Investigation of Antibiotic Resistance and Important Virulence Genes of *Escherichia coli* Isolated from Clinical Mastitic Bovine Milk

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ABSTRACT

Escherichia coli is one of the major bacterial pathogens causing bovine clinical mastitis worldwide. In this study, the isolation and identification of *E. coli* from clinical mastitic bovine milk samples, detection of enterovirulent pathogenic *E. coli*'s virulence genes [shiga toxin 1 (*stx*1), shiga toxin 2 (*stx*2), heat sensitive toxin (*lt*), heat resistant toxin (*st*), intimin (*eae*A), bundle forming pilus (*bfp*A), invasive antigen locus (*ial),* fimbrial antigen (*aaf*II)] and antibiotic resistance profiles of isolates were studied. A total of 390 milk samples were taken from 328 dairy cows, reared in a farm for one year period, having clinical mastitis symptoms. *E. coli* isolation was performed using conventional methods. Polymerase chain reaction (PCR) was used to confirm bacterial identification and to detect virulence genes. Antibiotic resistances of isolates to ten antimicrobials from eight antimicrobial families were examined by disk diffusion method. *E. coli* was isolated and identified in 17.2% (67/390) of mastitic milk samples. It was determined that out of 67 *E. coli* isolates, 13 (19.4%) carried at least one of the examined virulence genes. The most prevalent virulence genes were *stx*1 (5/13=38.5%), *stx*2 (2/13=15.4%), *st* (2/13=15.4%), *lt* (1/13=7.7%), *eae*A (3/13=23.1%) while there was no detection for *bfp*A, *ial*, and *aaf*II. It was determined that, 53.8% (7/13), 23.1% (3/13), and 23.1% (3/13) of the isolates were Shiga toxin producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC) and enteropathogenic *E. coli* (EPEC), respectively. It was also determined that 89.5% (60/67), 37.3% (25/67), 35.8% (24/67) of all isolates were resistant to chloramphenicol, ampicillin and tetracycline, respectively while 30.0% (20/67) were multiple resistant. It was concluded that the virulence genes of enterovirulent pathogenic *E. coli* were comparatively low, however it was considered that antimicrobial susceptibility should be monitored to detect changes in resistance profiles of mammary pathogen *E. coli* isolated from clinical mastitic bovine milk.

Keywords: Enterovirulent *Escherichia coli*; Clinical Mastitis; Virulence Gene; Antibiotic Resistance.

INTRODUCTION

The natural habitat of *Escherichia coli* is the mammalian gut and most of *E. coli* strains are harmless. However, some strains have acquired virulence genes that turn them into pathogens. Pathogenic strains can cause diseases in both healthy and immunosuppressed individuals (1, 2). Pathogenic *E. coli* strains can be classified into two large groups: intestinal (enterovirulent) and extraintestinal pathogenic. The most important human enterovirulent *E. coli* pathotypes are enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC) and enteroaggregative *E. coli* (EAEC) (1, 2).

EHEC is associated with foodborne outbreaks all over the world due to Shiga toxin secretion (1). Shiga toxin producing *E. coli* (STEC) produce toxins which can damage the digestive tract. Another important virulence factor of the EHEC subset is intimin that is a membrane protein encoded by the *eae* gene. ETEC is the most important bacteria that

cause diarrhea. These strains cause diarrhea with non-heat resistant (LT) and heat resistant (ST) enterotoxins. Some strains may only secrete LT, some may secrete only ST, but ETEC strains that produce both LT and ST toxins are known to cause severe diarrhea (1, 2).

The essential feature of EPEC is adhesion to specific cells on the intestinal mucosa, and this is done by a specific pathogenicity island. Some EPEC strains may also contain the EPEC adhesion plasmid, which also contains the gene cluster encoding the bundle forming pili (*bfp*) (1, 2). While EPEC strains with adhesion plasmids are classified as "typical EPECs", EPEC strains carrying only intimin genes without adhesion plasmids are classified as "atypical EPECs" and intimin, plays a role in the final stage of adhesion. *E. coli* having aggregative properties and is named as entroaggregative *E. coli* (EAEC) (2). The pathogenicity and clinical significance of these bacteria are still controversial. Another group with significantly lower prevalence is enteroinvasive *E. coli* (EIEC). Pathogenesis of these is based on phagocytosis and release from the activated complement system via the capsules. Currently, many researchers use molecular methods such as polymerase chain reaction (PCR) to identify these important pathotypes (1, 2).

Antimicrobial resistance is an important problem in both humans and animals all over the world. *E. coli* strains are considered to be excellent indicators of antimicrobial resistance because they are part of the normal microbiota, and also occur in the environment. The use and misuse of antimicrobial agents has led to the development of resistance. However, resistance to the majority of these antimicrobial agents is now developed. In recent years, antibiotic resistance studies have reported very different resistance rates in clinical *E. coli* mastitis in different countries (3-5).

E. coli is the most common bacterial agent causing mastitis in Turkey following *Staphylococcus aureus* (6). Clinical mastitis usually occurs when the udder is contaminated with feces and skin related bacteria microorganisms enter the mammary gland through the teat canal. In Turkey, there is no study about mammary pathogenic *E. coli* isolated from clinically mastitic bovine milk. For this purpose, it was aimed to isolate and identify *E. coli* strains from clinical mastitic bovine milk samples, to detect enterovirulent *E. coli*'s virulence genes (*stx*1, *stx*2, *lt, st, eae*A, *bfp*A, *ial, aaf*II) and to obtain antibiotic resistance profiles from all mammary pathogenic isolates.

MATERIAL AND METHODS

Clinical examination

Dairy cows were carefully examined for clinical mastitis by the veterinarian. Changes in the udder (such as swelling, heat, hardness, redness, or pain) and in milk (such as a watery appearance, flakes, clots, or pus) were noted. The animals with these findings were assessed as clinical mastitis (7).

Milk samples

Cows from which the samples were taken from were chosen from those cows which had not had any treatment since at least one month previously. In the study, a total of 390 milk samples were taken from 328 dairy cows with clinical mastitis reared in a private farm for a period of one year. One, 2, and 3 mastitic milk samples were obtained from 292, 10, and 26 cows, respectively. Milking on this farm was performed automatically. The age of the cows varied between 2 to 9 years.

Sample collection

On sampling, teat ends were cleaned 70% alcohol moistened swabs and allowed to dry. After discarding the first few streams, 2-5 ml of milk were collected into sterile 5 ml glass flasks. Samples were kept at 4°C during transportation and examined as soon as possible.

Microbiological examination

Milk samples were centrifuged at 3500 rpm for 5 min and the supernatant discarded. The sediment was vortexed and a loopful streaked on Eosin Methylene-Blue Agar (EMB) (Merck 1.01347, Germany). After incubation at 37°C for 24 hours, growing colonies were evaluated. The identification of the isolates was performed by oxidase test, EMB agar reproduction characteristics, Gram stain morphology and IMVIC reactions. *E. coli* identification was performed as previously described (7) and isolates were stored in Brain Heart Infusion Broth (Oxoid CM 1135, UK) containing glycerol 20% at -20C.

Antimicrobial susceptibility profiles

Ten antibiotics from 8 antimicrobial families were used in the antibiotic susceptibility test as previously described (8). The concentrations and abbreviations for the antimicrobials used in this study were given in Table 1. The results were inter-

Table 1: The antimicrobial agents used, disc contents and evaluation criteria

Antimicrobial Family	Antibiotic	Disc Content (µg)	Zones (Evaluation, mm)			References
			S	≥ (I)	R ≤	
β Lactam Inhibitor Combinations	Amoxicillin-clavulanic acid	20/10	18	14-17	13	8
β Lactam	Ampicillin	10	17	14-16	13	8
Aminoglycoside	Gentamicin	10	15	13-14	12	8
Folate pathway inhibitor	Trimethoprim/ sulfamethoxazole	1.25/23.75	16	11-15	10	8
Tetracycline	Tetracycline	30	15	12-14	11	8
Fenicol	Chloramphenicol	30	18	13-17	12	8
	Cefoperazone	75	21	16-20	15	8
Cephalosporins	Ceftriaxone	30	23	–	20	9
	Ciprofloxacin	5	21	16-20	15	8
Qinolons	Enrofloxacin	5	22	–	17	9

R: Resistant, **S:** Sensitive, **I:** Intermediate

preted as to the Clinical and Laboratory Standards Institute (8) and Comite de l'Antibiogramme de la Societe Française de Microbiologie (9) standards. *E. coli* ATCC 25922 strains were used as quality control for the antibiotic susceptibility tests.

Genotypic identification

The universal stress protein gene (*usp*A) was used in PCR examination for the verification of the *E. coli* isolates (10).

DNA extraction

DNA extraction from *E. coli* was performed using a commercial genomic DNA extraction kit (Fermentas, USA) as recommended by the manufacturer. DNA purity and quantity controls were also performed. The OD260/OD280 ratio indicated that the DNA was purely 1.8-2.0 (11).

PCR

PCR was used for the verification of the *E. coli* isolates and the detection of the most common intestinal pathogenic *E. coli*'s virulence genes. PCR, for each sample was carried out on a volume of 50 µl, final concentration was 10x Taq enzyme buffer solution 1x, 25 mM $MgCl_2$ 2 mM, 10 mM dNTP 0.2 mM, 100 ρmol primer (for each) 0.4 ρmol, 5 U Taq DNA polymerase 1.5 U (Fermentas, USA), 2 µl of each DNA. The prepared tubes were loaded in the thermalcycler (Boeco, Germany).

The DNA was amplified using the following protocol: an initial denaturation at 94˚C for 3 min, followed by 35 cycles of denaturation (94˚C for 30 s), annealing (52˚C for *lt* and *st*, 54˚C *usp*A and *ial*, 59˚C *stx*1, *stx*2 and *eae,* 60˚C *bfp*A and *aaf*II for 30 s) and extension (72˚C for 1 min), with a

Table 2: Primers used in the study

Pathotype	Target Gene	The virulence factor encoded by the target gene	Sequence (5'→3')	Tm	Product Size (bp)	References
E. coli	*usp*A	Universal stress protein	CCGATACGCTGCCAATCAG ACGCAGACCGTAGGCCAGAT	53.8 55.8	884	10
STEC	*stx*1	Shiga toxin 1	CTGGATTTAATGTCGCATAGTG AGAACGCCCACTGAGATCATC	58 61	150	12
	*stx*2	Shiga toxin 2	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	63 60	255	12
ETEC	*lt*	Heat sensitive toxin	GGCGACAGATTATACCGG CGGTCTCTATATTCCCTGT	60 56	450	12
	st	Heat-resistant toxin	ATTTTTCTTTCTGTATTGTCTT CACCCGGTACAAGCAGGATT	51 60	190	12
EIEC	*ial*	Invasive antigen locus	GGTATGATGATGATGAGTCCA GGAGGCCAACAATTATTTCC	57 56	650	12
	*eae*A	Intimin	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	63 63	384	12
EPEC	*bfp*A	Bundle forming pilus	AATGGTGCTTGCGCTTGCTGC GCCGCTTTATCCAACCTGGTA	66 64	324	12
EAEC	*aaf*II	Fimbrial antigen	CACAGGCAACTGAAATAAGTCTGG ATTCCCATGATGTCAAGCACTTC	64 61	378	13

Tm: Melting Temperature.

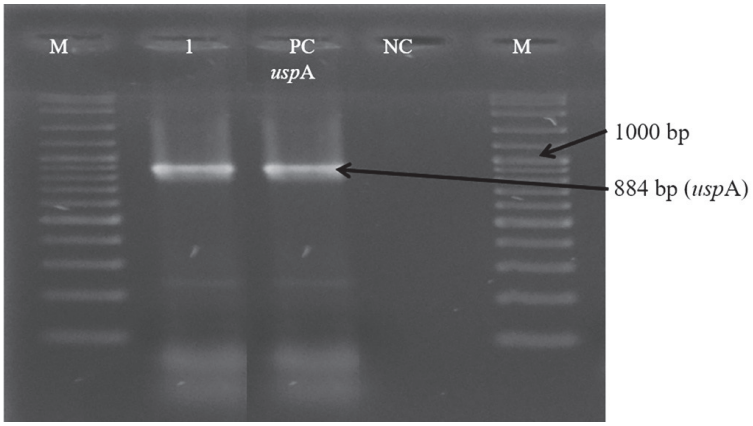

Figure 1: Gel electrophoresis of *E. coli* isolates **M:** 100 bp DNA Ladder **1:** Field *E. coli* isolate **PC:** Positive Control (*E. coli* ATCC 25922) **NC:** Negative Control (Master mix without DNA)

single final extension of 7 min at 72˚C. On electrophoresis, a 2% agarose gel stained with safe view (ABM, Canada) was used and the gel was exposed to 100 volts for 45 min. After electrophoresis, the gel was placed in the chamber of the transilluminator device connected to the computer and photographed under UV light. The primers used in the study were given in Table 2 (10, 12, 13). *E. coli* ATCC 35150 (*stx*1, *stx*2, *eae*A genes positive EHEC strain), ATCC 35401 (*lt*, *st* genes positive ETEC strain), ATCC 43893 (*ial* gene positive EIEC stain) and a sequenced field isolate (*bfp* gene positive EPEC strain) were used as positive controls.

RESULTS

Isolation and identification

In this study, 67 (17.2%) *E. coli* suspected isolates were obtained from 390 clinical mastitic bovine milk samples. Gram negative rod, oxidase -, indole +, MR +, VP -, citrate - isolates were identified as *E. coli*.

Genotypic identification

Following PCR with the *usp*A specific primers, 884 bp long product was obtained in all 67 isolates. It was molecularly confirmed that all isolates were *E. coli* (Figure 1). After this verification, antibiotic resistance and virulence genes of 67 isolates were examined.

Antibiotic resistance

The resistance profiles to 10 antibiotics from eight different antimicrobial families were studied. Out of 67 *E. coli* strains, 60 (89.5%) were found as resistant to chloramphenicol, 25 (37.3%) to ampicillin, 24 (35.8%) to tetracycline, 15 (22.4%) to trimethoprim/sulphametaxazole, 11 (16.4%) to enrofloxacin, 8 (11.9%) to ciprofloxacin, 7 (10.4%) to ceftriaxone and cefoperazone, 3 (4.5%) to gentamicin, 2 (3%) to amoxicillin/clavulanic (Table 3). Resistance to phenicol and β lactams group was the highest whereas resistance to beta-lactam inhibitor combinations and aminoglycoside group was lower.

The bacteria with three or more antimicrobial resistance were considered as multi-resistant (14). Accordingly, 3.0% of the isolates were found as susceptible to all antibiotics used, while 30.0% of the isolates were multi-resistant. A total of 10 antibiotic resistant phenotypes were found. It was determined that 10.5%, 4.5%, 4.5%, 9.0%, and 1.5% of the isolates were resistant to 3, 4, 5, 6, and 7 antimicrobial families, respectively (Table 4).

Virulence genes

The presence of eight important virulence genes (*stx*1, *stx*2, *st, lt*, *ial, eae*A, *bfp*A, *aaf*II) of *E. coli* from five pathotypes (EHEC, ETEC, EIEC, EPEC, EAEC) was investigated by PCR. The analysis of virulence genes of 67 isolates identified as *E. coli* revealed that 19.4% (13/67) of the isolates carried one virulence gene. It was determined that the most prevalent virulence genes were *stx*1 (5/13=38.5%), *stx*2 (2/13=15.4%), *st* (2/13=15.4%), *lt* (1/13=7.7%), *eae*A (3/13=23.1%). Results showed that 53.8% (7/13) of the

Table 3: Antibiotic resistance rates of isolates

Antibiotic	Suscebtible S (%)	Intermediate I (%)	Resistant R (%)
Chloramphenicol	2 (3.0)	5 (7.5)	60 (89.5)
Ampicillin	18 (26.9)	24 (35.8)	25 (37.3)
Tetracycline	42 (62.7)	1 (1.5)	24 (35.8)
Trimethoprim/ Sulphametaxazole	49 (73.1)	3 (4.5)	15 (22.4)
Enrofloxacin	48 (71.7)	8 (11.9)	11 (16.4)
Ciprofloxacin	50 (74.6)	9 (13.5)	8 (11.9)
Ceftriaxone	27 (40.3)	33 (49.3)	7 (10.4)
Cefoperazone	51 (76.2)	9 (13.4)	7 (10.4)
Gentamicin	56 (83.7)	8 (11.8)	3 (4.5)
Amoxicillin-Clavunic Acid	55 (82.1)	10 (14.9)	2 (3.0)

* Total Isolate Number: n=67.

Table 4: Multi-drug resistance patterns in *E. coli* isolated from clinical mastitis

Number	Antibiotics resistance patterns	Number (%) of multi-resistant isolates		
		All isolates (n=67)	Isolates carrying virulence gene (n=13)	
			STEC	ETEC
1	AMC, AMP, CHL	1 (1.5%)	1 (7.7%)	
2	CHL, TMS, TET	1 (1.5%)		
3	CHL, TET, CIP	2 (3.0%)		
4	AMP, TMS, CHL	3 (4.5%)		1 (7.7%)
5	AMP, CHL, TET, TMS	3 (4.5%)	1 (7.7%)	
6	AMP, CHL, TET, TMS, ENR	2 (3.0%)	1 (7.7%)	
7	AMP, CHL, TET, TMS, CIP	1 (1.5%)	1 (7.7%)	
8	AMP, CHL, TET, TMS, ENR, CEP, CEF	1 (1.5%)	1 (7.7%)	
9	AMP, CHL, TET, TMS, ENR, CIP, CEP, CEF	5 (7.5%)	1 (7.7%)	2 (15.4%)
10	AMC, AMP, CHL, TET, TMS, ENR, CIP, CEP, CEF	1 (1.5%)		

AMC: Amoxicillin-Clavulanic Acid, **AMP:** Ampicillin, **TMS:** Trimethoprim/Sulfametaxazole, **TET:** Tetracycline, **ENR:** Enrofloxacin, **CHL:** Chloramphenicol, **CEP:** Cefoperazone, **CIP:** Ciprofloxacin, **CEF:** Ceftriaxone
Note: *A STEC isolate is sensitive to all antibiotics used.
** One EPEC isolate 2, two EPEC isolates were resistant to 1 antibiotic.
***None of the isolates with multiple antibiotic resistances had resistance to gentamicin.

Table 5: Classification of enterovirulent *E. coli* isolates

Pathotype	Virulence gene	Number of isolates (%) (n=13)	Total number of isolates (%) (n=13)
STEC	*stx*1	5 (38.4)	7 (% 53.8)
	*stx*2	2 (15.4)	
ETEC	*st*	2 (15.4)	3 (% 23.1)
	lt	1 (7.7)	
EPEC	*eae*A	3 (23.1)	3 (% 23.1)
	*bfp*A	0 (0.0)	
EAEC	*ial*	0 (0.0)	0 (0.0)
EIEC	*aaf*II	0 (0.0)	0 (0.0)
TOTAL		13	

isolates were belonged to STEC, 23.1% (3/13) to ETEC, and 23.1% (3/13) to atypical EPEC. And all these 13 isolates were carrying the single virulence gene (Table 5, Figure 2). It also noted that there was no isolates carrying the virulence genes of the typical EPEC, EIEC and EAEC pathotypes.

DISCUSSION

Clinical mastitis usually occurs when the udder is contaminated with feces and microorganisms entry the mammary gland through the teat canal. Actually, most of mastitis cases in most of regions are caused by pathogens colonizing the teat and udder skin (7). In recent years, the increase of *E. coli* mastitis may be associated with virulence gene diversity and increased antibiotic resistance (15). In this study, *E. coli* was isolated at a rate of 17.2% from clinical mastitic milk samples. In studies conducted in different countries, it has been reported that *E. coli* was isolated from mastitic bovine milk at various rates ranging from 21% to 69% from India and Swedish, respectively (16,17). The difference in isolation rates may be depend on the collection of materials in different geographical regions and seasons, differences in sampling methods, and farm hygiene.

Shiga toxin-producing *E. coli* is a major source of foodborne infections. Dairy cows are the most important reservoir of STEC. STEC colonization in adult ruminants which is asymptomatic, unlike humans (18). One of the most important transmission routes of STECs to humans is unpasteurized milk (19). In this study, the *stx*1 frequency was found higher than the *stx*2 frequency. This can be accepted as parallel with reports showing that most STEC strains from cattle harbor the *stx*1 gene (20) but contrasts to another study (21) in Brazil. Jenkins *et al.* (22) reported that the rates of *stx*2 were higher among cattle except in the winter season. Studies have reported that *stx*2 was a more important virulence factor than *stx*1, which is associated with human and animal diseases (23).

Although ETEC isolates originating from mastitis are generally reported to harbor *st*, the isolate ratios of ETECs isolated from bovine milk are relatively low (24). In this study, 4.5% of all *E. coli* isolates were identified as ETEC. The results were consistent with the findings that ETECs with bovine mastitis originated generally at low rates and produced more *st* (24).

The *eae*A and *bfp* genes are used to identify EPEC (1,2). The virulence factor *eae*A, when present in *stx*-negative *E. coli* strains, is known as the atypical EPEC

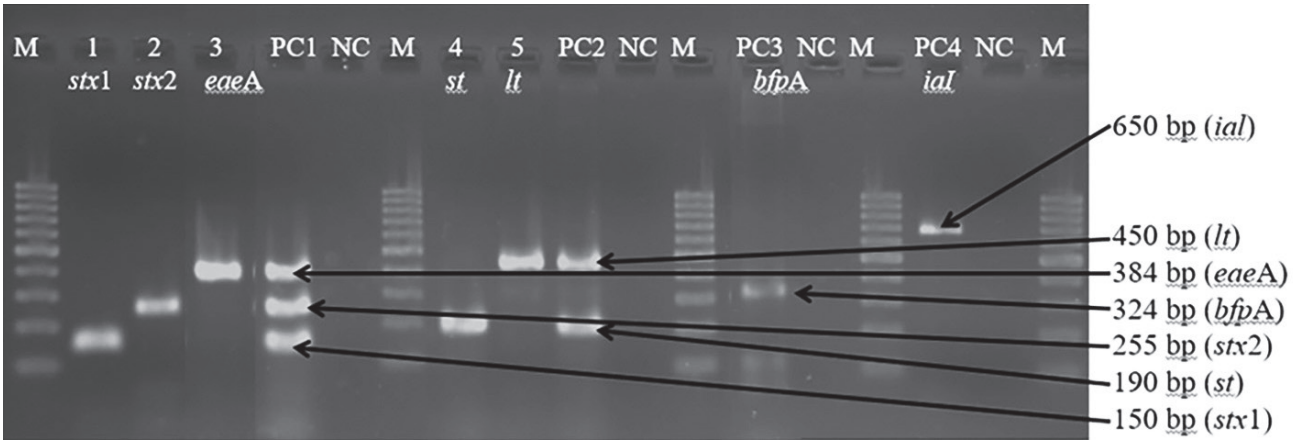

Figure 2. Gel electrophoresis images of virulence genes **M:** 100 bp DNA Ladder **1.** *stx*1 gene positive field isolate **2.** *stx*2 gene positive field isolate **3.** *eae*A gene positive field isolate **4.** *iaI* gene positive field isolate **5.** *lt* gene positive field isolate **PC1:** *E. coli* ATCC 35150 (EHEC) **PC2:** *E. coli* ATCC 35401 (ETEC) **PC3:** Sequenced EPEC field isolate **PC4:** *E. coli* ATCC 43893 (EIEC) **NC:** Negative Control (Master mix without DNA)

(1,2). In this study 4.5% of 67 *E. coli* isolates were identified as atypical EPEC. Caine *et al.* (25) investigated two farms in South Africa and reported the ratios as 24.5% and 18% for atypical EPEC. In the same study, typical EPEC isolation was not reported. In other studies, the presence of EPEC in milk also varied in different regions (0.8%-33%) (26, 27).

Antibiotic resistance is a major problem for human and animal health (28). Because of the resistance, the treatment of infectious diseases results in economic losses. Due to overuse and inappropriate antimicrobial use, resistance to the majority of antibiotics has developed. In this study, *E. coli* strains were found resistant to chloramphenicol (89.5%), ampicillin (37.3%) and tetracycline (35.8%); gentamycin (4.5%) and amoxicillin-clavulanic acid (3.0%). Previous studies reported different resistances to antibiotics, especially penicillin (4,21), tetracycline (21), trimethoprim-sulphamethoxazole (29), fluoroquinolones (30) and cephalosporin (31). In these studies, the resistance profiles of *E. coli* isolates to antimicrobial agents appeared to be different from each other. This difference is thought to be due to the fact that the antimicrobial agents commonly used between countries or between enterprises are dissimilar.

Chloramphenicol is a broad spectrum bacteriostatic drug. It is especially suitable for conjunctivitis treatment in cattle. Due to side effects such as anemia caused by bone marrow suppression, it should not routinely be used as a first choice, especially in food animals, in infectious diseases of cattle (32). Although chloramphenicol has been banned for use in food animals, a similar high level of resistance was reported in China (33,34) and Turkey (35). At first glance, there was no logical explanation for these high levels of resistance but it might be related to the use of florfenicol, a fluorinated derivative of chloramphenicol, which was approved in 1996 to treat bovine respiratory infections and thus could have been introduced into many livestock operations (36). Also, this might be due to the fact that chloramphenicol (or florfenicol), β lactam and tetracycline were often widely used for the prevention or treatment of bovine mastitis in the past in our region. For this reason, follow-up of antibiotic resistance profiles on a regular basis is needed for successful treatment.

Antibiotic resistance and virulence genes can be carried on the same chromosomal structures or plasmids (37). In this study, 29.9% of all *E. coli* isolates and 69.2% of the isolates having virulence gene were found as multiple resistant. This was an important finding for this study indicating that isolates with a virulence gene might also be multiple resistant. However, antibiotic resistance at the genomic level was not evaluated.

In this study, it was determined that 19.4% of isolates were *E. coli* for the investigation virulence genes. It is recommended that in order to distinguish mammary pathogen *E. coli* from environmental *E. coli* it's essential to investigate different virulence genes (long polar fimbriae, increased serum resistance and enteroaggregative *E. coli* heat-stable enterotoxin 1) (38) and main determinants of pathogenicity (the ferric dicitrate locus) (39).

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