C, which includes low-virulence commensal groups, is closely related to phylogroup B1 (11). Very little is known about phylogroups C and F (11).

Studies on colistin resistance in broilers in Türkiye are relatively new and limited in number. The first report of colistin resistance in broilers in Türkiye came in 2018 when it was found that one out of four *E. coli* isolates obtained from chicken meat samples in the southern region of Türkiye carried the *mcr*-1 gene (15). Two years later, another study detected the *mcr*-1 gene in one out of eleven phenotypically colistin-resistant isolates obtained from raw chicken meat samples (16). However, in a study conducted in 2021, no *mcr*-1 gene was detected in any of the fifteen phenotypically colistin-resistant *E. coli* isolates obtained from chicken fecal samples (17).

The aims of this study were to investigate the antibiotic resistance profiles of *E. coli* isolates obtained from broilers with colibacillosis, the presence of the *mcr*-1 gene, which is the most common plasmid-mediated colistin resistance gene, the phylogenetic diversity of colistin-resistant isolates and their clonal relationships.

MATERIAL AND METHODS

Ethical Statement

The isolates used in the study are derived from previous works of the author, and in accordance with article 8(o) of our university's ethics committee regulations, there is no requirement for ethical committee approval for clinical practices for diagnostic and therapeutic purposes.

Material

The material of this study consisted of samples collected from the diseased livers of 243 broilers of the Ross 308 breed, obtained from seven farms belonging to the same poultry integration company, and sent to the routine diagnostic laboratory approximately one year (January-December 2022) of Aydın Adnan Menderes University, Faculty of Veterinary Medicine. The collected samples were stored in Stuart Medium (Oxoid, UK) at refrigeration temperatures (2-8°C) until they were sent to the laboratory. Samples were mostly taken during slaughter, and in cases where broilers died before slaughter, necropsies were performed to collect samples. The broilers sampled were taken from broilers of between 14-40 days old. Clinical symptoms observed in broilers included recurrent unresponsive cough, anorexia, dyspnea, diarrhea, weight loss, lameness, and mortality starting from the first week of life. It was noted that colistin sulfate was occasionally used for the treatment of upper respiratory tract and gastrointestinal infections in all the sampled flocks, for the purpose of infection treatment.

Bacterial isolation and identification

Isolation was performed using standard bacteriological methods. Swabs were streaked onto MacConkey Agar (Merck 105465, Germany) and incubated aerobically at 37°C for 24 hours. The next day, a single lactose-positive colony on MacConkey agar was sub-cultured onto EMB agar (Merck 101347, Germany). After another 24 hours of incubation at 37°C, *E. coli* showing characteristic green metallic sheen colony was collected. These colonies were subjected to biochemical tests (motility, oxidase test, citrate utilization, indole test, methyl red, Voges-Proskauer, etc.). For the confirmation of bacterial identification, an automated system (BD Phoenix, Becton-Dickinson, USA) was used for evaluation according to the manufacturer's instructions. The isolates were stored in Brain Heart Infusion Broth (BHIB) containing 20% glycerol (Merck 110493, Germany) at -20°C.

Antibiotic resistance

Antibiotic susceptibility test:

Antibiotic susceptibility testing (AST) of the isolates identified as *E. coli* was performed using the BD Phoenix (Becton-Dickinson, USA) automated system with NMIC/ID 433 panels. The isolates were tested against 20 antibiotics belonging to nine different antimicrobial families (Lipopeptide: amikacin (AN), gentamicin (GM); Carbapenem: ertapenem (E), imipenem (IPM), meropenem (MEM); Cephem: cefazolin (CFZ), cefuroxime (CXM), ceftazidime (CAZ), ceftriaxone (CRO), cefepime (FEP); Penicillin: ampicillin (AMP); Beta Lactam: ceftolozane-tazobactam (CT), amoxicillin clavulanate (AMC), ampicillin sulbactam (AS), piperacillin-tazobactam (PT); Lipopeptide: colistin (COL); Folate: trimethoprim-sulfamethoxazole (TS); Quinolone: ciprofloxacin (CIP), levofloxacin (LF), Tetracycline: tigecycline (TIG)). The resistance status of the isolates against these antibiotics was examined. The results were evaluated according to the criteria of the "European Committee on Antimicrobial Susceptibility Testing (EUCAST)" (18). *E. coli* ATCC 25922 strains were used as quality control organisms.

Phenotypic detection of colistin resistance:

During the study, colistin resistance in all isolates was examined using an automated system. The interpretation of colistin minimum inhibitory concentration (MIC) results was carried out using the EUCAST clinical breakpoints (susceptible ≤2mg/L; resistant >2mg/L) (18).

Multidrug resistance (MDR) and multidrug resistance index (MAR):

MDR was defined as resistance to three or more antimicrobial classes (19).

The MAR index, which indicates the spread of resistance in the examined bacterial populations, was calculated as the ratio of the number of antibiotics to which the bacterium is resistant to the total number of antibiotics analyzed. A MAR index result greater than 0.2 indicated the presence of media-borne bacterial strains in which more than one antibiotic was used (20).

Table 1. All primers used in this study.

	Target gene	Primer sequence (5′→3′)	Tm (°C)	Size (bp)	Reference
Colistin resistance	*mcr*1-F	CGGTCAGTCCGTTTGTTC	56.0	309	3
	*mcr*1-R	CTTGGTCGGTCTGTAGGG	58.2		
Clonality	ERIC-F	ATGTAAGCTCCTGGGGATTCAC	62.1	Variable	9
	ERIC-R	AAGTAAGTGACTGGGGTGAGCG	64.0		
Phyloyping	*chu*A.1b	ATGGTACCGGACGAACCAAC	60.5	288	11
	*chu*A.2	TGCCGCCAGTACCAAAGACA	60.5		24
	yjaA.1b	CAAACGTGAAGTGTCAGGAG	58.4	211	11
	yjaA.2b	AATGCGTTCCTCAACCTGTG	58.4		
	TspE4C2.1b	CACTATTCGTAAGGTCATCC	56.4	152	11
	TspE4C2.2b	AGTTTATCGCTGCGGGTCGC	62.5		
	AceK F	AACGCTATTCGCCAGCTTGC	60.5	400	11
	ArpA1 R	TCTCCCCATACCGTACGCTA	60.5		25
Group E	*ArpAgpE* F	GATTCCATCTTGTCAAAATATGCC	60.1	301	26
	ArpAgpE R	GAAAAGAAAAAGAATTCCCAAGAG	58.4		
Group C	*trpAgpC*.1	AGTTTTATGCCCAGTGCGAG	58.4	219	26
	trpAgpC.2	TCTGCGCCGGTCACGCCCC	68.1		
Internal Control	*trpBA* F	CGGCGATAAAGACATCTTCAC	59.4	489	27
	trpBA R	GCAACGCGGCCTGGCGGAAG	68.7		

T_m: Annealing temperature.

PCR

DNA extraction, purity, and quantification controls:

In this study, DNA extraction was performed using the sonication method (21). For this purpose, *E. coli* stock cultures were passaged onto EMB agar and incubated at 37°C for 24 hours. A single colony from this bacterial culture was picked and transferred to 5 ml of nutrient BHIB broth, followed by incubation at 37°C for 18-24 hours. After incubation, the culture was centrifuged at 13.500 rpm for 5 minutes, and the supernatant was discarded. The pellet was resuspended in 200 µl of PBS in an eppendorf tube (~10^8/ml). The suspension was sonicated at 40 Hz for 10 minutes and then centrifuged at 13.500 rpm for 5 minutes. The supernatant was transferred to another eppendorf tube, and the DNA concentration was measured using a nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) for purity and quantification controls. DNA samples with OD260/280 values between 1.6 and 2.0 were considered to have sufficient purity (22), and a 3µl volume of template DNA was used in each PCR reaction.

Genotypic detection of colistin resistance:

The presence of the *mcr*-1 gene was examined using PCR. Genomic DNA from *E. coli* NCTC 13846 strain was used as a positive control, and genomic DNA from *E. coli* ATCC 25922 strain was used as a negative control.

Phylogenetic typing:

The distribution of colistin-resistant *E. coli* isolates into phylogenetic groups was determined using the quadruplex PCR method (11). This method targets seven coding genes, namely *chuA, yjaA, TspE4.C2, arpA, arpAgpEh, trpA* and t*rpBA*. It classifies *E. coli* into eight phylogenetic groups (A, B1, B2, C, D, E, F, clade I). The quadruplex PCR was performed using the primer sequences shown in Table 1 (11).

PCR reactions were carried out in a volume of 25 µl. The final concentrations were as follows: 1x Taq enzyme buffer solution, 2mM 25mM $MgCl_2$, 0.2mM 10mM dNTPs, 0.4 ρmol of each primer, and 1.5 U Taq DNA polymerase (Fermentas, Massachusetts, USA). Each reaction contained

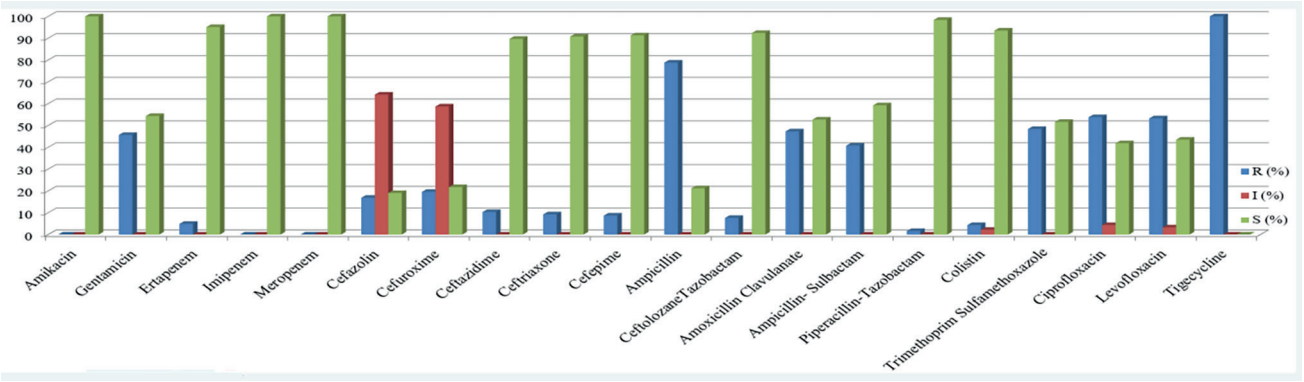

Figure 1. Antibiotic resistance and susceptibility using the all *E. coli*.

3 µl of DNA. The prepared tubes were loaded into a thermalcycler (Boeco, Hamburg, Germany).

The DNA was amplified using the following protocol: an initial denaturation at 95˚C for 5 minutes, followed by 30 cycles of denaturation at 95˚C for 30 seconds, annealing at 56˚C for 30 seconds (for *mcr*-1 and phylogroup PCR), and extension at 72˚C for 60 seconds, with a final extension at 72˚C for 10 minutes. A 2% agarose gel stained with Safe View (100 ml/6 µl) (ABM, Richmond, Canada) was used for gel electrophoresis, and the gel was subjected to 100 volts for 60 minutes. After the electrophoresis process, the gel was placed in the chamber of a transilluminator device (Vilbert Lourmat, Collegien, France) connected to a computer and photographed under UV light. When the amplified product formed a band of the expected size (Table 1.), it was assumed to carry the target gene.

ERIC-PCR:

The PCR reaction consisted of an initial denaturation at 95˚C for 5 minutes, followed by 30 cycles of denaturation at 95˚C for 1 minute, annealing at 37˚C for 1 minute, extension at 72˚C for 3 minutes, and a final extension at 72˚C for 10 minutes. A negative control with only master mix (lacking DNA) was used. Genetic relationships were calculated and a dendrogram was constructed based on the presence of bands using the Bio-1D program. The gel images were digitized, and PC-assisted genomic fingerprint analysis was performed using the Bio-Gene software program (Version 11.02). Similarity matrices of the complete densitometric curves of the gel bands were calculated using the Dice coefficient. Cluster analysis of the similarity matrices was performed using the unweighted pair group method with arithmetic mean (UPGMA) algorithm (23). All amplification products were subjected to electrophoresis on a 2% agarose gel at 100 volts for two hours.

RESULTS

Bacterial isolation and identification

In this study, a total of 184 (75.7%) *E. coli* isolates were identified from 243 broiler chickens suspected of colibacillosis. The biochemical test results of *E. coli* isolates showing pink colonies on MacConkey agar and metallic green sheen on EMB agar, with Gram-negative rod morphology, were as follows: motility +, indole +, oxidase -, MR +, VP -, citrate -.

Antibiotic resistance

Antibiotic susceptibility test:

Using an automated system, the resistance profiles of the 184 *E. coli* isolates to 20 antimicrobial drugs belonging to nine antibiotic families were examined.

The *E. coli* isolates exhibited high resistance rates to certain antibiotics, including tigecycline (100.0%), ampicillin (78.8%), ciprofloxacin (53.8%), and levofloxacin (53.2%). They showed moderate resistance rates to trimethoprim-sulfamethoxazole (48.4%), amoxicillin-clavulanate (47.3%), gentamicin (45.6%), and ampicillin-sulbactam (40.8%). However, they displayed low resistance rates to cefuroxime (19.6%), cefazolin (16.7%), ceftazidime (10.3%), ceftriaxone (9.2%), cefepime (8.7%), ceftolozane-tazobactam (7.6%), ertapenem (4.9%), colistin (4.4%), and piperacillin-tazobactam (1.6%). All isolates remained susceptible to amikacin, imipenem, and meropenem (Table 2, Figure 1.).

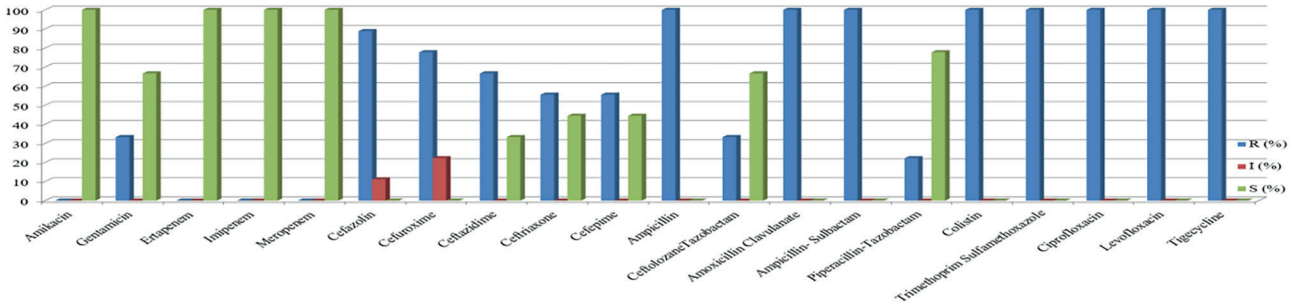

Figure 2. Antibiotic resistance and susceptibility of colistin-resistant *E. coli* isolates.

Table 2. Antibiotic susceptibility test results.

Antimicrobial Family-Antibiotic Name	All isolates (n=184)		*mcr*-1 gene (+) isolates (n=9)	
	R (%)	S (%)	R (%)	S (%)
Aminoglycoside				
Amikacin	0 (0.0)	184 (100.0)	0 (0.0)	9 (100.0)
Gentamicin	84 (45.6)	100 (54.4)	3 (33.3)	6 (66.7)
Carbapenem				
Ertapenem	9 (4.9)	175 (95.1)	0 (0.0)	9 (100.0)
Imipenem	0 (0.0)	184 (100.0)	0 (0.0)	9 (100.0)
Meropenem	0 (0.0)	184 (100.0)	0 (0.0)	9 (100.0)
Cephem				
Cefazolin	31 (16.7)	35 (19.0)	8 (88.8)	0 (0.0)
Cefuroxime	36 (19.6)	40 (21.7)	7 (77.7)	0 (0.0)
Ceftazidime	19 (10.3)	165 (89.7)	6 (66.6)	3 (33.4)
Ceftriaxone	17 (9.2)	167 (90.8)	5 (55.5)	4 (44.5)
Cefepime	16 (8.7)	168 891.3)	5 (55.5)	4 (44.5)
Penicillin				
Ampicillin	145 (78.8)	39 (21.2)	9 (100.0)	0 (0.0)
Beta Lactam				
CeftolozaneTazobactan	14 (7.6)	170 (9.4)	3 (33.3)	6 (66.7)
Amoxicillin Clavulanate	87 (47.3)	97 (52.7)	9 (100.0)	0 (0.0)
Ampicillin Sulbactam	75 (40.8)	109 (59.2)	9 (100.0)	0 (0.0)
Piperacilli Tazobactam	3 (1.6)	181 (98.4)	2 (22.2)	7 (77.8)
Lipopeptid				
Colistin	8 (4.4)	172 (93.4)	9 (100.0)	0 (0.0)
Folate				
Trimethoprim Sulfamethoxazole	89 (48.4)	95 (51.6)	9 (100.0)	0 (0.0)
Quinolone				
Ciprofloxacin	99 (53.8)	77 (41.8)	9 (100.0)	0 (0.0)
Levofloxacin	98 (53.2)	80 (43.5)	9 (100.0)	0 (0.0)
Tetracycline				
Tigecycline	184 (100.0)	0 (0.0)	9 (100.0)	0 (0.0)

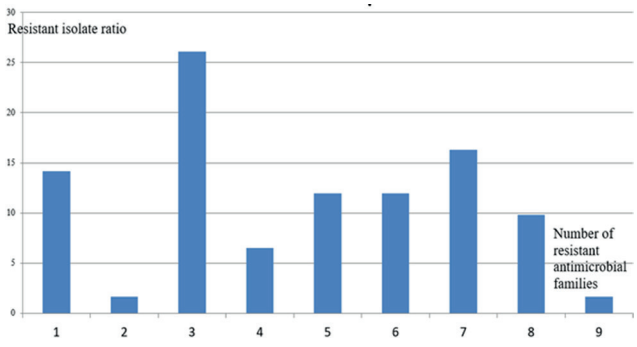

Figure 3. Distribution of MDR status of isolates.

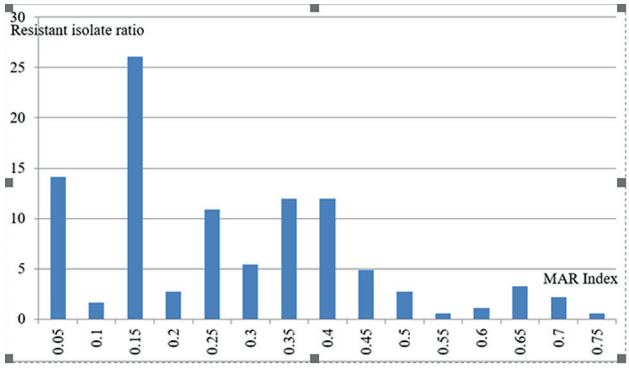

Figure 4. Distribution of MAR index.

Table 3. MDR status of isolates.

Number of antimicrobial families	Number of resistant isolates (%)	
1	26 (14.2)	29 (15.8%)
2	3 (1.6)	
3	48 (26.0)	155 (84.2%)
4	12 (6.5)	
5	22 (12.0)	
6	22 (12.0)	
7	30 (16.3)	
8	18 (9.8)	
9	3 (1.6)	

(n=184)

Table 4. MAR status of isolates.

Number of antibiotics	Number of resistant isolates (%)		MAR index	
1	26 (14.1)	77 (41.9)	0.05	<0.2
2	3 (1.6)		0.1	
3	48 (26.0)		0.15	
4	5 (2.8)	107 (58.1)	0.2	≥0.2
5	20 (10.9)		0.25	
6	10 (5.4)		0.3	
7	22 (12.0)		0.35	
8	22 (12.0)		0.4	
9	9 (4.9)		0.45	
10	5 (2.8)		0.5	
11	1 (0.5)		0.55	
12	2 (1.0)		0.6	
13	6 (3.3)		0.65	
14	4 (2.2)		0.7	
15	1 (0.5)		0.75	
Total	184 (100.0)	184 (100.0)		

Phenotypic determination of colistin resistance:

Throughout the study, colistin resistance was screened in all *E. coli* isolates using an automated system. Among the isolates, 8 out of 184 (4.4%) were identified as colistin-resistant, while one isolate showed intermediate susceptibility (Table 2, Figure 1, Figure 2.).

Regarding antibiotic classes, it was determined that the highest antibiotic resistance was observed against tetracyclines, followed by penicillins, quinolones, folates, aminoglycosides, cephalosporins, carbapenems, and lipopeptides.

MDR:

Among the *E. coli* isolates, 84.2% (155/184) were determined to be MDR, while 15.8% (29/184) were non-multidrug-resistant (NMDR). The resistance rates to 9, 8, 7, 6, 5, 4, 3, 2, and 1 antimicrobial classes were determined as 1.6%, 9.8%, 16.3%, 12.0%, 12.0%, 6.5%, 26.0%, 1.6%, and 14.2%, respectively (Table 3, Figure 3). All colistin-resistant isolates were MDR, with the number of resistant antimicrobial classes ranging from 7 to 9.

MAR Index:

It was determined that 58.1% (107/184) of all *E. coli* isolates had a MAR index greater than 0.2, while 41.9% (77/184) had a MAR index less than or equal to 0.2 (Table 4., Figure 4.). The MAR indices of colistin-resistant isolates ranged from 0.45 to 0.75.

PCR

Detection of colistin resistance:

DNA samples obtained from all isolates were examined by PCR for the presence of the *mcr*-1 gene. The *mcr*-1 gene was identified in 4.9% (9/184) of the isolates. Phenotypically, all colistin-resistant or intermediate isolates harbored the *mcr*-1 gene on a plasmid (Figure 5.).

Phylogrouping:

According to Clermont's phylogenetic method, eight out of nine *E. coli* isolates (88.9%) were classified into four phylogroups (B1, C, F, E), while one isolate (11.1%) could not be classified. The dominant phylogenetic group was B1 (33.3%; 3/9), followed by C and F (22.2%; 2/9), E (11.1%; 1/9), and an unknown group (11.1%; 1/9) (Figure 6, Figure 7.)

ERIC Typing:

The genomic diversity of nine *E. coli* isolates with genotypic colistin resistance, obtained from seven farms, was determined using the ERIC-PCR fingerprinting method. Electrophoretic analysis of PCR reaction products showed

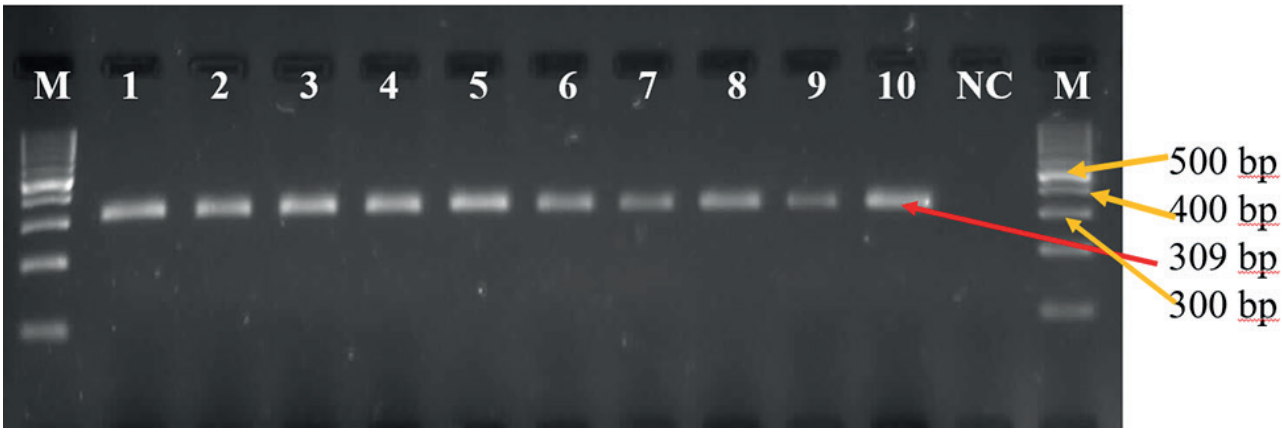

Figure 5. Gel electrophoresis for colistin resistance encoded by the *mcr*-1 gene. M: 100 bp DNA ladder (Fermentas, USA), Lane 1-9: Field isolates harboring the *mcr*-1 gene (309 bp), Lane 10: Positive Control (*E. coli* NCTC 13846), NC: Negative Control (*E. coli* 25922).

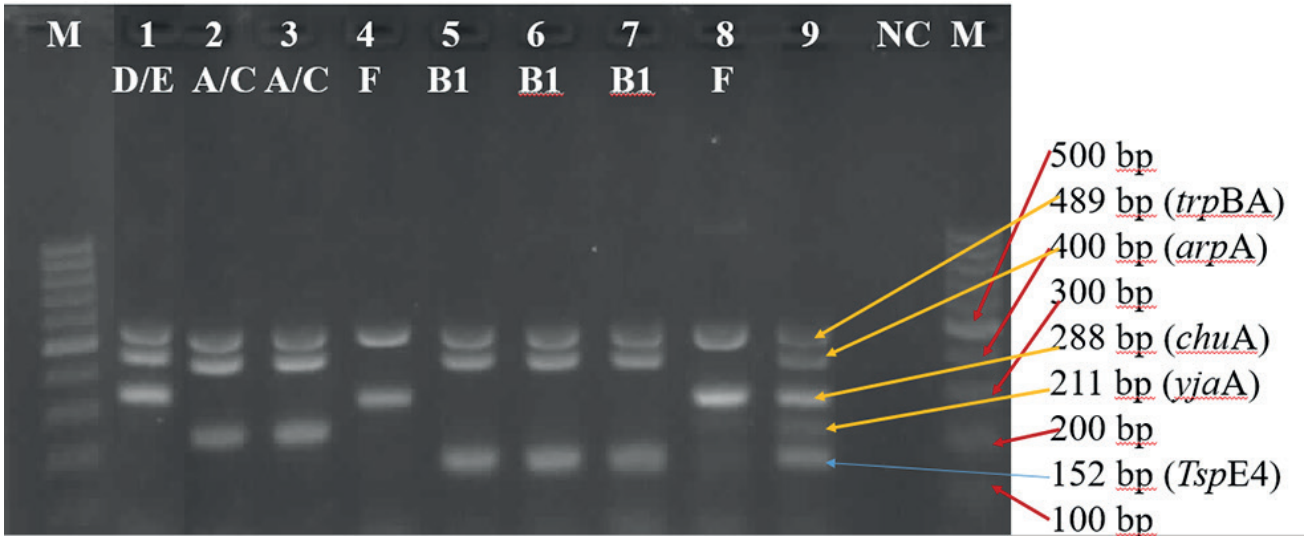

Figure 6. Quadruplex PCR profiles of new Clermont phylotyping method. Lane 1: Group D/E (+ + + – –), Lane 2 and Lane 3: Group A/C (+ + – + –), Lane 4, Lane 8: Group F (+ – + – –), Lane 5, Lane 6, Lane 7: Group B1 (+ + – – +), Lane 9: Group unknown (+ + + + +) (152 bp, 211 bp, 288 bp, 400 bp, 489 bp), NC: Mastermix without DNA M: Marker (100 bp).

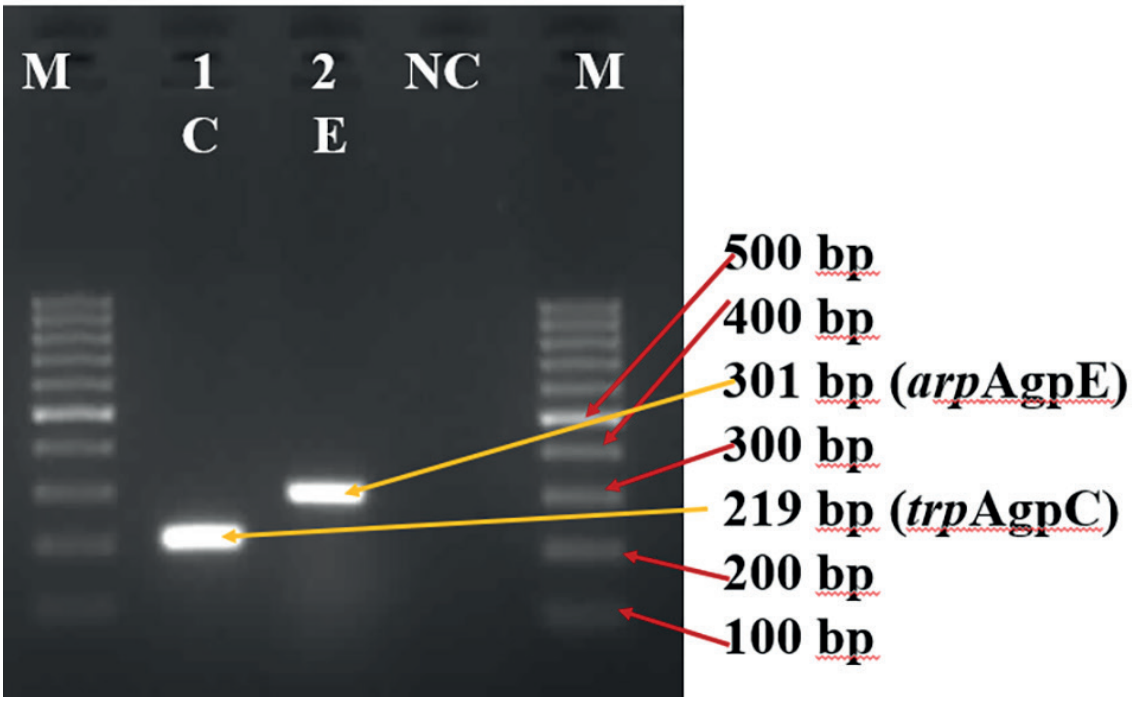

Figure 7. PCR profiles of new Clermont phylotyping method of group C and group E. Lane 1: Group A (219 bp), Lane 2: Group E (301 bp) NC: Mastermix without DNA M: Marker (100 bp).

a variation in the number of bands, ranging from 4 to 9, with sizes ranging from approximately 100 bp to 1,500 bp. The obtained ERIC types were named from A to G. Based on the UPGMA analysis with a similarity coefficient of 15%, seven main clusters were identified, with six being single clusters (A, B, D, E, F, G) and one being a multiple cluster (C). However, samples 6 and 7 from the C1 clone obtained from farm 4 exhibited the same genetic background and were closely related to isolate 4 from farm 3. Different fingerprinting patterns were obtained for all other isolates (Figure 8).

The farms, from which the nine isolates carrying the genotypic *mcr*-1 gene were obtained, along with their phylogroups, ERIC types, MDR status and antibiotic resistance profiles, are shown in Table 5.

DISCUSSION

Plasmid-mediated resistance mechanisms are of clinical and epidemiological significance due to their ability to rapidly spread among bacteria through horizontal gene transfer and their potential to cause outbreaks (3). The *mcr*-1 gene, which is one of the mobile colistin resistance genes, was discovered in 2015 (3), and in many cases, the exact mechanism of resistance development has not been fully elucidated (28).

The use of the standard broth microdilution (BMD) method is recommended for colistin minimum inhibitory concentration (MIC) testing (18). Colistin showed weak diffusion on agar, making the interpretation errors of the disk diffusion method high and thus reducing its reliability (18). Reference BMD requires experienced personnel as it involves manual preparation of antibiotic solutions, making it challenging to implement in laboratories. Additionally, it is a time-consuming method, rendering it impractical. Recent studies have reported that the use of automated systems for determining colistin resistance in *E. coli* isolates does not lead to significant errors and demonstrates acceptable performance (29, 30). In this study, the use of an automated system was preferred for the phenotypic determination of colistin resistance. Eight isolates (4.4%) were found to be colistin-resistant, while one isolate showed intermediate susceptibility. Furthermore, to elucidate the genetic mechanism of colistin resistance, all isolates were also examined by PCR for the presence of the *mcr*-1 gene. It was found that all *E. coli* isolates carrying the *mcr*-1 gene had MIC values >2 µg/ml for the AST performed using the automated system.

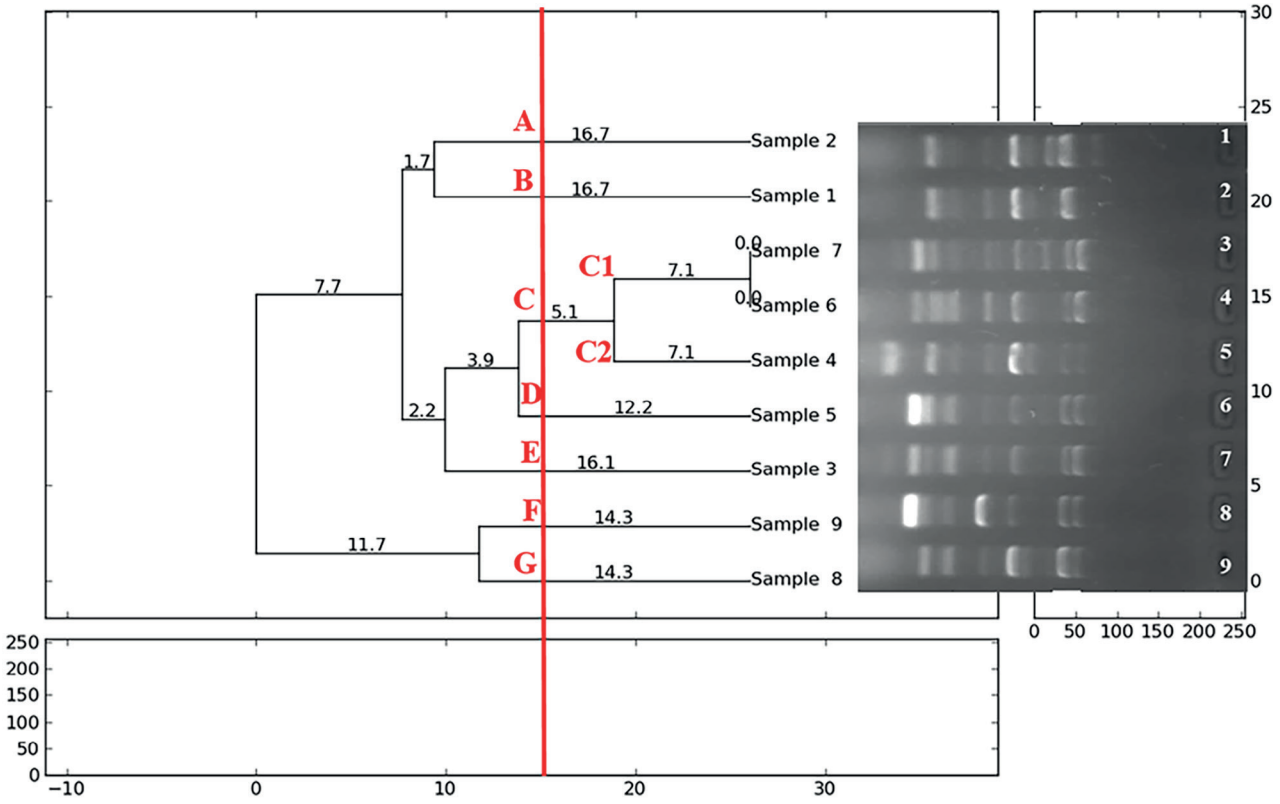

Figure 8. Dendrogram of ERIC-PCR profiles of nine *E. coli* isolates. When the dendrogram based on ERIC-PCR profiles of nine *E. coli* isolates was evaluated using the UPGMA analysis in the PyElph 1.4 program, a total of seven main clusters were identified, including six single clusters (A, B, D, E, F, and G) and one multiple cluster (C), with a 15% similarity coefficient. Two isolates (6, 7) from the C1 clone obtained from the same farm shared the same genetic background and were closely related to C2 clone an isolate (4) obtained from a different farm.

Table 5. Farms, phylotypes, ERIC types, MDR status and antibiotic resistance profiles of the isolates.

Isolate no	Farm	Phylogroups	ERIC type	MDR status	Resistance profiles
1	1	E	B	9	*, GM, CFZ, CXM, CAZ, CRO, FEP
2	1	C	A	9	*, GM, CFZ, CXM, CAZ, CRO, FEP
3	2	C	E	8	*, CFZ
4	3	F	C2	8	*, CFZ
5	4	B1	D	9	*, GM, CXM
6	5	B1	C1	8	*, CFZ, CXM, CAZ, CT
7	5	B1	C1	8	*, CFZ, CXM, CAZ, CRO, FEP, CT
8	6	F	G	8	*, CFZ, CXM, CAZ, CRO, FEP, CT, PT
9	7	? (MLST)	F	7	*, CFZ, CXM, CAZ, CRO, FEP, PT

*: Isolates are AMP, AMC, AS, TS, CIP, LF, TIG resistant.

One isolate without phenotypic colistin resistance was found to carry the *mcr*-1 gene. Similar cases, where isolates carry resistance genes but do not exhibit phenotypic resistance, have been reported previously (31, 32). The detection of the *mcr*-1 gene in colistin-susceptible isolates indicates the potential for silent dissemination of this gene. These studies highlight the importance of confirming phenotypic methods with molecular methods.

The *E. coli* isolates carrying the *mcr*-1 gene obtained in this study were resistant to most antibiotics commonly

used in human medicine and poultry farming, including ampicillin, amoxicillin clavulanate, ampicillin sulbactam, trimethoprim sulfamethoxazole, ciprofloxacin, levofloxacin, and tigecycline. Similar multidrug resistance has been reported in *mcr*-1 gene carrying *E. coli* isolated from chicken meat (33), chicken environmental samples (34) and humans (30). Studies have suggested that treatment options for colistin-resistant isolates may involve combination therapy with various antibiotic classes (35).

In Turkiye, colistin sulfate is used to treat digestive and respiratory system infections caused by *E. coli* and *Salmonella* species in poultry. In this study, it was noted that colistin had been used for therapeutic purposes in broiler chickens on the farms where the samples were collected. The first identification of colistin-resistant *E. coli* from poultry meat samples in Türkiye was reported in 2018 (15). One of the isolates obtained in this study showed similarity of (ST3941) to *mcr*-1 positive *E. coli* isolated from humans in Italy, indicating that ColR *E. coli* can be transmitted to humans through contaminated animal products. Two years later, the presence of the *mcr*-1 gene was reported in one of the 11 phenotypically colistin-resistant isolates from chicken meat samples (16). Subsequently, in another study, although phenotypic colistin resistance was detected in 7.5% of *E. coli* isolates obtained from chicken fecal samples, no genotypic resistance was found (17). In this study, eight colistin-resistant *E. coli* isolates were obtained phenotypically from APEC isolates, and it was found that nine isolates carried the *mcr*-1 gene genotypically. The detection of the *mcr*-1 gene in all phenotypically colistin-resistant isolates indicated that colistin resistance was plasmid-mediated.

However, the high prevalence of *mcr*-1 gene carrying *E. coli* isolates in this and other studies could be attributed to cross-contamination due to hygiene deficiencies in slaughterhouses (36).

In this study, all isolates were found to be resistant to tigecycline. Tigecycline is a member of the tetracycline class of antibiotics and is commonly used in the treatment of serious bacterial infections in humans. The emergence and spread of strains of *E. coli* isolated from chickens carrying the tigecycline resistance gene *Tet*(X4) and the colistin resistance gene *mcr*-1 have been reported in previous studies, posing a potential threat to public health by reducing the effectiveness of colistin and tigecycline, which are important for the treatment of severe clinical infections (37). In the study conducted by Li *et al*. (2022), it was emphasized that the combination of the *Tet*(X4) and *mcr*-1 genes in *E. coli* through conjugative or mobilization capable plasmids could potentially lead to widespread dissemination of these resistance genes and contribute to a global antibiotic resistance crisis (37).

The transfer of antibiotic resistance genes through plasmids is an important mechanism that allows rapid spread of antibiotic resistance among bacteria. In our study, it was found that none of the broiler chickens in the sampled farms had been exposed to tigecycline, unlike colistin. However, all of our isolates exhibited tigecycline resistance. There could be two possible explanations for this observation. Alterations in ribosomal targets or transport systems in tetracycline resistant bacteria could also trigger tigecycline resistance. Tetracycline has commonly been used as an antibiotic in the sampled farms. Tetracycline and tigecycline have the same mechanism of action, binding to the same ribosomal targets to inhibit protein synthesis in bacterial cells. Therefore, the use of tetracycline antibiotics may have contributed to the development of tigecycline resistance. Resistance genes can be transferred through plasmids or other genetic elements, and their presence and transfer could contribute to the spread of resistance to different antibiotics. Further research and analysis of resistance mechanisms is needed to better understand this phenomenon.

The MAR index has been reported as an effective method for tracking the source of antibiotic resistant organisms (38). In this study, it was found that the MAR index of 107 isolates (58.1%) ranged from 0.2 to 0.75, and all nine isolates resistant to colistin had MAR index values greater than 0.2. In general, samples exceeding a MAR index of 0.2 can pose a serious concern for public health as they carry a high risk of contamination (20) and are considered an indicator of isolates originating from an environment where antibiotics are frequently used (19). These findings indicate the frequent use of antibiotics in the animals of the sampled farms. The emergence of MDR *E. coli* isolates carrying the *mcr*-1 gene in APEC isolates may be attributed to selective pressure caused by the indiscriminate use of antibiotics (39).

Phylogenetic classification is important for understanding the pathogenesis of *E. coli* and its relationship with hosts. It has been reported that *E. coli* strains belonging to the same phylogenetic group share similar phenotypic and genotypic characteristics, pathogenicity and ecological traits (24). In this study, three out of the nine isolates carrying the *mcr*-1

gene (33.3%) were classified as commensal or environmental phylogroup B1, and two isolates (22.2%) were closely related to phylogroup C, which is known to be associated with phylogroup B1. Previous studies, have also reported that phylogroup B1 is the dominant phylogroup isolated from various environmental samples of animals (40, 41). The dominance of phylogroup B1 can be explained by its ability to survive in the environment for extended periods, possibly due to unique stress tolerance characteristics (41), or to the emergence of certain lineages within B1 that easily colonize sediment and/or soil habitats (13). Isolates belonging to phylogroups B1 and C may be environmental or relatively low-virulence strains; however, they can acquire plasmid-mediated resistance genes from other bacterial species in their surroundings through horizontal gene transfer. For instance, environmental factors such as contact with animals, farm environments, or wastewater can play a role in the transmission of these genes to environmental commensal isolates. The dissemination of such isolates in poultry environments, their acquisition of plasmid-mediated resistance genes, and the increase in their numbers within the poultry environment can contribute to the spread of antibiotic resistance, negative impacts on human health and the emergence of environmental effects. Therefore, monitoring, control and implementation of appropriate measures for these isolates is crucial. In this study, two isolates (22.2%) were associated with the pathogenic B2 phylogroup, specifically within the F phylogroup. These findings are significant because extra-intestinal and virulent *E. coli* are classified under the B2 and D phylogenetic groups, and their carriage of multidrug resistance can exacerbate infections caused by pathogenic strains. Additionally, the F phylogenetic group is characterized as the sister group of B2 and therefore, can be considered pathogenic. However, further studies focusing on specific virulence factors are important to better understand the pathogenic properties of strains within these phylogroups.

In this context, another notable study, conducted within a similar scope, examined the emergence and dissemination of plasmid-mediated colistin resistance in *E. coli* strains obtained from broiler flocks in Türkiye between 2013 and 2014 (42). The findings of this study indicate the significant role of plasmid mediated mechanisms in colistin resistance, as evidenced by the detection of the *mcr*-1 gene. Furthermore, phylogenetic analysis revealed isolates belonging to four distinct phylogroups, demonstrating the widespread presence of resistance in isolates with different genetic backgrounds. This underscores the prevalence of resistance in isolates with diverse genetic backgrounds (42).

In this study, using the ERIC typing method, a total of seven ERIC types were identified, including six individual genotypes (A, B, D, E, F, and G) from seven farms and one multiple genotype (C). Two isolates obtained from the same farm had the same genetic background, while an isolate from a different farm was closely related. This may indicate the presence of genetic similarities among poultry within the same farm and the possibility of genetic relationships between different farms, suggesting potential clonal dissemination of colistin-resistant isolates. However, it has also been reported in different studies, as in this study, that a significant proportion of colistin-resistant isolates may be clonally unrelated (32), indicating that the spread of other isolates is not solely attributed to a clonal outbreak.

The results of this study indicate that *E. coli* isolates obtained from broiler farms in western Türkiye exhibit low levels of colistin resistance but high levels of tetracyclines resistance. The high prevalence of MDR *E. coli* isolates suggests inappropriate antibiotic usage in the farms. The detection of the *mcr*-1 gene in all phenotypically colistin resistant isolates indicates the significant role of plasmid mediated mechanisms in colistin resistance. Filogroup analysis revealed that colistin resistant isolates belonged to four different filogroups, indicating the widespread presence of resistance in isolates with different genetic backgrounds. While clonal dissemination was observed between farms based on ERIC typing results, a significant proportion of colistin resistant isolates were clonally unrelated. These findings emphasize the need for careful evaluation and control of antibiotic usage in farm environments.

The emergence of mobile colistin resistance genes can facilitate the rapidly spreads of antibiotic resistance genes in animals living in crowded poultry farm environments, such as broiler chickens. This can lead to increased antibiotic resistance, zoonotic disease risks, treatment challenges and problems in the pharmaceutical industry. The obtained results highlight the need for strict control of antibiotic usage in the poultry sector and the monitoring of the phylogenetic and clonal relationships of isolates. They emphasize the importance of further research on colistin resistance and its genetic dissemination.

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