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Determination of virulence factors and antimicrobial resistance of *E. coli* isolated from calf diarrhea, part of eastern Turkey

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Abstract: Microorganisms have a primary role in the formation of calf diarrhea. *Escherichia coli* pose an environmental risk to young animals caused by fecal excretion. In this study, rectal swab samples (n= 133) were collected from calves with diarrhea aged from 1 day to 3 months, between August 2017 and August 2018. The samples were cultured on MacConkey agar, and then antimicrobial susceptibility and virulence genes for *Escherichia coli* isolates (n= 133) were investigated by disk diffusion method according to clinical and laboratory standards institute standards and multiplex polymerase chain reaction, respectively. The isolates were found to be highly resistant to oxytetracycline (78.9%), trimethoprim-sulfamethoxazole (69.2%), neomycin (60.9%), and erythromycin (58.6%). Besides, multidrug resistance was determined in 71.4% of isolates. Thirty-three of 133 (24.81%) isolates were positive for at least one virulence factor. The pathotypes of enterotoxigenic *Escherichia coli* (F5 and/or F41 fimbria and STa), enterohemorrhagic *Escherichia coli* (*Stx* and *eae*), enteropathogenic *Escherichia coli* (*eae*) and Shiga toxin-producing *Escherichia coli* (*Stx-eae*) were found in 51.5%, 6.1%, 15.2%, and 12.1%, respectively. However, the virulence properties were detected as; *Stx*1 (3.03%), *Stx*2 (9.09%), STa (21.21%), and *eae* (15.15%); the F41 and F5 were not detected. Also, the fifteen-point two percent of strains (5/33) were the hybrid type that carried both *Stx* (either *Stx*1 or *Stx*2) and enterotoxigenic *Escherichia coli* specific enterotoxin gene STa. The existence of different virulence factors found in this study supports the statement that calves are possible bearers of pathogens that are dangerous to public health.

Keywords: Antimicrobial resistance, diarrhoea, *Escherichia coli*, hybrid strain, virulence gene.

Türkiye'nin doğusunda buzağı ishallerinden izole edilen *E.coli*'lerin virulens faktörlerinin ve antimikrobiyel direncinin belirlenmesi

Özet: Buzağı ishallerinin oluşumunda mikroorganizmalar primer role sahiptir. *Escherichia coli*, genç hayvanlarda fekal atılıma bağlı olarak çevresel bir risk oluşturmaktadır. Bu çalışmada, Ağustos 2017-Ağustos 2018 tarihleri arasında 1gün ile 3 aylık yaştaki ishalli buzağıların rektal svap örnekleri toplandı (n=133). Toplanan örnekler MacConkey agara ekilerek kültüre edildi ve Klinik Laboratuvar Standartları Enstitüsünün bildirdiği standartlara göre antibiyotik duyarlılıkları belirlendi, multipleks polimeraz zincir reaksiyonu ile de virulens özellikleri incelendi. İzolatlar oksitetrasikline (%78,9), trimethoprim-sulfamethoxazole (%69,2), neomisine (%60,9) ve eritromisine (%58,6) yüksek oranda dirençli bulundu. Aynı zamanda, izolatların %71,4'ünde çoklu direnç saptandı. 133 izolatın 33'ünde (%24,81) en az bir virulens faktör pozitif bulundu. Enterotoksijenik *E. coli* (F5 ve/veya F41 fimbria ve STa), enterohemorajik *Escherichia coli* (*Stx* ve *eae*), enteropatojenik *E. coli* (*eae*) ve Shiga toksin oluşturan *E. coli* (*Stx-eae*) patotipleri sırasıyla %51,5, %6,1, %15,2 ve %12,1 oranlarında bulundu. Virulens özellikleri *Stx*1 %3,03, *Stx*2 %9,09, STa %21,21 ve *eae* %15,15 oranlarında bulunurken, F41 ve F5 bulunamadı. Suşların %15,2'si hem *Stx* (*Stx*1 ve *Stx*2) hem de enterotoksijenik *E. coli*'ye spesifik enterotoksin geni STa bulundurduğu için hibrid suş olarak tespit edildi.

Bu çalışmada farklı virulens faktörlerin varlığının belirlenmesi, buzağıların halk sağlığı açısından tehlikeli olan patojenlerin taşıyıcısı olabileceğini desteklemektedir.

Anahtar sözcükler: Antimikrobiyel direnç, *Escherichia coli*, hibrit suş, ishal, virulens geni.

Introduction

Calf diarrhea, which causes serious economic losses, is an important issue in cattle breeding in Turkey and worldwide. Microorganisms, variable environmental conditions, and farming-dependent issues primarily affect the formation of infection (4, 10, 13). *Escherichia coli* has environmental epidemiology that causes important risk to young animals. Diarrhea in calves is a common issue in the early years of life and occurs in almost every farm, affecting animal welfare worldwide. Moreover, diarrhea can frequently lead to death in animals in less than one month old. In addition to death, treatment, veterinary costs

are a crucial issue of economic loss to farmers because of the colibacillosis. Infectious and noninfectious agents play an important role in calf diarrhea. Effective control of calf diarrhea with a multifactorial structure is difficult. *Escherichia coli* is the most frequently isolated bacteria in calves less than 2 months old. The prevalence of these bacteria in farms depends on the geographical status, farm management, and herd size (14, 19, 27).

Strains of *E. coli* colonize the host's intestine with different virulence factors and induce diarrhea by escaping the immune system. The virulence factors of bacteria have an important role in colonization and adhesion (F2-F6; F17; F18; F41 fimbria and intimin; LT, STa, and STb; and verotoxin). Virulence factors can be combined, particularly in persistent infections. *Escherichia coli* pathotypes, such as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), verotoxin- and Shiga toxin-producing *E. coli* (STEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli*, and enteroadherent *E. coli* (EAEC) strains, may responsible for diarrhea in farm animals and humans. When ETEC infection occurs in young calves, it is called colibacillosis (19, 23, 27).

Adhesion factors (F17 fimbria, S fimbria, P fimbria, a fimbrial adhesin, and capsule-like adhesin structures) that are involved in the binding of bacteria to cells can be found in the chromosomal structures of *E. coli* and are encoded by plasmids. Various toxin structures of *E. coli* (Shiga toxin, CNF1, CNF2, labile toxin, and stable toxin) are effective in the pathogenesis of infection with different features. The severity of infection caused by *E. coli* strains with more than one virulence factor may vary depending on the host's immune system (15, 19, 24). The development of molecular techniques has facilitated the identification of virulence factors; however, it is difficult to determine the virulence factors phenotypically and erroneous results often occur (15, 24). Shiga toxin-producing *E. coli* can cause significant acute illnesses, such as severe food-mediated gastrointestinal infections and hemolytic uremic syndrome, and are effective for a long time in humans, causing diarrhea in both animals and humans (2, 9).

This study was aimed to reveal the antimicrobial susceptibility and virulence genes of *E. coli* that lead to calf diarrhea in various cattle farms.

Materials and Methods

Sampling E. coli isolation and identification: Between August 2017 and August 2018, 133 diarrheal calves (<3 months of age) samples were cultured to *E. coli* isolation. The rectal swab samples of calves were collected from Atatürk University Faculty of Veterinary Medicine Animal Hospital that was located in Erzurum, Turkey. It was not known whether the animals were given antimicrobials before sampling. Rectal samples were collected by using sterile swabs containing Stuart medium. The samples were delivered to the laboratory as soon as possible in cold containers and examined bacteriologically without any delay. Samples were directly streaked on the MacConkey agar for the isolation of *E. coli*. The media were incubated at 37°C for 24 hrs. Lactose positive colonies from each culture were selected and confirmed to be *E. coli* by species-specific PCR after sub-culturing in tryptic soya broth (TSB). All strains were stored in TSB containing 10% (v/v) glycerol at -20°C until further use (13).

Detection of virulence factors of E. coli strains: *E. coli* strains were sub-cultured on TSB for 16-18 hrs. To extract genomic DNA, the supernatant was discarded after centrifugation of one ml of the broth culture placed in a 1.5 mL tube, pellet resuspended in 100µL sterile distilled water, boiled at 100°C for 15 min, and centrifuged at 12.000×g for 15 min. All of isolates were confirmed as *E. coli* by PCR (29). Then, multiplex PCR (mPCR) was performed to detect the virulence genes (Table 1), (*Stx*1,

Table 1. Primer sequences, predicted size of PCR products.

Primer	Oligonucleotid sequences	Size of product	References
PhoA F	GGTAACGTTTCTACCGCAGAGTTG	468 bp	29
PhoA R	CAGGGTTGGTACACTGTCATTACG		
*Stx*1 F	TTC GCT CTG CAA TAG GTA	555 bp	15
*Stx*1 R	TTC CCC AGT TCA ATG TAA GAT		
*Stx*2 F	GTG CCT GTT ACT GGG TTT TTC TTC	118 bp	15
*Stx*2 R	AGG GGT CGA TAT CTC TGT CC		
Intimin F	ATA TCC GTT TTA ATG GCT ATC T	425 bp	15
Intimin R	AAT CTT CTG CGT ACT GTG TTC A		
F41 F	GCA TCA GCG GCA GTA TCT	380 bp	15
F41 R	GTC CCT AGC TCA GTA TTA TCA CCT		
K99 F	TAT TAT CTT AGG TGG TAT GG	314 bp	15
K99 R	GGT ATC CTT TAG CAG CAG TAT TTC		
STa F	GCT AAT GTT GGC AAT TTT TAT TTC TGT A	190 bp	15
STa R	AGG ATT ACA ACA AAG TTC ACA GCA GTA A		

*Stx*2, STa, F5, F41, and *eae*) of the isolates (12, 15, 24). The 50 µL of PCR mixture was contained 5 µL 10X PCR buffer, 1.5 mmol $MgCl_2$, 2 µL dNTP mix (2.5 mM each of dNTPs), 1 µL forward and reverse primers, 0.2 µL Taq DNA polymerase (5 U/µL, Thermo Scientific), 5 µL templateDNA, and up of molecular grade distilled water. Amplification was performed with 25 cycles of amplification at 95°C for 60 sec initial denaturation, 94°C for 30 sec of denaturation, 50°C for 45 sec of annealing, 70°C for 90 sec of extension, and 10 min of final extension step at 72°C. The PCR products were subjected to 1% agarose gel electrophoresis at 130 volts for 30 min by being stained with ethidium bromide (0.5 µg/mL). *E. coli* ATCC 25922 DNA was used as the quality control strain for species-specific PCR.

Antimicrobial susceptibility testing: Antimicrobial susceptibilities test for isolates were performed by disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guideline (7). The antimicrobial discs (Oxoid, UK) tests were trimethoprim-sulfamethoxazole, gentamicin, oxytetracycline, enrofloxacin, chloramphenicol, ofloxacin, ciprofloxacin, marbofloxacin, erythromycin, neomycin, cefoperazone, cefuroxime, ampicillin-sulbactam, and ceftiofur. *Escherichia coli* ATCC 25922 was used as a control strains. The strains were recorded as susceptible, intermediate, or resistant according to the zone diameter interpretative standards recommended by CLSI. Isolates, which are resistant to three or more antimicrobial classes were defined as multidrug-resistant (MDR) isolate (1).

Statistical analysis: Rates of antimicrobial resistance between carrying virulence genes *E. coli* and non-carrying virulence genes *E. coli* were compared by Pearson's Chi-squared (x^2) tests using the statistical package SPSS version 20. $P<0.05$ were considered statistically significant for comparisons (9).

Results

In total, a hundred thirty-three *E. coli* isolated from rectal swabs were confirmed by PCR. Multiplex PCR result showed that 24.81% (33/133) of *E. coli* isolates had various virulence genes (Table 2). The virulence genes were detected *Stx*1 (3.03%), *Stx*2 (9.09%), STa (21.21%),

Table 2. Distribution of antimicrobial resistance class pattern for carrying virulence genes of *E. coli* isolates.

***E. coli* patotypes**	**Virulence gene patterns**	**Frequency (n= 33)**	**Antimicrobial resistance class patterns***
HYBRID	STa -*Stx*2-F41- INTIMIN	1	MCRs, TETs
HYBRID	STa-*Stx*2-F41	1	MCRs, AMGs, PHs, CEPs
HYBRID	STa-*Stx*1-INTIMIN	1	MCRs, TETs
ETEC	STa-F41-K99	2	MCRs, PHs, TETs
ETEC	STa-F41-K99	1	MCRs, TETs
ETEC	STa-F41-K99	2	Qs, MCRs, AMGs, PHs, TETs, FPIs
ETEC	STa-F41-K99	1	Qs, MCRs, AMGs, PHs, TETs, FPIs, CEPs
ETEC	STa-F41-K99	1	Qs, MCRs
HYBRID	STa-F41-INTIMIN	1	Qs, MCRs, AMGs, PHs, TETs, FPIs, CEPs
HYBRID	STa-F41-INTIMIN	1	Qs, MCRs, AMGs, PHs, TETs, FPIs
ETEC	STa -K99	1	MCRs, AMGs, PHs, TETs
ETEC	STa -K99	2	Qs, MCRs, AMGs, TETs, FPIs
EHEC	INTIMIN-*Stx*1	1	Qs, TETs, FPIs
EHEC	INTIMIN-*Stx*1	1	Qs, MCRs, AMGs, PHs, TETs, FPIs
EPEC	INTIMIN	2	Qs, MCRs, AMGs, PHs, TETs, FPIs
EPEC	INTIMIN	1	MCRs, AMGs, TETs, CEPs, FPIs
EPEC	INTIMIN	1	Qs, MCRs, AMGs, TETs, CEPs, FPIs
EPEC	INTIMIN	1	-
ETEC	STa	2	MCRs
ETEC	STa	2	Qs, MCRs, AMGs, PHs, TETs, FPIs
ETEC	STa	1	MCRs, AMGs, TETs, FPIs
ETEC	STa	1	Qs, TETs, FPIs
ETEC	STa	1	Qs
STEC	*Stx*1	1	TETs, FPIs
STEC	*Stx*2	2	Qs, MCRs, AMGs, PHs, TETs, FPIs
STEC	*Stx*2	1	FPIs

*The isolate, which was resistant to one of the antimicrobials in a group, was considered as resistant for that group. Qs: quinolones, MACs: macrolide, AMGs: aminoglycosides, PHs: phenicols, TETs: tetracyclines, CEPs: cephems, FPIs: folate pathway inhibitors.

and *eae* (15.15%); however, none of them were carried F41 or F5. *Escherichia coli* with pathotypes ETEC (F5 and/or F41 fimbria and STa), EHEC (*Stx* and *eae*), EPEC (*eae*), and STEC-EHEC (*Stx-eae*) were found to be 51.5%, 6.1%, 15.2%, and 12.1%, respectively. In addition, five of the 33 (15.1%) toxigenic strains, which are harbored 4 strains *eae* and one strain F41, were hybrid. The isolates showed high rates of resistance to oxytetracycline, trimethoprim-sulfamethoxazole, neomycin, and erythromycin; however, they showed high rates of susceptibility to cefoperazone, ceftiofur, and cefuroxime (Table 3).

The multidrug-resistant *E. coli* strains were determined as 71.4%. Three of five hybrids and two of four STEC strains were MDR. Oxytetracycline resistance was detected at the highest rate of*E. coli* isolates both with and without virulence genes (Table 4). One isolate was susceptible to all antibiotics tested in the study (Figure 1). The relationship between the isolates of carrying and non-carrying virulence genes was statistically non-significant (Table 4).

Table 3. Antimicrobial susceptibility pattern of *E. coli* isolates.

Antibiotics (µg)	Resistance breakpoint (mm)	S (%)	I (%)	R (%)
Ampicillin-sulbactam (20 µg)	≤11	108 (81.2)	10 (7.5)	15 (11.3)
Cefoperazone (75 µg)	≤15	120 (90.2)	2 (1.5)	11 (8.3)
Ceftiofur (30 µg)	≤19	123 (92.5)	0 (0.0)	10 (7.5)
Cefuroxime (30 µg)	≤14	120 (90.2)	1 (0.8)	12 (9.0)
Chloramphenicol (30 µg)	≤12	56 (42.1)	2 (1.5)	75 (56.4)
Ciprofloxacin (5 µg)	≤15	64 (48.1)	3 (2.3)	66 (49.6)
Enrofloxacin(5 µg)	≤16	61 (45.9)	2 (1.5)	70 (5.6)
Erythromycin (5 µg)	≤13	37 (27.8)	18 (13.5)	78 (58.6)
Gentamicin (30 µg)	≤12	80 (60.2)	13 (9.8)	40 (30.1)
Marbofloxacin (5 µg)	≤14	62 (46.6)	5 (3.8)	66 (49.6)
Neomycin (30 µg)	≤12	42 (31.6)	10 (7.5)	81 (60.9)
Ofloxacin (5 µg)	≤12	55 (41.4)	1 (0.8)	77 (57.9)
Oxytetracycline(30 µg)	≤11	27 (20.3)	1 (08.)	105 (78.9)
Sulfamethoxazole-trimethoprim (25 µg)	≤10	40 (30.1)	0 (0.0)	93 (69.9)

S: Sensitive, I: Intermediate, R: Resistance.

Table 4. Antimicrobial resistance rates between carrying and non-carrying virulence gene *E. coli* isolates*.

Antibiotics	% Resistant (number of resistant isolates)		P
	Carrying virulence genes (n= 33)	Non-carrying virulence genes (n= 100)	
Ampicillin-sulbactam	6.1% (2)	13.0% (13)	0.274
Cefoperazone	9.1% (3)	7.0% (7)	0.692
Ceftiofur	6.1% (2)	8.0% (8)	0.714
Cefuroxime	6.1% (2)	10.0% (10)	0.493
Chloramphenicol	45.5% (15)	60.0% (60)	0.144
Ciprofloxacin	42.4% (14)	52.0% (52)	0.340
Enrofloxacin	42.4% (14)	56.0% (56)	0.175
Erythromycin	75.8% (25)	57.0% (57)	0.054
Gentamicin	18.2% (6)	34.0% (34)	0.085
Marbofloxacin	39.4% (13)	53.0% (53)	0.175
Neomycin	48.5% (16)	65.0% (65)	0.091
Ofloxacin	54.5% (18)	59.0% (59)	0.653
Oxytetracycline	78.8% (26)	79.0% (79)	0.979
Trimethoprim–sulfamethoxazole	63.6% (21)	72.0% (72)	0.363

*: The statistical package SPSS 20 version was used for the description of antimicrobial patterns.

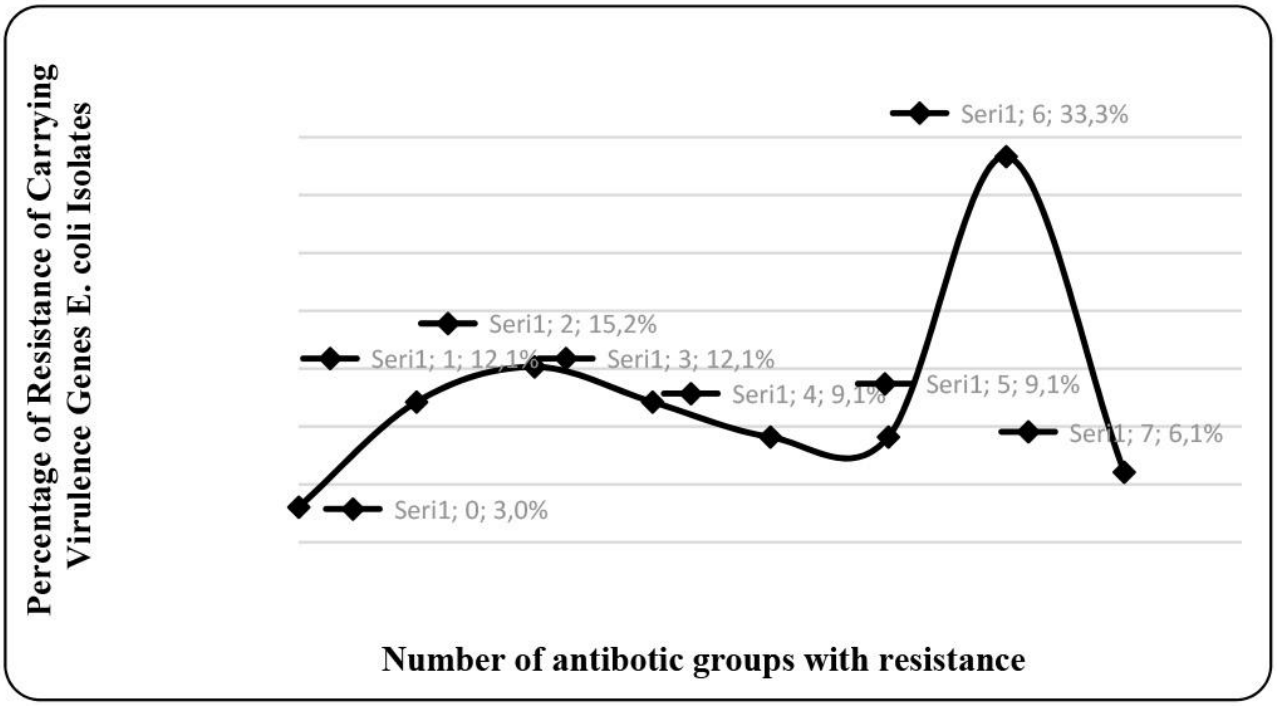

Figure 1. Multidrug resistance rates of carrying virulence gene *E. coli* isolates.

Discussion and Conclusion

Calf diarrhea is commonly associated with more than one infectious agent, and most outbreaks are caused by multiple factors, including hygiene conditions, nutrition, and the environment. *Escherichia coli* is the most important bacterial cause of diarrhea in calves. Diarrheagenic *E. coli* (DEC) is recognized as the major cause of neonatal calf diarrhea with severe lethal outcomes. Virulence factors from several pathogenic *E. coli* strains may predispose calves to diarrhea. Simultaneously, antimicrobial resistance in *E. coli* strains causes infections that are difficult to treat. Various studies have reported the virulence factors and antimicrobial resistance of such infections in calves (9, 15, 24, 28).

Coura et al. (8) determined that the most common virulence profile of *E. coli* strains were *Stx*2, *Stx*1, *eae*, and STa. Hashish et al. (16) reported the most common virulence genes to be STa, *Stx*1, *Stx*2, F41, and F5. The F41 virulence gene was determined in 6 and 17 isolates in studies by Andrade et al. (3) and Nguyen, et al. (24), respectively. In previous studies in Turkey, K99 fimbriae were found at a prevalence of 9.4%–30.2% in calves (11, 17, 26, 30). Furthermore, Güler et al. (15) isolated 12 ETEC strains with K99, F41 and STa combinations in Turkey. However, it was determined that the F41 structure with K99 was found only in F41-producing strains and may cause diarrhea. Moreover, the K99 virulence gene was reported to be found in combination with intimin and/or *Stx* (3, 24, 26). In this study was in agreement on STa and *Stx* virulence genes with previous studies (3, 16, 24) but different on only F5 and F41 carrying strains with the same studies. These differences were may have been caused by some virulence factors, including phage-encoded and plasmid-encoded factors, which are related to the pathogenesis of *E. coli* strains. The presence of fimbrial genes with other virulence genes is considered to increase the virulence of the strains.

Coura et al. (8) reported the pathotypes of *E. coli* to be ETEC (6.8%), EHEC (37.9%), EPEC (6.8%), and STEC (48.5%). Other studies have reported an association between STEC and diarrhea (16, 28). *E. coli* with the pathotypes ETEC, EHEC, EPEC, and STEC-EHEC were found to be 51.5%, 6.1%, 15.2%, and 12.1%, respectively, in this study. These virulence genes have already been reported to be associated with diarrhea (6). The transfer of virulence-related genes between different virulence-bearing *E. coli* strains results in the development of different pathotypes. These developing pathotypes result in the emergence of the term “hybrid,” which was defined as the combination of virulence genes (18). Some researchers have described strains that include the characteristics of EHEC and EPEC pathotypes as hybrid strains (5, 22). Nyholm et al. (25) reported 14% of hybrid strains from animal *E. coli* strains. This ratio is similar to the results of this study (15.1%). Hybrid strains have been associated with the hemolytic uremic syndrome, particularly in humans; therefore, the presence of these strains is important not only for animal health but also for

human health. Although no data exist on the virulence potential of STEC–ETEC hybrid strains isolated from calves and if we consider that patients in this study to our clinic come from different regions of Erzurum, the widespread distribution and clinical relevance might indicate their virulence potential.

In the present study, no virulence factor was detected in 100 *E. coli* strains isolated from diarrhea samples. This result is in accordance with the results of previous studies (24, 28). A possible explanation for this finding is that these strains are nonpathogenic and the diarrhea may cause by another infectious agent like virus and parasite.

Although calf diarrhea associated with *E. coli* infection is often treated with antimicrobials, treatment may be unsuccessful because of resistant isolates in animals. *E. coli* isolates acquired from diarrhea were found to be resistant to amoxicillin, tetracycline, and cefotaxime in Bangladesh (4) and to penicillin, streptomycin, tetracycline, lincomycin, and sulfamethoxazole in Iran (28). In Turkey, *E. coli* isolates were found to be resistant to ampicillin, trimethoprim-sulfamethoxazole, kanamycin, tetracycline, nalidixic acid, and enrofloxacin (15). In this study, the isolates were remarkably resistant to oxytetracycline, trimethoprim-sulfamethoxazole, and neomycin, with prophylactic and therapeutic usages in calves with diarrhea. Some reports detected MDR strains and it was determined that resistance developed particularly against commonly used antimicrobials such as ampicillin, amoxicillin, clavulanic acid, oxytetracycline, and streptomycin (1, 21, 32). In this study, MDR strains were found similar to those in other studies (9, 20).

The use of antimicrobials in the treatment of bacterial calf diarrhea may be necessary; however, uncontrolled and unconscious use of antimicrobials creates resistance to common antimicrobials and causes MDR in bacteria. It should be noted that *E. coli* strains with MDR may be present in farms that do not use antimicrobials. Walk et al. (31) reported that, irrespective of antimicrobial use, tetracycline resistance is adopted in animals by an undetermined helpful mutation.

In conclusion, we know that the ETEC, EHEC, EPEC and STEC-EHEC strains are really important for calves. On the other hand, STEC-EHEC strains a big concern associated with severe diarrhoea and HUS in human health. Therefore, the defense against these strains is crucial for both animal and human health. Multidrug resistant strains are a global problem. The existence of MDR hybrid type *E. coli* strains in livestock poses a potential health threat to humans. Consequently, antimicrobial choosing during the infections should base on antimicrobial susceptibility tests. In this study, we provide a data source for an antimicrobial approach to calf diarrhea in our region.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

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