

PATHOTYPING AND ANTIBIOTIC RESISTANCE
CHARACTERISTICS OF *ESCHERICHIA COLI* ISOLATES
FROM CALF DIARRHEA

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Abstract

Calf diarrhea is an important disease that causes economic losses and *E. coli* is the primary pathogen. In this study, stool samples were collected from a total of 100 calves with diarrhea. As a result of 16S rRNA PCR analysis, it was confirmed that 50 (96.15%) of 52 *E. coli* isolates were molecularly *E. coli*. As a result of molecular pathotyping studies of 50 *E. coli* isolates, the pathotypes of 21 (42.0%) *E. coli* strains were identified. *E. coli* ($n = 50$) isolates were found to be resistant to amoxicillin-clavulanate by 34%, to ciprofloxacin by 38%, and to trimethoprim-sulfamethoxazole by 60%. Different degrees of antimicrobial resistance genes were detected in 26 (52%) *E. coli* isolates. In conclusion, a data source was provided for the antimicrobial treatment procedure in calf diarrhea caused by *E. coli* in our region, and the results that could guide future studies were also brought to the literature.

Key words: *E. coli*, isolation, pathotyping, antimicrobial resistance

Introduction. Bacterial digestive system diseases pose a significant health problem for humans and animals worldwide. Among the causative agents, *E. coli*

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is responsible for diarrhea in farm animals, especially young ones. Calf diarrhea occurs in calves between 2–10 days after birth and can result in economic loss and death [1]. *E. coli* is a Gram-negative bacillus found in the normal intestinal flora of mammals and poultry, with different pathotypes causing various infections. These can be split into intestinal and extraintestinal pathogenic *E. coli*, with the former causing diarrhea, severe colitis, and dysentery. *E. coli* are divided into five groups as enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), entero-aggregative (EAEC), and enterohemorrhagic (EHEC) considering the infection and serological differences they cause [2]. Antimicrobial resistance often develops in *E. coli* due to the unconscious use of antimicrobial agents. Most resistance genes are found in massive conjugative plasmids, which also contain genes for certain virulence factors. Antibiotic medication can be affected by alterations in the antibiotic resistance patterns of microbial populations in the animal's environment [3].

The aim of this study is to identify pathotyping, antibiotic susceptibility and antibiotic resistance genes of *E. coli* pathogen that plays a role in calf diarrhea.

Materials and methods. Sampling. In our study, stool samples were collected from a total of 100 calves with diarrhea from farms located within the borders of Western Aegean province and were brought to ADU Veterinary Faculty Microbiology Department research laboratory under cold chain.

Isolation of *E. coli*. Specimens were streaked into blood agar (5% sheep) and EMB agar for bacteriological examination and incubated at 37 °C for 24–48 h. *E. coli* colonies were evaluated in terms of colony morphology, Gram staining character and metallic green formation on Eosin Methylene Blue (EMB) agar [4].

Phenotypic identification of *E. coli* with BD Phoenix™. *E. coli* colonies seen as Gram-negative bacilli under the microscope were inoculated into Tryptic Soy agar for pure culture. TSA were incubated for 24 h at 37 °C. The BD Phoenix NMIC/ID-400 panel kit was used for *E. coli* suspected isolates. Biochemical identification data obtained from the device were evaluated.

DNA extraction of *E. coli*. DNA extractions from *E. coli* isolates were performed using a Bacterial DNA Purification Kit (HibriGen™) according to the kit methodology. The DNA samples were stored at –20 in a freezer until they were ready for use in PCR.

Primers used in *E. coli* pathotyping. Primer sequences to be used in the identification and pathotyping of *E. coli* isolates are given in Table 1 [5].

Genotypic identification of *E. coli* isolates. For the species-specific identification of DNA samples obtained from *E. coli* isolates, PCR was performed with uidA gene specific primers obtained from the Beta-d-glucuronidase chromosome gene region [5].

All PCR reactions were examined at a total volume of 25 µl, the final concentration was 1X Taq buffer solution, 1.25 U Taq DNA polymerase, 2 mM magnesium chloride (MgCl₂), 160 µmol/l each dNTP, and 1 µl of 100 ng of template

T a b l e 1

Primers and their concentrations used in *E. coli* pathotyping [5]

No	<i>E. coli</i> pathotype	Target gene(s)	Product	Primer 5' – 3'	Product (bp)	Primer concn. (μM)
1	<i>E. coli</i>	uidA	β-d-glucuronidase	ATGCCAGTCCAGCGTTTTTGC AAAGTGTGGGTCAATAATCAGGAAGTG	1487	0.2
2	EPEC	bfpB	Bundle forming pilus protein BfpB	GACACCTCATTGCTGAAGTCG CCAGAACACCTCCGTTATGC	910	0.2
3	EPEC/EHEC	eae	Intimin	TCAATGCAGTCCGTTATCAGTT GTAAAGTCCGTTACCCCAACCTG	482	0.2
4	EHEC	stx ₁	Shiga toxin 1	CGATGTTACGGTTTGTACTGTGACAGC AATGCCACGCTTCCAGAATTG	244	0.2
		stx ₂	Shiga toxin 2	GTTTTCACCATCTTCGTCTGATTATTGAG AGCGTAAGGCTTCTGCTGTGAC	324	0.2
5	ETEC	est1b	Heat stable toxin	TGTCTTTTTCACCTTTCGCTC CGGTACAAGCAGGATTACAACAC	171	0.2
6	EIEC	ipaH	Invasion plasmid antigen H	CTCGGCACGTTTAAATAGTCTGG GTGGAGAGCTGAAGTTTCTCTGC	933	0.3
		virF	Transcriptional regulator VirF	AGCTCAGGCAATGAACTTTTGAC TGGGCTTGATATTCCGATAAGTC	618	0.2
7	EAEC	pic	Serine protease autotransporter	AGCCGTTTCCGCAAGAAGCC AAATGTCAGTGAACCGACGATTGG	1111	0.2
		aafII	Aggregative adherence fimbria II protein AafB	CACAGGCAACTGAAATAAGTCTGG ATTCCCATGATGTCAAGCACTTC	378	0.1
8	DAEC	daaE	Dr adhesin family protein DaaE	GAACGTTGGTTAATGTGGGGTAA TATTACCCGGTCGGTTATCAGT	542	0.1
9	Other <i>E. coli</i> pathotypes	astA	entero-aggregative toxin	TGCCATCAACACAGTATATCCG ACGGCTTTGTAGTCCTTCCAT	102	0.3

DNA. The uidA primer concentrations to be put into the prepared master mixes are prepared 0.2 μ M. PCR amplification was done using a thermal cycler (Eppendorf, Netheler, Hinz GmbH, Hamburg, Germany). The amplification conditions used were as follows: an initial denaturation step at 94 °C for 5 min was performed, followed by 35 cycles of 94 °C for 30 s, annealing at 62 °C for 30 s and elongation at 72 °C for 1 min. A final elongation step was executed at 72 °C for 5 min [5]. The PCR products were visualized using a gel documentation system (specifically, the Infinity VX2 from France) and positive results were scanned at a length of 1487 bp. Isolates displaying in this band range were genotypically confirmed as *E. coli*.

Pathotyping of *E. coli* isolates. The multiplex PCR was done with primers specific to gene regions to define the pathotypes of *E. coli* strains confirmed with the uidA gene. Multiplex PCR were done according to CHANDRA et al. [5] and the PCR products were visualized using a gel documentation system (specifically, the Infinity VX2 from France). DNA samples with positive results were evaluated according to the amplicon sizes indicated by Chandra et al. [5].

Determination of antibiotic susceptibility of *E. coli* isolates with BD Phoenix™. Antimicrobial susceptibility tests were also initiated using the BD Phoenix™NMIC/ID-400 kit of *E. coli* isolates. Purified 24-hour fresh *E. coli* cultures were loaded into the BD Phoenix M50 device. In the antibiogram susceptibility profile made with the device, ampicillin, ampicillin-sulbactam, amoxicillin-clavulanic acid, amikacin, cefepime, cefazolin, ceftazidime, cefuroxime, ceftriaxone, ceftazone-tazobactam, ciprofloxacin, colistin, ertapenem-sulbactam, gentamycin, imipracenemia-tazobactam, ertapenem-ertapenem, tigecycline, trimethoprim-sulfamethoxazole antibiotics were studied. These antibiotics are available in the packages of BD Phoenix™ kits for the diagnosis of Gram-negative (NMIC/ID-400) bacteria and panels for antibiotic susceptibility.

Identification of antibiotic resistance genes. The primers to be used to identify antibiotic resistance genes of *E. coli* pathotypes are presented in Table 2 [6].

Multiplex PCR amplification for *E. coli* samples was done in a total volume of 25 μ l, 10X Taq enzyme buffer solution 5 μ l, 2 U Taq DNA polymerase, 25 mM MgCl₂, 200 μ mol each dNTP and 1 μ l of 100 ng of template DNA. The concentration of each primer to be put into the prepared master mixes is 0.5 μ M. PCR amplification was carried out using a thermal cycler (Eppendorf, Netheler, Hinz GmbH, Hamburg, Germany). The amplification conditions used were as follows: an initial denaturation step at 94 °C for 8 min was performed, followed by 32 cycles of 95 °C for 60 s, annealing at 55 °C for 70 s and elongation at 72 °C for 2 min. A final elongation step was executed at 72 °C for 8 min [6]. PCR products underwent analysis via 1.5% agarose gel electrophoresis and amplicon bands with appropriate amplicon sizes were evaluated in the gel imaging system according to SHAHRANI et al. [6].

T a b l e 2
Primers of the antibiotic resistance genes [6]

No	Genes	Primer (5' – 3')	Product (bp)	Antibiotic resistance
1	aadA1-F	TATCCAGCTAAGCGCGAACT	447	Streptomycin
	aadA1-R	ATTTGCCGACTACCTTGGTC		
2	tetA-F	GGTTCACCTCGAACGACGTCA	577	Tetracycline
	tetA-R	CTGTCCGACAAGTTGCATGA		
3	tetB-F	CCTCAGCTTCTCAACGCGTG	634	Tetracycline
	tetB-R	GCACCTTGCTGATGACTCTT		
4	dfrA1-F	GGAGTGCCAAAGGTGAACAGC	367	Trimethoprim
	dfrA1-R	GAGGCGAAGTCTTGGGTAAAAAC		
5	Sul1-F	TTCGGCATTCTGAATCTCAC	822	Sulfonamide
	Sul1-R	ATGATCTAACCCTCGGTCTC		
6	aac[3]-IV-F	CTTCAGGATGGCAAGTTGGT	286	Gentamicin
	aac[3]-IV-R	TCATCTCGTTCTCCGCTCAT		
7	CITM-F	TGGCCAGAACTGACAGGCAAA	462	Ampicilline
	CITM-R	TTTCTCCTGAACGTGGCTGGC		
8	blaSHV-F	TCGCCTGTGTATTATCTCCC	768	Cephalotin
	blaSHV-R	CGCAGATAAATCACCACAATG		
9	ereA-F	GCCGGTGCTCATGAACTTGAG	419	Erytromycin
	ereA-R	CGACTCTATTCGATCAGAGGC		
10	Qnr-F	GGGTATGGATATTATTGATAAAG	670	Fluoroquinolone
	Qnr-R	CTAATCCGGCAGCACTATTTA		

Results and discussion. In our study, *E. coli* was isolated from 52 (52%) of 100 stool samples from calves with diarrhea in the farms located in the Western Aegean region. *E. coli* isolates ($n = 52$) were confirmed by using BD Phoenix M50 device and all isolates identified as *E. coli*. As a result of 16S rRNA PCR analysis with uidA gene specific primers, 50 (96.2%) of 52 *E. coli* isolates were confirmed as *E. coli*.

Molecular pathotyping of 50 (96.2%) *E. coli* were examined using the method of Chandra et al. [5]. As a result of molecular pathotyping studies, the pathotypes of 21 (42.0%) *E. coli* strains were identified, while 29 (58.0%) *E. coli* strains could not be typed. Of 21 (42.0%) *E. coli* strains were identified as a single pathotype to 6 (28.5%) EPEC, to 4 (19.0%) EIEC, to 1 (4.76%) EHEC, to 1 (4.76%) DAEC; were identified as two pathotypes to 2 (9.52%) EHEC and EPEC, to 2 (9.52%) DAEC and EIEC, to 1 (4.76%) EHEC and DAEC, to 1 (4.76%) EIEC and EAEC; were identified as three pathotypes to 2 (9.52%) DAEC, EIEC and EAEC, and to 1 (4.76%) EHEC, EPEC and EIEC (Table 3).

T a b l e 3

Pathotype distributions of *E. coli* strains

Isolate No	Sample No	<i>E. coli</i> pathotypes	Genes
1	18	EPEC	<i>eae</i>
2	33		
3	38		
4	40		
5	41		
6	42		
7	28	EIEC	<i>virF</i>
8	29		
9	43		
10	45		
11	15	EHEC	<i>stx₁</i>
12	21	DAEC	<i>daaE</i>
13	17	EHEC and EPEC	<i>stx₁+eae</i>
14	39		<i>stx₂+eae</i>
15	26	DAEC and EIEC	<i>daaE+virF</i>
16	27		
17	25	EHEC and DAEC	<i>stx₂+daaE</i>
18	60	EIEC and EAEC	<i>virF+pic</i>
19	51	DAEC, EIEC and EAEC	<i>daaE+virF+pic</i>
20	52		
21	45	EHEC,EPEC and EIEC	<i>stx₁+eae+virF</i>

E. coli ($n = 50$) isolates were determined sensitive to aztreonam, cefepime, ceftriaxone, cefuroxime and gentamicin at a rate of 90, 84, 82, 80, 66, respectively. However, *E. coli* ($n = 50$) isolates were found resistant to amoxicillin-clavulanate by 34%, to ciprofloxacin by 38% and to trimethoprim sulfamethoxazole by 60%. In addition, it is important that the *E. coli* isolates of trimethoprim sulfamethoxazole show 60% resistance.

Our analysis of antibiotic resistance genes in *E. coli* isolates ($n = 50$) revealed the presence of resistance genes at varying degrees in 26 isolates (52.0%), while no resistance genes were detected in 24 isolates (48.0%). Of the isolates, 8 (30.8%) had resistance genes belonging to one antibiotic group, 13 (50.0%) had resistance genes belonging to two antibiotic groups, 4 (15.4%) had resistance genes belonging to three antibiotic groups, and 1 (3.8%) had resistance genes belonging to four antibiotic groups (Fig. 1).

E. coli strains were examined according to antibiotic groups; it was determined that tetracycline resistance was observed in different degrees in 22 (84.6%) isolates. In addition, 14 (54%) isolates were found to have varying degrees of

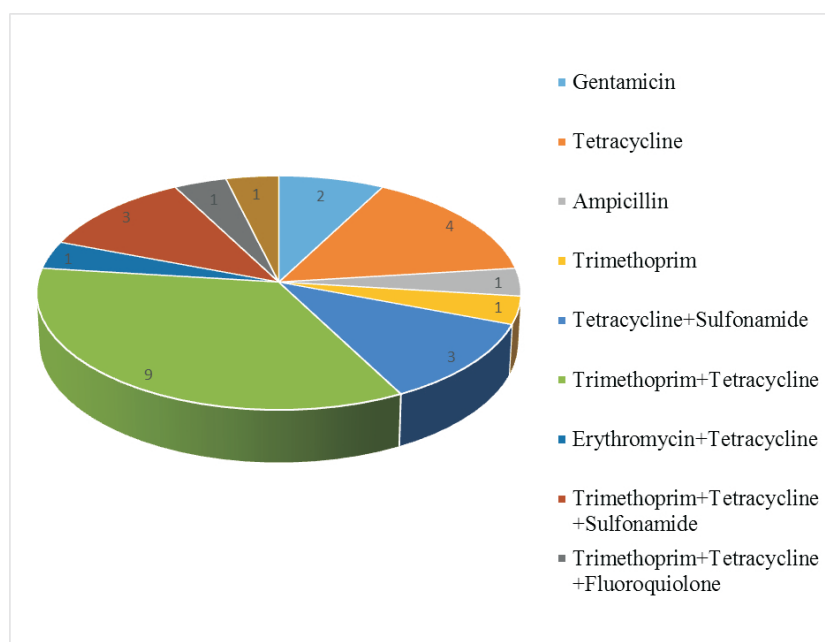


Fig. 1. Antibiotic resistance gene distribution of *E. coli* isolates ($n = 26$)

trimethoprim resistance.

In this study, *E. coli* virulence genes were detected using multiplex PCR method. Prior research has demonstrated that PCR has a 100% specificity and a 98% sensitivity [7]. As a result of molecular verification of 52 isolates identified by biochemical tests, 50 (96.15%) of them were determined as *E. coli*. The isolation data obtained are similar to the rates stated in earlier surveillance research of calves with diarrhea in Turkey [8].

In this study, virulence genes were not detected in 24 (48.0%) *E. coli* strains and this result is compatible with the results of a previous study [6]. ETEC pathotype was not found in our study and this is thought to be as a result of a commonly available commercialized vaccine routinely administered to pregnant cattle in Turkey.

In this study, the EPEC defined 11.5% of *E. coli* isolates and was determined based on the detection of the bfbB gene from calves with diarrhea, indicating that EPEC is a pathogen for calves as stated by MOXLEY and SMITH [9]. In addition, EPEC is reported in various parts of the world to a lesser extent by GÜLER et al. [10] in Turkey.

In this study, 21 (42.0%) *E. coli* strains were identified as a single pathotypes to 6 (28.5%) EPEC, to 4 (19.0%) EIEC, to 1 (4.76%) EHEC, to 1 (4.76%) DAEC; were identified as two pathotypes to 2 (9.52%) EHEC and EPEC, to 2 (9.52%) DAEC and EIEC, to 1 (4.76%) EHEC and DAEC, to 1 (4.76%) EIEC and EAEC;

were identified as three pathotypes to 2 (9.52%) DAEC, EIEC and EAEC, and to 1 (4.76%) EHEC, EPEC and EIEC. These results were consistent with earlier findings that identified combined strains of diverse *E. coli* pathotypes [11]. In addition, JOHURA et al. [12] reported that 34% of pathogenic *E. coli* isolates obtained from various farm animals carried both ETEC and STEC toxin genes and called them crossover strains.

Antibiograms are thought to be more trustworthy than genotypic resistance gene identification for identifying antibiotic resistance [13]. In our study, 34.6% development of resistance was observed against tetracycline and trimethoprim mixture antibiotics due to their widespread use in the veterinary field. In addition, both tetA and tetB genes were identified in 2 isolates in the determination of trimethoprim and tetracycline resistance.

The development of antimicrobial resistance in pathogenic *E. coli* strains is associated with the misuse of antibiotics. Multi-antibiotic resistance in *E. coli* strains is often associated with many genes such as bla-TEM, blaCTX (B-lactamase genes), B (aminoglycoside resistance gene) and sul1 (sulfonamide resistance gene) genes [14]. In our study, tetA, tetB and dfrA1 were found to be the most frequent antibiotic resistance genes and multidrug resistant isolates have been identified. In this study, it was determined that amoxicillin-clavulanic acid, trimethoprim sulfamethoxazole and ciprofloxacin were surprisingly ineffective in the treatment against *E. coli* isolates in calves with diarrhea.

Conclusion. Calf diarrhea is one of the most important problems of breeding and causes economic losses in farms. *E. coli* is the most important pathogen that plays a role in calf diarrhea. As a result of the intensive use of antibiotics in the treatment protocols of *E. coli*-induced calf diarrhea, multi-antibiotic resistance develops and thus pathogens that threaten both calf and human health occur. As a result of this research, the pathotyping, antibiotic resistance and resistance genes of *E. coli* were revealed. With this study, a data source was provided for the antimicrobial treatment procedure in calf diarrhea caused by *E. coli* in our region and the results that could guide future studies were also brought to the literature.

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