The Relationship of Phylogenetic Distribution and Integron Gene Presence with Multiple Antibiotic Resistance in *Escherichia coli* Isolates Obtained from Bovine Milk with Mastitis

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ABSTRACT

Multiple antibiotic resistant (MDR) bacteria are one of the most important current threats to public health. Integrons are important mobile genetic mechanisms involved in the spread of resistance. *Escherichia coli* includes both commensal and pathogenic clones found in different phylogenetic groups. Revealing the distribution of phylogenetic groups and the presence of integrons in *E. coli*, which causes clinical mastitis (CM), may assist in the development of specific strategies for the treatment and control of this pathogen. In this study, it was aimed to examine the relationship between phylogenetic distribution and integron related gene presence with multi-antibiotic resistance of bacteria in *E. coli* isolates obtained from bovine milk with clinical mastitis. The material of the study consisted of 73 *E. coli* (17.3%) isolates obtained from 422 milk samples. After bacterial isolation was performed with conventional biochemical tests; Identification, phylogeny and presence of integron genes were examined by Polymerase Chain Reaction (PCR). The resistance patterns of the isolates against nine antibiotics belonging to nine antimicrobial families were analyzed using the Kirby-Bauer disc diffusion method. Isolates resistant to at least three drugs from various antimicrobial drug classes were defined as multi-drug resistant (MDR). Chi-square ($\chi 2$) test was used to calculate the relationship between multidrug resistance of isolates, their phylogenetic distribution and presence of integron genes ($p<0.05$). According to the antibiotic test results, the highest resistance was found against ampicillin (79.4%) and amoxicillin clavulanate (61.6%), while the highest sensitivity was against ciprofloxacin (86.3%) and cefoperazone (84.9%). 52.1% (38/73) of the isolates were MDR. The majority of isolated *E. coli* strains belonged to group A (37.0%), followed by group B1 (24.7%), group C (15.0%), group D (5.5%), group E (% 8.2%) and then group F (4.1%). It was determined that 31.5% of the isolates carried integron related genes. There was no significant relationship between MDR and phylogenetic distribution of the isolates. However, the relationship between MDR and the prevalence of integron related genes was significant ($p=0.001$). The high rate of multi-antibiotic resistance of isolates belonging to phylogroups A and B1, which are generally considered commensal, is a concern. The increase presence of MDR bacteria in animals can lead to the emergence of resistant bacteria that can be transferred to humans through direct contact or through the food chain, reducing the effectiveness of antibiotics used to treat human diseases.

Key words: *Escherichia coli*; Multiple Antibiotic Resistance; Phylogenetic Distribution; Integron.

INTRODUCTION

Bacterial pathogens that cause mastitis can be contagious or environmental. Contagious mastitis agents have the feature of being easily transmitted from animal to animal. Environmental pathogens such as *Escherichia coli* are important because they are frequently isolated from bovine mastitis (1, 2) and can cause toxic or fatal effects in affected cattle (3). While commensal strains of *E. coli* are bacteria of the intestinal microbiota of humans and animals, virulent strains are pathogens that cause intestinal or extra-intestinal diseases (1). The barrier between commensalism and virulence of *E. coli* largely depends on the balance between the host's immune status and the bacteria's ability to elicit virulence factors (4). Recently, the term mammary pathogenic *E. coli* has been proposed to classify mastitis-associated pathogens (5), however, specific virulence factors that may reveal the relationship between these strains and mastitis have not been identified (6). Therefore, the determination of pathogenic *E. coli* strains is important in terms of determining the risk profile of the isolate.

Phylotyping of *E. coli* isolates is very useful as it gives an idea about the source and pathogenicity of the isolate obtained. Clermont *et al.* (2013) analyzed four genes (*arp*A, *chu*A, *yja*A, and *Tsp*E4.C2) and classified *E. coli* isolates into eight phylogroups (A, B1, B2, C, D, E, F, and clade I) (7). According to this, birds, reptiles, fish and some mammals carry phylogroup A. The B1 phylogroup is generally environmental. This type of *E. coli* has a low risk to human health. Humans predominantly carry phylogroup B2. This type of *E. coli* has a high risk to human health. Recently, phylogroup C has been identified, which includes a group of strains closely related to but distinct from phylogroup B1. Phylogroup C and phyogroup F, about which little is known are acknowledged to have a low risk to human health, unlike Clade 1 strains, which include extraintestinal pathogenic strains. Phylogroup D is associated with humans and omnivores (pigs) while the phylogroup E is linked to herbivores and cattle. These two phylotypes pose a moderate risk to human health (7, 8). Phylogenetic analyzes have shown that virulent extraintestinal *E. coli* strains are mostly associated with group B2, while commensal strains are mostly associated with A or group B1 phylogroups (9). The mastitis-causing *E. coli* has great intraspecific diversity and has been classified in different phylogenetic groups (mostly A, B1, B2 or D) (10). It has been reported that the majority of *E. coli* strains causing bovine mastitis belong to phylogroups A and B1 (9-13). Information on the C, E and F groups that cause CM is quite scarce (14, 15).

Integrons, which are very effective in the capture, integration and expression of gene cassettes, play an important role in the spread of multi-antibiotic resistance among bacteria (16). Expression of an integron can induce bacterial multidrug resistance and cause the resistance gene to spread in the same or different bacteria by a conjugative plasmid (16). It has been reported that class I integrons in *E. coli* may play a role in the spread of trimethoprim and aminoglycoside resistance genes to other microorganisms (17, 18). Among the five different classes of integrons, class I and class II integrons are the most common and clinically important (16). Integrons are very common (22-95%) in enteric bacteria isolated from various infections, but they have also been found in commensal bacteria (12, 19).

Various antimicrobial agents (tetracyclines, sulfonamides, quinolones, macrolides, β-lactams) are used in the treatment of bovine mastitis cases caused by *E. coli* (20). Unfortunately, it has been reported that antibiotic therapy is not always successful in the treatment of mastitis caused by coliforms in cattle (21). In recent years, the incidence of multiple antibiotic resistant (MDR) bacteria has increased considerably both in Türkiye (22) and in many countries (12, 23) around the world (17, 18). While multidrug resistant virulent *E. coli* may be responsible for the failure of antibiotic treatments in bovine mastitis, it may also pose a potential threat to the transmission and development of antibiotic resistance in humans.

Antimicrobial resistance and phylogroups in *E. coli* isolated from cow milk with mastitis have been investigated in previous studies (23, 24, 25). In studies conducted on *E. coli* mastitis in Türkiye, either antibiogram (22) or the distribution of phylogenetic groups (14) has been examined.

In this study, it was aimed to examine the relationship between phylogenetic distribution and integron related genes presence with multi-antibiotic resistance of bacteria in *E. coli* isolates obtained from bovine milk with clinical mastitis.

MATERIAL AND METHOD

Bacterial Isolates

In this study, 422 milk samples from 17 farms in western Türkiye were collected over a period of approximately one

Table 1. All primers used in this study.

Primers	Target Gene	Sequence (5'-3')	Amplicon (bp)	T_m	Reference
Universal	16S rRNA	AGAGTTTGATCCTGGCTCAG GACGGGCGGTGTGTACAA	1371	58.4 58.4	(28, 29)
Integron	*int*1	CCTCCCGCACGATGATC TCCACGCATCGTCAGGC	280	57.2 57.2	(16)
	*int*2	TTATTGCTGGGATTAGGC ACGGCTACCCTCTGTTATC	233	51.6 57.3	(30)
Quadruplex	*chu*A	ATGGTACCGGACGAACCAAC TGCCGCCAGTACCAAAGACA	288	60.5 60.5	(7)
	*yja*A	CAAACGTGAAGTGTCAGGAG AATGCGTTCCTCAACCTGTG	211	58.4 58.4	
	*Tsp*E4C2	CACTATTCGTAAGGTCATCC AGTTTATCGCTGCGGGTCGC	152	56.4 62.5	
	*arp*A	AACGCTATTCGCCAGCTTGC TCTCCCCATACCGTACGCTA	400	60.5 60.5	
Group E	*arp*A	GATTCCATCTTGTCAAAATATGCC GAAAAGAAAAAGAATTCCCAAGAG	301	60.1 58.4	
Group C	*trp*A	AGTTTTATGCCCAGTGCGAG TCTGCGCCGGTCACGCCC	219	58.4 68.1	
Internal control	*trp*A	CGGCGATAAAGACATCTTCAC GCAACGCGGCCTGGCGGAAG	489	59.4 68.7	

year (January 2020 to January 2021). Milk samples from 16 to 41 were obtained from each farm. Milk samples were taken from animals that had not been treated with antibiotics for at least two weeks prior to sampling. The age of the cows from which the material was taken varied between 3-11 years of age and the number of cows in each farm varied between 22-67. Automatic milking was used for milking in all farms.

Clinical mastitis was defined by the presence of inflammatory symptoms (swelling, warmth, redness, and pain) of the udder and/or changes in color, fluidity and odor of the milk during routine examination of the mammary lobes just before milking by veterinarians. Milk samples were taken from the affected mammary lobes under aseptic conditions, one from each animal. While collecting milk, the first few drops of milk were discarded after cleaning the nipples with 70% alcohol.

Approximately 5-10 ml of milk sample was taken into sterile tubes and delivered to the laboratory under cold chain conditions on the same day. Milk samples were stored at minus 20˚C until the isolation and identification process was completed.

Isolation and Identification

Milk samples were centrifuged at 3500 rpm for 5 minutes and the supernatant was discarded. The residue was vortexed. A loopful (0.01 ml) of milk was inoculated on Eosin Methylene Blue (EMB) agar (Merck, 103858, Germany) and incubated aerobically at 37˚C for 24 to 48 hours. A single colony (flat colonies with a dark center and metallic green sheen) was selected from EMB agar plates. Each isolated strain was evaluated for purity on MacConkey agar (Merck, 146066, Germany). Colonies fermenting ≥3 mm of pink lactose were identified as *E. coli* suspect colonies and passaged onto tryptic soy agar (TSA) (Merck, 105458, Germany). Gram staining and biochemical confirmation tests (oxidase, catalase, motility, indole, methyl red, Voges-Proskauer, urease, sugar fermentation tests, etc.) were performed (2).

Polymerase Chain Reaction (PCR)

DNA extraction, purity and quantity control: In this study, DNA extraction was performed by the sonication method as previously reported (26). For this purpose, isolates were passaged from stock cultures to blood agar and incubated at 37˚C for 24 hours. A colony was then taken from this bacte-

Table 2. Antimicrobial agents and results.

Antimicrobial family	Antimicrobial agent	Disc content (µg)	Zone diameter (mm)		Resılts	
			≥S	≤R	S (%)	R (%)
Penicillins	Ampicilin	10	17	13	12 (16.4)	58 (79.4)
Beta Lactam	Amoxicillin clavulanate	20/10	18	13	24 (32.8)	45 (61.6)
Tetracyclines	Tetracycline	30	15	11	44 (60.3)	27 (36.9)
Folate pathways	Trimetoprim/sulfamethoxazole	1.25/23.75	16	10	48 (65.8)	17 (23.3)
Aminoglycosides	Streptomisin	10	15	11	56 (76.7)	14 (19.2)
Phenicols	Chloramphenicol	30	18	12	58 (79.4)	13 (17.8)
Nitrofurans	Nitrofurantoin	300	17	14	57 (78.0)	11 (15.0)
Cephems	Cefoperazone	75	21	15	62 (84.9)	9 (12.3)
Qinolones	Ciprofloxacin	5	26	21	63 (86.3)	8 (10.9)

Multiple Antibiotic Resistance (MDR) and Multiple Antibiotic Resistance Index (MAR).

rial culture and transferred to 5 ml Tryptic Soy Broth (TSB) (Merck 105459, Germany). Thereafter the TSB was incubated at 37˚C for 18 to 24 hours. The broth was centrifuged at 13500 rpm for 5 min. The supernatant was discarded. The residue was diluted with 200 µl PBS in an eppendorf tube (~10^8/ml). The suspension was sonicated at 40 Hz for 10 minutes then centrifuged at 13500 rpm for 5 min. Three microliters of supernatant were used as template DNA in each PCR reaction. DNA purity and quantity controls were also performed. The ratio of OD260/OD280 was between 1.6-2.0 indicating that the DNA was pure (27). Then, DNA was electrophoresed on 1% agarose gel and the presence of DNA bands in the UV transilluminator were investigated.

Primers: Firstly, the bacterial presence and DNA extraction was confirmed by amplification of the 16S rRNA gene. For the PCR performed using 16S rRNA universal primers, *Escherichia coli* ATCC 25922 strain was used as positive control, and mastermix without DNA was used as negative control. Target genes, sequences, product lengths, melting temperature (Tm), references of primers used in this study and the results are shown in Table 1.

To amplify the genes, 50 µl of reaction mixture was made containing 2 mM $MgCl_2$, 0.4 mM of each of the four dNTPs, 0.1 mM oligonucleotide primers, 1.5 U Taq polymerase (Fermentas, Massachusetts, USA) and 20 ηg template DNA. The prepared tubes were loaded in the thermal cycler (Boeco, Hamburg, Germany). The DNA was amplified using the following protocol: initial denaturation at 95˚C for 5 min, followed by 30 cycles of denaturation (95˚C for 30 s), annealing for 30 s [51˚C (*int*2), 56˚C (*int*1, *int*3, 16S rRNA, *chu*A, *yja*A, *Tsp*E4C2, *arp*A, *trp*A)] and extension (72˚C for 1 min), with a single final extension for 7 min at 72˚C. Determination of phylogenetic groups of the *E. coli* isolates were carried out by reference Clermont's multiplex PCR method (7). The amplified PCR products were analysed in 1.7% agarose gel stained with with Safe View (100 ml/6 µl) (ABM, Richmond, Canada) and the gel was exposed to 100 volts for 45 min. After electrophoresis, the gel was placed in the chamber of the transilluminator device, which was connected to the computer and photographed under UV light (Vilbert Lourmat, Collegien, France). When the amplified product formed a band of the expected size (Table 1.), it was assumed to carry the gene examined. In the negative control of water was used instead of the DNA template.

Antimicrobial Resistance

For each *E. coli* isolate confirmed by PCR, the antimicrobial resistance against nine antibiotics (Oxoid, United Kingdom) belonging to nine different antibiotic families was tested by the disk diffusion method (Table 2.). The tested antibiotics belonged to the most important antibiotic families frequently recommended for the treatment of *E. coli* mastitis infections. Susceptibility testing was determined by the Kirby-Bauer disk diffusion method. A bacterial suspension of 0.5 McFarland standard turbidity was first prepared using a 24h culture. A sterile cotton swab was dipped into the bacterial suspension, and the swab was pressed and twisted against the inner surface of the test tube to remove excess fluid. The swab was streaked across a Mueller-Hinton agar (MHA) (Oxoid,

Table 3. The relationship between antibiotic resistance and phylogroups.

Antibiotics	Phylogenetic Groups, n (%)							
	A	B1	C	D	E	F	*	Total
	27 (37.0)	**18 (24.7)**	**11 (15.0)**	**4 (5.5)**	**6 (8.2)**	**3 (4.1)**	**4 (5.5)**	**73 (100.0)**
Ampicilin	20 (27.4)	15 (20.5)	8 (10.1)	3 (4.1)	6 (8.2)	3 (4.1)	3 (4.1)	58 (79.4)
Amoxicillin clavulanate	18 (24.7)	13 (17.8)	4 (5.6)	3 (4.1)	4 (5.6)	1 (1.4)	2 (2.7)	45 (61.6)
Tetracycline	10 (13.7)	8 (10.9)	4 (5.6)	0 (0.0)	3 (4.1)	2 (2.7)	0 (0.0)	27 (36.9)
Trimetoprim/sulfamethoxazole	7 (9.6)	5 (6.9)	1 (1.4)	2 (2.7)	1 (1.4)	1 (1.4)	0 (0.0)	17 (23.3)
Streptomisin	9 (12.4)	1 (1.4)	1 (1.4)	1 (1.4)	0 (0.0)	1 (1.4)	0 (0.0)	13 (17.8)
Chloramphenicol	6 (8.2)	3 (4.1)	0 (0.0)	1 (1.4)	3 (4.1)	0 (0.0)	0 (0.0)	13 (17.8)
Nitrofurantoin	4 (5.6)	3 (4.1)	1 (1.4)	1 (1.4)	2 (2.7)	0 (0.0)	0 (0.0)	11 (15.0)
Cefoperazone	4 (5.6)	3 (4.1)	0 (0.0)	0 (0.0)	1 (1.4)	1 (1.4)	0 (0.0)	9 (12.4)
Ciprofloxacin	3 (4.1)	1 (1.4)	1 (1.4)	0 (0.0)	2 (2.7)	0 (0.0)	1 (1.4)	8 (10.1)

*: Non-phylotypeable isolates.

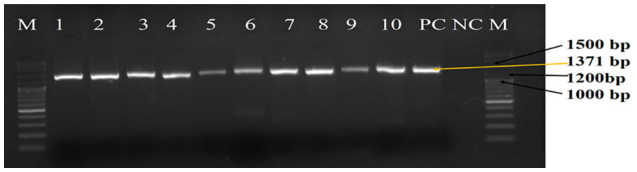

Figure 1. PCR performed by using 16S rRNA universal primers. M: Marker (100 bp DNA Ladder, Fermentas) 1-10: PCR performed by using isolated microorganism's DNA. PC: Positive Control (*E. coli* ATCC 25922) NC: Negative Control (Mastermix without DNA).

103872, United Kingdom) surface in a zigzag manner. The MHA plate was turned 45 degrees clockwise and streaked again using the same swab, and this step was repeated one more time so that the swab had been streaked across the agar a total of three times. The antibiotic discs were placed onto the agar using a pair of sterile forceps. Antibiotics disks were placed onto the same *E. coli* inoculated MHA plate, and the plates were incubated at 37°C for 18 to 24 hours. Zone diameters of susceptibility testing results were categorized as sensitive (S), intermediate (I), or resistant (R) and evaluated as previously reported (31).

Isolates resistant to at least three drug classes from various antimicrobial classes were evaluated as multi-antibiotic resistant (MDR) and isolates resistant to two or fewer antimicrobial classes were evaluated as non-multi-antibiotic resistant (NMDR) (32). The MAR for each isolate was determined by dividing the number of antibiotics to which the isolate was resistant by the total number of antibiotics tested (33).

Statistical Analysis

SPSS (Statistical Package for Social Sciences) version 23.0 (SPSS Inc., Chicago, IL, USA) package program was used for statistical analysis of the data obtained. Pearson Chi-square ($\chi 2$) test (the Fisher exact test) was used to compare frequency data. The $\chi 2$ test was used to reveal statistical difference between MDR and integron related gene prevalances as well as phylogenetic groups of isolates. A p-value of <0.05 was considered statistically significant at the 95% confidence interval.

RESULTS

Biochemical Tests and Phenotypic Identification

A total of 73 *E. coli* isolates (17.3%) were obtained from 422 milk samples collected from cows with clinical mastitis. Biochemical test results of isolated Gram-negative bacilli: oxidase, urease, citrate, hydrogen sulfide were negative and motility, catalase, indole, lactose fermentation, gas were positive.

PCR

Firstly, bacterial presence and DNA extraction were confirmed with amplification of the 16S rRNA gene (Figure 1.).

Integron Genes

Twenty-three *E. coli* isolates (31.5%, 23/73) were found to contain integron related genes. Eighteen (24.6%) isolates

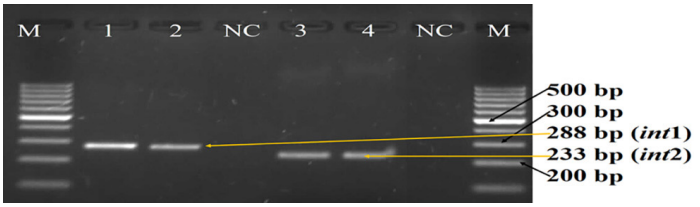

Figure 2. Agarose gel electrophoresis of integron related genes. 1. 2. *int*1 gene positive *E. coli* isolates (280 bp) 4. 5. *int*2 gene positive *E. coli* isolate (233 bp) 3. 6. NC: Master mix without DNA M: 100 bp DNA Ladder (Fermentas, USA).

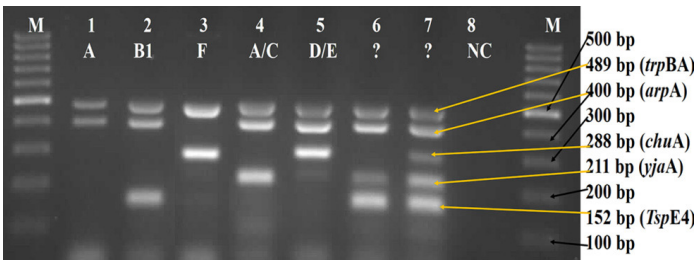

Figure 3. Quadruplex PCR profiles of new Clermont phylotyping method. Lane 1: Group A (+ + – – –), Lane 2: Group B1 (+ + – – +), Lane 3: Group F (+ – + – –), Lane 4: Group A/C (+ + – + –), Lane 5: Group D/E (+ + + – –), Lane 6: Group unknown (+ + – + +), Lane 7: Group unknown (+ + ++ +), Lane 8: NC: Mastermix without DNA M: Molecular weight marker (100 bp, Fermentas).

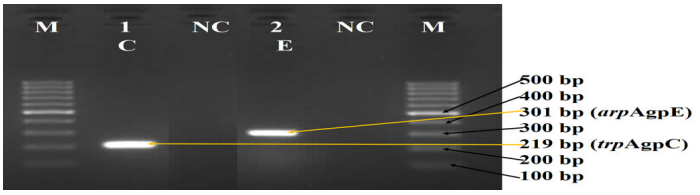

Figure 4. PCR profiles of new Clermont phylotyping method of group C and group E. Lane 1: Group A (219 bp), Lane 2: Group E (301 bp) NC: Mastermix without DNA M: Molecular weight marker (100 bp, Fermentas).

carried class I and five (6.9%) class II integron related genes. There were no isolates carrying both classes of integron genes jointly (Figure 2.).

Phylogenetic Typing

Of 73 *E. coli* isolates 69 (94.5%) were classified into six phylogroups according to Clermont's phylogenetic method (7) (Table 3.). Four isolates (5.5%) could not be classified. Phylotyped *E. coli* isolates belonged to group A (27/73; 37.0%), followed by group B1 (18/73, 24.7%), group C (11/73, 15.0%), group D (4/73, 5.5%), group E (6/73, 8.2%) and group F (3/73, 4.1%) (Figure 3 and Figure 4.). B2 and clade I phylogroups were absent in our isolates.

Antibiogram

The analysis of the antibiotic susceptibility tests for all tested isolates showed high rates of resistance for ampicillin (79.4%) and amoxicillin clavulanate (61.6%). Moderate to low rates of resistance were observed for tetracycline (36.9%), trimethoprim/sulfamethoxazole (23.3%), streptomycin (19.2%), chloramphenicol (17.8%), nitrofurantoin (15.0%), cefoperazone (12.3%) and ciprofloxacin (10.9) (Table 1 and Figure 5).

Thirty-eight (58.1%) isolates showed multi-drug resistance phenotypes. The most effective antibiotics against isolates are ciprofloxacin and cefoperazone (86.3% and 84.9% susceptibility rate, respectively).

Antibiotic resistance of *E. coli* isolates according to phylogenetic groups is shown in Table 3.

The isolates in phylogroup A were resistant to all antibiotics used (ampicillin (27.4%), amoxicillin clavulanate (24.7), tetracycline (13.7%), trimethoprim/sulfamethoxazole (9.6%), streptomycin (12.4), chloramphenicol (8.2%), nitrofurantoine (5.6%), cefoperazone (5.6%) and ciprofloxacin (4.1%)) were resistant at higher rates than isolates found in other phylotypes. The phylogroup with the second highest antibiotic resistance was phylogroup B1 (Table 3).

MDR AND MAR INDEX

Of the 73 isolates, 38 (52.1%) were multi-antibiotic resistant. While fifteen of the isolates (20.5%) were resistant to three and four antibiotics; seven isolates (9.6%) were resistant to five antibiotics and one isolate (1.3%) to six antibiotics. The MAR indexes of multiple antibiotic resistant isolates ranged from 0.3 to 0.6 (Table 4).

Of the 73 isolates examined in this study, 35 (47.9%) were not multi-antibiotic resistant: three (4.1%) isolates were susceptible to all antibiotics used. Eight isolates (10.9%) were resistant to at least one of the antibiotics used and 24 isolates (39.9%) were resistant to two antibiotic. The MAR index of these isolates also ranged from 0 to 0.2 (Table 4.).

Statistical Analysis

The relationship between MDR with the prevalence of phylogenetic groups and integron related genes is shown in Table 5.

Table 4. MAR index and resistance phenotype of *E. coli* isolates.

Number of isolate (%)	Number of resistant antibiotic	MAR index	Resistance Phenotype
3 (4.1)	0	0	NMDR
8 (10.9)	1	0.1	NMDR
24 (39.9)	2	0.2	NMDR
15 (20.5)	3	0.3	MDR
15 (20.5)	4	0.4	MDR
7 (9.6)	5	0.5	MDR
1 (1.3)	6	0.6	MDR

There was no significant relationship between MDR and phylogenetic distribution of the isolates. However, the relationship between MDR and the prevalence of integron genes was significant ($\chi2$=11.18, p=0.001). When the integron classes were examined separately, it was shown that class I integron genes appeared to be extremely important for the emergence and transmission of MDR among bacteria ($\chi2$=6.33, p=0.02). However, no significant relationship was found between MDR and class II integron genes ($\chi2$=1.68, p=0.36).

Table 5. The relationship between MDR with the prevalence of phylogenetic groups and integron related genes.

		MDR	NMDR	P	$\chi2$
Phylogenetic groups	A+	15	12	0.14	2.36
	A-	13	23		
	B1+	11	7	0.43	0.79
	B1-	27	28		
	C+	3	8	0.10	3.19
	C-	35	27		
	D+	1	3	0.34	1.24
	D-	37	32		
	E+	5	1	0.20	2.56
	E-	33	34		
	F+	2	1	1.00	0.29
	F-	36	34		
Integron related genes	*int*1+	14	4	0.02*	6.33
	int 1-	24	31		
	int 2+	4	1	0.36	1.68
	int 2-	34	34		
	int +	18	4	0.001***	11.18
	int -	20	31		

DISCUSSION

While *E. coli* can cause temporary infections in the mammary gland, it can also cause enduring infections with a high risk of recurrence due to its adhesion ability and invasion into the mammary epithelium (34). Antimicrobial resistance is a potential factor that may play a role in the persistence of *E. coli in vivo*, leading to failure of antimicrobial therapy. The emergence of virulent strains of *E. coli* with zoonotic properties is a serious threat to public health.

To the best of our knowledge, this is the first study in Türkiye examining the relationship between the phylogenetic groups and the presence of integron genes and multi-antibiotic resistance of the bacterium of *E. coli* isolated from cow milk with bovine clinical mastitis.

In studies conducted in different countries (Pakistan, Iran, China, Türkiye, Bangladesh) in recent years, *E. coli* isolation rates in cow milk with mastitis vary between 7.8% and 35.8% (12, 15, 22, 23, 24). The highest *E. coli* isolation rate was reported in the study conducted in Bangladesh. Since the samples were taken in the present study from farms with persistent mastitis, it is not surprising that moderately high rates of *E. coli* isolation were reported. The differences between the isolation rates in the above-mentioned studies may vary depending on the different antibiotic usage habits in the farms or the different geographical characteristics of the regions where the studies were conducted.

Understanding the genomic structure of *E. coli* showed that strains belonging to different phylogroups were not randomly distributed, but were related to the source of isolation (7). Phylogenetic characterization is an important tool for improving understanding of the *E. coli* population and the relationship between strains and disease (35). In a study conducted in Türkiye in 2017, the most common phylogroups were B1 (45.2%) and C (37.4%), while the other phylogroups A (5.7%), D (1.3%), E (8.9%) and F (0.6%) were found at very low rates (14). In this study, a total of 73 *E. coli* were isolated and 69 (94.5%) of the isolates were phylotyped whereas four (5.5%) could not be phylotyped.

In this study, similar to other studies, phylogroup A (37.0%) and phylogroup B (24.7%) were the two phylo-

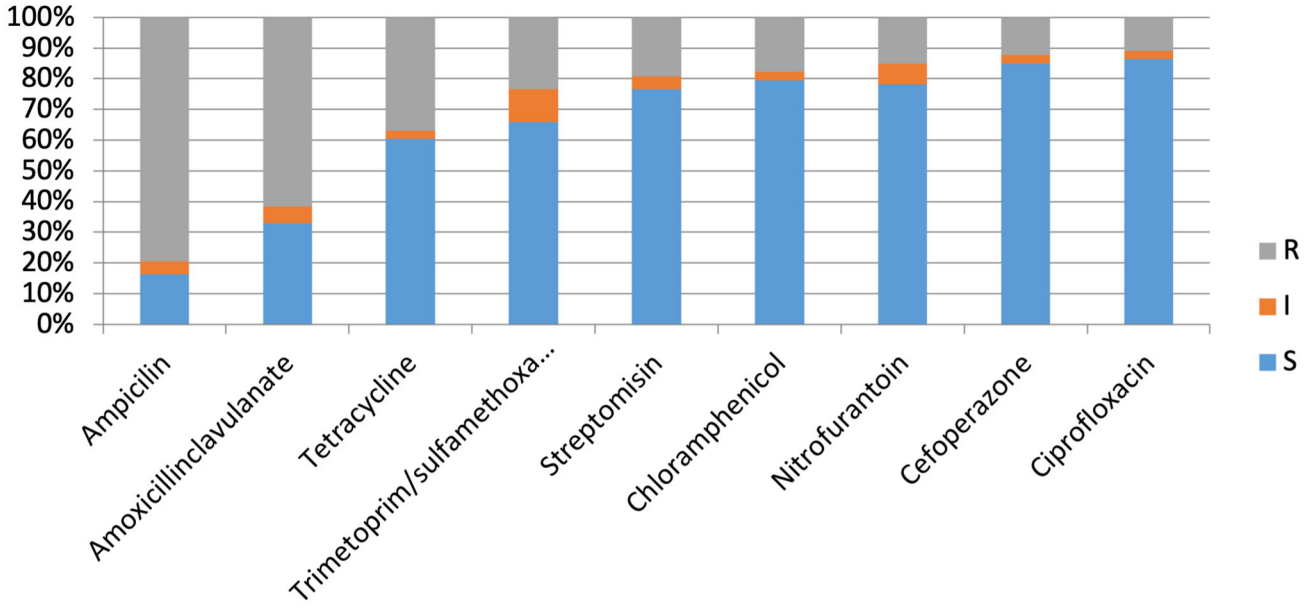

Figure 5. Antimicrobial susceptibility and resistance profiles of *E. coli* isolates.

types detected with the highest rates (64.7%). Therefore, it can be considered that our isolates are mostly commensals transmitted from the gastrointestinal tract of cattle to the udder. This may suggest that udder hygiene has not deserved enough attention during and after milking in farms. In other studies, evaluating the phylogeny of *E. coli* isolated from mastitic bovine milk, phylogroups A and B1 were detected at high rates (10, 12, 13, 15, 23, 24, 36). In our study, lower rates (32.8% in total) were detected in other phylogroups C (15.0%), D (5.5%), E (8.2%) and F (4.1%). The fact that 32.8% of our isolates were phylotypes other than commensal phylotypes found in the gastrointestinal tract also showed the potential of extraintestinal phylotypes to cause clinical mastitis remains potentially possible.

In this study, parallel results were obtained with studies showing that phylogroup E strains can cause clinical mastitis at relatively low rates (8.2%) (10, 14). The best-known strains of phylogroup E belong to group O157:H7, which has been involved in several fatal outbreaks (37). The newly identified phylogroup F was named closely related to phylogroup B2 (7, 8). In this study, phylotype F was determined at the lowest rate (4.1%) compared to other phylotypes. However, the B2 phylotype, which is the phylotype usually carried by humans, was not found. B2 phylotype has been reported by a limited number of researchers in Brazil, Iran and Pakistan (13, 23, 24); In studies conducted in France, Türkiye, China, Mexico and Bangladesh (10, 12, 14, 15, 36), it was not detected as in this study.

In the present study, it was determined that extra-intestinal phylotypes phylotype D (5.5%) and phylotype F (4.1%) may also cause mastitis, albeit at low rates. Although it has been reported in previous studies that phylotype D may cause acute bovine mastitis (10, 13, 23, 24, 36), there is very limited information about phylotype F (14).

Integrons are genetic elements that play a vital role in the development and spread of multi-antibiotic resistance in clinical isolates due to their ability to capture and integrate gene cassettes. Integrons may contribute to the multidrug resistance phenotype of integron-positive strains, but other resistance mechanisms may also be involved (16, 19). Evaluation of antimicrobial resistance pattern by genotypic and phenotypic methods in epidemiological studies is beneficial in terms of controlling antimicrobial resistance and improving the results of treatments.

The number of studies examining the frequency of integrons in *E. coli* isolated from animals is quite limited. To the best of the knowledge of the authors, integrons were detected for the first time in Türkiye in clinical bovine mastitis caused by *E. coli* in the present study. In a study conducted in Tunisia, it was reported that class 1 integrons were detected in 50% (18) of extended spectrum-lactamase positive *E. coli*,

and in 83.3% in China (17). In this study, on the other hand, lower rates of integron genes (31.5%) were detected than the two studies mentioned. Since both of the above-mentioned studies (17, 18) examined multi-antibiotic-resistant extended spectrum-lactamase positive *E. coli* isolates, the integron gene carrying rates of these isolates may have been found to be high.

Bacterial species seen in farms, antibiotic preferences used by employees, regional and administrative differences lead to different results in antibiogram studies. In a study conducted in Bangladesh, the highest resistance was observed against amoxicillin (94.5%), ampicillin (89.5%) and tetracycline (89.5%) in *E. coli* isolates (12). In a study conducted in Iran, the highest resistance was seen against streptomycin (97.2%) amoxicillin (66.1%) and tetracycline (42.2%) (23). In this study, a high level of resistance was observed against ampicillin (79.4%) and amoxicillin clavulanate (61.6%) furthermore, the rate of chloramphenicol resistant isolates was 17.8%. Chloramphenicol is a broad-spectrum bacteriostatic drug. It is especially suitable for the treatment of conjunctivitis in cattle. Due to side effects such as anemia caused by bone marrow suppression, it should not be routinely used as the first choice in infectious diseases of cattle, especially in livestock (38). In a study conducted in Türkiye in 2019, the rate of chloramphenicol resistance was reported as 89.5% in *E. coli* isolates obtained from cow's milk with clinical mastitis (39). It is signficant that the rate of chloramphenicol resistance has decreased over the past three years.

In the majority of mastitis cases, antimicrobial therapy may fail for various reasons (use of antimicrobials without antibiogram testing, use of suboptimal doses and durations, etc.). The increase in the number of antimicrobial resistant isolates in recent years may be a sign of treatment failures. Multi-drug resistant *E. coli* from bovine mastitis has been reported frequently (12, 23, 40, 41). In a study conducted in Türkiye in recent years, it was shown that 48.3% of *E. coli* isolates developed resistance to all antimicrobial agents used (22). In this study, the rate of multi-antibiotic resistant strains was high (52.1%). Again, high levels of MDR (100%, 84.2%, and 40.6%) have been reported in Iran (23), Bangladesh (12) and Canada (40). These results point out that there is an increasing resistance to conventional antibiotics among *E. coli* strains that cause clinical mastitis.

Many antibiotics are prescribed and widely dosed in the treatment of bacterial infections; this contributes to the emergence of resistant strains.The MAR index is an effective, valid and cost-effective method used to trace the source of antibiotic-resistant organisms (42). In this study, MAR indexes of 35 isolates (47.9%) that were not resistant to multiple antibiotics also ranged from zero to 0.2. However, MAR indexes of 38 multi-antibiotic resistant isolates (52.1%) ranged from 0.3 to 0.6. In general, samples with a MAR index exceeding 0.2 can always pose a problem for public health because they carry a high risk of contamination (33), and are considered to be an indication of isolates originating from an environment where antibiotics are frequently used (32). These findings show that antibiotics are frequently used in animals.

Integrons and resistance genes in plasmids can spread easily among bacterial species (16, 19). Bacteria can thus act as a reservoir for the spread of multiple antibiotic resistance. The presence of class I and class II integron genes in isolates obtained from animal clinical samples in Türkiye was previously detected (43, 44). Different studies have shown that the prevalence of class I integron genes is higher than that of other integron gene classes. In a study conducted in Türkiye in 2022, it was reported that 74% of 50 uropathogen *E. coli* isolates carried the class I integron gene and 44% carried the class II integron gene; and, as previously emphasized that antibiotic resistance rates are higher in strains containing integron (46). Similarly, in this study, it was determined that 31.5% of the isolates carried integron genes (class 1, 24%; class 2, 6.9%). In Syria, 54.6% of the uropathogen *E. coli* isolates carried the class I integron gene and there was a strong correlation between multidrug resistance and class I integrons, as in this study (47).

In this study, it was determined that the rate of multi-antibiotic resistance was high (52.1%) in *E. coli* isolates obtained from clinical mastitis in western Türkiye. Although it has been determined environmental *E. coli* pathotypes cause clinical mastitis at higher rates; extra-intestinal pathotypes have also been isolated from clinical mastitis at a substantial rate. An environmental pathogen, such as *E. coli*, infecting the udder may suggest that these infections maybe caused by poor hygienic conditions; however, other phylotypes that are not found in the gastrointestinal tract also have the potential to cause clinical mastitis at substantial rates.

The findings of this study showed that commensal *E. coli* has a high potential to develop antimicrobial resistance. In this study, it was determined that 31.5% of the isolates

carried the integron related gene. In particular, it has been shown that class I integrons are extremely important for the emergence and transmission of multi-antibiotic resistance among bacteria. To improve current treatment strategies and expand our therapeutic options, there is a need for better understanding of all the characteristics of multiple antibiotic resistant isolates and studies using more samples.

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