Examination of Pathotypes, Phylogroups, and Antibiotic Resistance of *Escherichia coli* Isolates Obtained from Diarrheic Pet Dogs

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ABSTRACT

Intestinal pathogenic *Escherichia coli* (DEC) remains a significant zoonotic etiological agent causing diarrhea leading to fatalities in both humans and animals. This study aimed to assess the pathotypes, phylogroups, and antimicrobial resistance profiles of DEC isolates obtained from pet dogs and analyze their interrelationships. Two hundred *E. coli* isolates were collected from rectal swab samples of 40 diarrheic and 40 apparently clinically healthy (non-diarrheic) pet dogs puppies aged between 1-6 months, between January and June 2023. After *E. coli* isolation using classical conventional methods, their identification was performed phenotypically using the BD Phoenix 100 automatic microbiology system and confirmed genotypically using PCR. Pathotypes (EHEC, EPEC, ETEC, EIEC) and phylogenetic groups (A, B1, B2, C, D, E, F, Clade 1) of isolates were investigated via multiplex PCR. Resistance profiles to 19 antibiotics belonging to ten antimicrobial families were determined using the BD Phoenix 100 automated microbiology system with NMIC/ID 400 Gram negative identification cards. The relationship between the clinical status of the sampled dogs (diarrheic and healthy) and the antibiotic resistance profiles, multi-drug resistance (MDR), pathotypes, and phylogroups of *E. coli* isolates was analyzed using the Chi-square (χ^2) test. Pathotyping revealed that all *E. coli* isolates belonged to EHEC (47.2%), EPEC (34.5%), ETEC (12.8%), and EIEC (5.5%) pathotypes, while phylogenetic analysis indicated that the isolates were distributed among filogroups B2 and C (23.7%), D (20.0%), B1 (12.7%), E (9.0%), F (3.6%), and A (1.8%). Antibiotic susceptibility test results revealed that 78.2% of isolates exhibited MDR. Significant statistical associations were observed between the clinical status of dogs and resistance to ampicillin, amoxicillin-clavulanic acid, ciprofloxacin and tigecycline. However, no significant statistical relationship was found between multi-drug resistance profiles, pathotypes and phylogroups of the isolates. The presence of virulence genes specific to DEC pathotypes in canine isolates indicated that apparently healthy pet dogs could serve as a potential source of human infections, similar to diarrheic dogs. The presence of diverse phylogroups highlights the population diversity of *E. coli*, while the high prevalence of multi-drug resistant isolates underscores the necessity for careful antibiotic selection in the treatment and control of infections.

Keywords: Antibiogram; *Escherichia coli*; Phylogroup; Diarrhea; Dog; Pathotype.

INTRODUCTION

Escherichia coli, as a member of the *Enterobacteriaceae* family, is a significant part of the normal commensal biota of both humans and animals (1). However, certain *E. coli* strains are associated with numerous clinical diarrhea cases in both humans and animals (2). It has been documented that these

strains carry a wide range of virulence genes responsible for pathogenicity. In this context, seven different *E. coli* pathotypes have been identified: enteropathogenic (EPEC), enterohemorrhagic (EHEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAEC), diffusely adherent (DAEC), adherent invasive (AIEC) (1).

The EPEC, which commonly causes diarrhea in both humans and animals, contains the intimin (*eae*) gene and is classified into typical and atypical strains based on the presence of the *bfp*A gene located on a plasmid. Typical EPEC (tEPEC) strains are *eae*+ and *bfp*A+, while atypical EPEC (aEPEC) strains carry only the *eae*+ gene as they lack the plasmid (3). The EHEC pathotype, responsible for symptoms such as abdominal pain, bloody diarrhea and mild fever, produces two types of Shiga toxins, *stx*1 and *stx*2 (4). The pathogenicity of ETEC is determined by the production of heat-stable (*st*) and heat-labile (*lt*) enterotoxins (1). EIEC carries the invasive plasmid antigen H (*ipa*H) gene (5).

E. coli strains can be classified into different categories based on their phylogenetic characteristics and genetic backgrounds. It has been determined that *E. coli* strains belonging to different phylogenetic groups exhibit distinct phenotypic and genotypic characteristics (6). Clermont and colleagues (2013) divided *E. coli* isolates into eight phylogroups (A, B1, B2, C, D, E, F, and Clade I) by analyzing four genes (*arp*A, *chu*A, *yja*A, and *TspE4*.C2) (6). In recent years, a new phylogroup (G) has been reported between B2 and F phylogroups (7). Phylogroup A, which includes isolates with low virulence, is considered commensal, while phylogroup B1, commonly found in the intestinal flora, represents environmental strains (8). The more pathogenic phylogroups include B2 and D (9). The B2 phylogroup is highly virulent and poses a significant risk to human health. Phylogroup F, which typically contains enterohemorrhagic isolates, is the sister group of phylogroup B2. Phylogroup C, encompassing commensal groups with low virulence, is closely related to phylogroup B1 (6). Very little is known about the virulence of phylogroups C and F (6).

E. coli excreted in feces by domestic animals constitutes a significant source for the zoonotic transmission of pathogenic agents (10). Diarrheic animals, due to their frequent and uncontrolled defecation, contribute to a higher dissemination of *E. coli* compared to non-diarrheic animals. Pathogenic and non-pathogenic *E. coli* strains are considered potential reservoirs for antimicrobial resistance genes, and their presence in dog feces poses a serious threat to public health (2). This situation places individuals in direct contact with these animals, such as dog owners, caregivers, children and veterinarians, at a higher risk.

Pets hold a significant place in people's lives, and the number of individuals living with pets is steadily increasing. Therefore, the potential for pets to carry zoonotic diseases is an important public health concern. Phenotypic and genotypic markers of aEPEC isolated from diarrheic and non-diarrheic dogs have been reported to be similar to those found in isolates from human disease (11). Similarly, some dog aEPEC strains have been shown to share virulence genes commonly found in human pathogenic strains. Strains of the same serotype isolated from dogs and children share virulence genes and are phylogenetically closely related, indicating a potential zoonotic risk (12). In general, studies suggest that enteropathogenic *E. coli* isolates in dogs may have the potential to transmit to humans, and further research is needed to fully understand the potential risks (10,11,12).

In countries that do not have good regulations regarding antibiotic use, the arbitrary use of antimicrobial drugs in pets is a common problem. This practice leads to an increase in antimicrobial-resistant *E. coli* strains. Especially in dogs, treatment with antimicrobial agents such as β-lactams, fluoroquinolones and sulfonamides is becoming a significant public health problem (13).

As antimicrobial resistance continuously evolves, regular monitoring studies of resistance are crucial to guide treatment decisions and develop up-to-date control strategies. Overall, the variability in antibiotic resistance complicates the selection of antimicrobial agents and increases the need for culture and sensitivity testing. Additionally, while disease-causing microorganisms in pets tend to differ from those in humans, there is always potential for antibiotic resistance genes to transfer between humans and pets (14). It has been documented that multi-resistant *E. coli* strains are shared between dogs and their owners (15).

In Türkiye, there is no available information about the antimicrobial resistance profiles, pathotypes, and phylogroups of *E. coli* isolates obtained from clinical samples of pet animals. For this reason, this study aimed to evaluate the pathotypes, phylogroups, and antimicrobial resistance profiles of intestinal pathogenic *E. coli* isolates obtained from pet dogs, and analyze their interrelationships.

MATERIAL AND METHODS

Ethical Approval

This study was conducted with the approval of the Aydın Adnan Menderes University Animal Experiments Local Ethics Committee, dated 18.05.2023, and numbered 64583101/2023/76.

Animal Material

The rectal swab samples required for *E. coli* isolation were obtained between January and June 2023 from materials brought to the Department of Microbiology at the Faculty of Veterinary Medicine, Aydın Adnan Menderes University, by clinics and from a private clinic. All the dogs included in the study were either sheltered or home-raised. Many of them were considered mixed bred and all dogs were puppies aged between 1-6 months. None of the dogs had received antibiotic treatment in the last two weeks, with 40 exhibiting diarrhea symptoms and another 40 serving as the control group, showing no signs of diarrhea. During the study, the medical history of each dog was documented and recorded. In addition, informed consent was obtained from the owners, indicating their permission for their pets to participate in the study.

Fecal samples were collected directly from the rectum by a veterinarian using a rectal swab. For this purpose, swabs were inserted approximately one centimeter byond the anal sphincter and rotated to obtain visible fecal material. Once it was ensured that a fecal sample had been collected, it was immediately placed in a semi-solid transport medium (Carry-Blair medium, Micropoint Diagnostics, USA) to prevent moisture loss. Information such as the animal's identification details, sampling date, etc., was recorded on the sample. The samples were stored in a refrigerator (4-8°C) until they were cultured, ensuring that the cultures were performed as soon as possible (within a maximum of 72 hours).

Bacterial Isolation

For the isolation of *E. coli*, differential and selective agar media including EMB and MacConkey agar were used. In aseptic conditions, rectal swab samples were streaked onto EMB agar. After incubation under aerobic conditions at 37°C for 18-24 hours, five colonies with a greenish metallic sheen on the EMB agar were selected and subcultured onto MacConkey agar. Subsequently, in order to increase the likelihood of detecting virulence genes, at least three colonies from each petri dish were taken from the lactose-fermenting pink colonies after incubation under aerobic conditions at 37°C for 18-24 hours. Colonies were passaged onto blood agar for purification purposes. Following that, Gram staining and standard biochemical tests (oxidase, catalase, indole) were performed. Isolates that exhibited Gram-negative rod morphology, lactose fermentation within one day, negative oxidase, and positive catalase and indole tests were considered as suspected *E. coli* isolates (16). Isolates were stored in BHIB containing 15% glycerol at -20°C until bacterial identification, antibiotic susceptibility testing, and molecular tests were conducted. Bacterial identification and antibiotic susceptibility testing of isolates were carried out using the BD Phoenix 100 automated microbiology system.

Reference Strains

For molecular studies, *E. coli* ATCC 35150 (EHEC; *stx*1, *stx*2, *eae*A), ATCC 35401 (ETEC; *lt*, *st*), ATCC 43893 (EIEC; *ial*) were used as positive controls, and *E. coli* ATCC 25922 strain was used as a negative control; for antibiotic susceptibility testing, *E. coli* ATCC 25922 strain was used as a quality control strain.

Antibiotic Susceptibility Tests

Antibiotic susceptibility tests of *E. coli* isolates were performed using the BD Phoenix 100 automated microbiology system (Becton-Dickinson, USA) with the NMIC/ID 400 Gram-negative identification card. The NMIC/ID 400 panel evaluates 19 antibiotics from ten antimicrobial families (Aminoglycosides: amikacin (AN), gentamicin (GM), netilmicin (NET); Carbapenems: ertapenem (ETP), imipenem (IMP), meropenem (MEM); Cephalosporins: cefuroxime (CXM), ceftazidime (CAZ), ceftriaxone (CRO), cefepime (FEP); Monobactam: aztreonam (ATM); Penicillin: ampicillin (AMP), piperacillin (PIP); β-Lactam: amoxicillin clavulanate (AXC), piperacillin tazobactam (TZP); Lipopeptide: colistin (COL); Folates: trimethoprim sulfamethoxazole (SXT); Quinolone: ciprofloxacin (CIP); Tetracycline: tigecycline (TGC)).

Multi-drug resistance (MDR) was defined as the condition where bacteria were resistant to at least one antibiotic in three or more antibiotic classes (17).

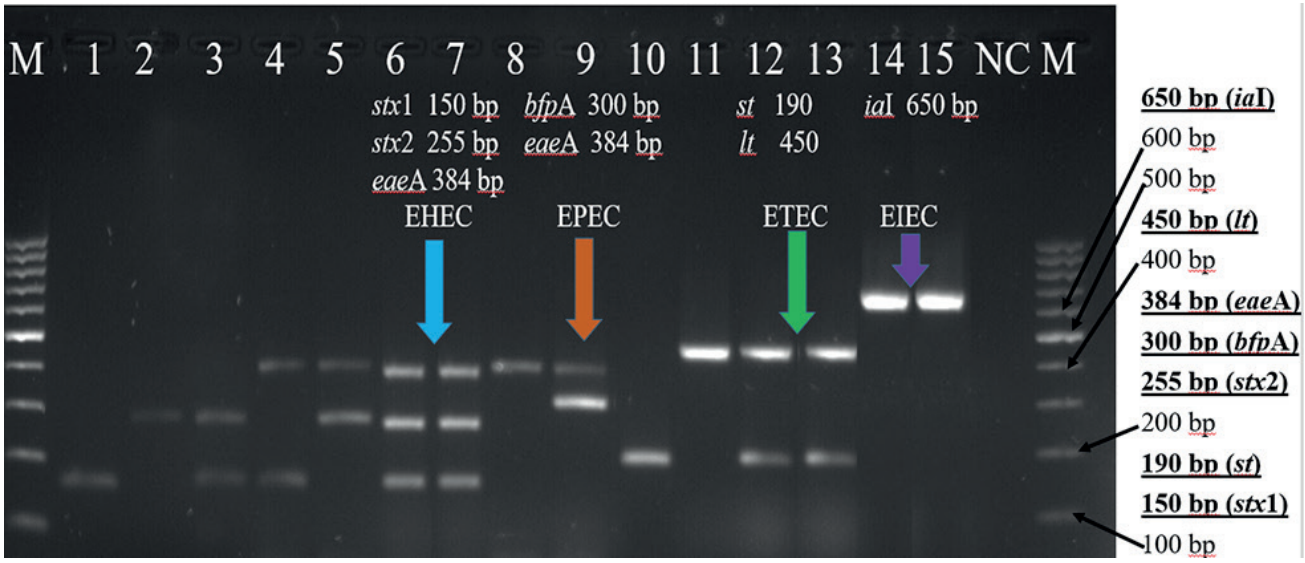

Figure 1. Agarose gel electrophoresis of virulence gene PCR products associated with the pathotype. **1.** *stx*1 (150 bp), **2.** *stx*2 (255 bp) **3.** *stx*1+ *stx*2 (150 bp+255 bp) **4.** *stx*1+*eae*A (150 bp+384 bp) **5.** *stx*2+ *eae*A 2 (255 bp+384 bp) **6**. *stx*1+*stx*2+*eae*A (150 bp+255 bp+384 bp) 7. Positive Control EHEC (*E. coli* ATCC 35150) 8. *eae*A (384 bp) 9. *bfp*A+*eae*A (300 bp+384 bp) **10.** *st* (190 bp) **11.** *lt* (450 bp) **12**. *st*+*lt* (190 bp+450 bp) **13.** Positive Control ETEC (*E. coli* ATCC 35401) **14**. *iaI* (650 bp) **15.** Positive Control EIEC (*E. coli* ATCC 43890) (*ial*) **NC:** Negative Control (master mix without DNA) **M:** 100 bp DNA Ladder (Fermentas).

Polymerase Chain Reaction (PCR)

DNA Extraction, Purity and Quantity Control: In this study, DNA extraction was performed using the sonication method (18). After DNA extractions were completed, their concentrations were checked for purity and quantity using a nanodrop (Thermo Fisher Scientific, Waltham, MA, USA). DNA with OD260/280 values between 1.6 and 2.0 were considered to have sufficient purity (19) and used at a volume of 3 µl as template DNA in each PCR reaction.

Primers: To identify the *E. coli* pathotypes causing diarrhea, the target genes *stx*1, *stx*2, *eae*A (EHEC) (20); *lt* and *st* (ETEC) (21); *eae*A and *bfp* (EPEC) (21); and ial (EIEC) (22) were examined. The phylogenetic distribution of *E. coli* isolates was determined using the quadruplex PCR method (6). This method targets the *chu*A, *yja*A, *TspE4*.C2, *arp*A, and *trp*A genes and classifies *E. coli* into eight phylogroups (A, B1, B2, C, D, E, F, clade I). The quadruplex PCR was performed using the primer sequences shown in Table 1 (6, 23, 24, 25).

PCRs were conducted in a volume of 25 µl. The final concentrations were adjusted as follows: 1x Taq enzyme buffer solution 1x, 25 mM $MgCl_2$ 2 mM, 10 mM dNTP 0.2 mM, 100 ρmol of each primer 0.4 ρmol, 5 U Taq DNA polymerase 1.5 U (Fermentas, Massachusetts, USA), and 3µl of each DNA sample. After preparing the tubes, they were loaded into a thermal cycling device (Boeco, Hamburg, Germany).

Once the master mixes were prepared, the PCR tubes were labeled with the corresponding sample numbers, and 22 µl of master mix was added for each sample. Subsequently, 3 µl of the extracted DNA was added, the tube mouths were tightly closed, and then they were loaded into the thermal cycling devices and programmed. For DNA amplification, the device was set to perform initial denaturation at 95°C for 5 min: 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C (*stx*1, *stx*2, *eae*A, *bfp*A, *lt*, *st*, *iaI*) and 56°C (*chu*A, *yja*A, *TspE4*C2, *arp*A *trp*A) for 30 sec, extension at 72°C for 60 sec, and a final extension at 72°C for 10 min.

Statistical Analysis

The statistical analysis of the obtained data was performed using SPSS (Statistical Package for Social Sciences) version 23.0 (SPSS Inc., Chicago, IL, USA) software package. The Pearson Chi-square (χ^2) test (Fisher's Exact χ^2 Test) was used to compare frequency data. The χ^2 test was used to examine the relationship between the clinical status of sampled dogs

Table 1. Primers used in the study.

	Primer	Target Gene	Sequence (5'-3')	Amplicon size (bp)	Tm
	EHEC	*stx*1	CTGGATTTAATGTCGCATAGTG AGAACGCCCACTGAGATCATC	150	58.0 61.0
	EHEC	*stx*2	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	255	63.0 60.0
	ETEC	*lt*	GGCGACAGATTATACCGTG CGGTCTCTATATTCCCTGTT	450	60.0 56.0
	ETEC	*st*	ATTTTTCTTTCTGTATTGTCTT CACCCGGTACAAGCAGGATT	190	51.0 60.0
	EPEC	*eae*	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	384	63.0 63.0
	EPEC	*bfp*A	GGAAGTCAAATTCATGGGGGTAT GGAATCAGACGCAGACTGGTA GT	300	61.0 65.0
	EIEC	*ial*	GGTATGATGATGATGAGTCCA GGAGGCCAACAATTATTTCC	650	57.0 56.0
Quadruplex PCR	*chu*A.1b *chu*A.2	*chu*A	ATGGTACCGGACGAACCAAC TGCCGCCAGTACCAAAGACA	288	60.5 60.5
Quadruplex PCR	*yja*A.1b *yja*A.2b	*yja*A	CAAACGTGAAGTGTCAGGAG AATGCGTTCCTCAACCTGTG	211	58.4 58.4
Quadruplex PCR	*TspE*4C2.1b *TspE*4C2.2b	*TspE*4.C2	CACTATTCGTAAGGTCATCC AGTTTATCGCTGCGGGTCGC	152	56.4 62.5
Quadruplex PCR	*Ace*K F *Arp*A1 R	*arp*A	AACGCTATTCGCCAGCTTGC TCTCCCCATACCGTACGCTA	400	60.5 60.5
Group E	*Arp*AgpE F *Arp*AgpE R	*arp*A	GATTCCATCTTGTCAAAATATGCC GAAAAGAAAAAGAATTCCCAAGAG	301	60.1 58.4
Group C	*trp*AgpC.1 *trp*AgpC.2	*trp*A	AGTTTTATGCCCAGTGCGAG TCTGCGCCGGTCACGCCCC	219	58.4 68.1
Internal Control	*trp*BA.F *trp*BA.R	*trp*A	CGGCGATAAAGACATCTTCAC GCAACGCGGCCTGGCGGAAG	489	59.4 68.7

(diarrhea or healthy) and the resistance of *E. coli* isolates to antibiotics, multidrug resistance status, pathotypes, and phylogroups. Results were considered statistically significant when the difference between means was $p<0.05$, with a 95% confidence interval.

RESULTS

Bacterial Isolation and Identification

A total of 80 rectal swab samples were obtained from dogs in this study, comprising 40 clinical cases (with diarrhea) and 40 control samples (without diarrhea). Suspected *E. coli* colonies were obtained from 55 (68.7%; 55/80) dogs, showing metallic green sheen on EMB agar and lactose fermentation on MacConkey agar (29 diarrhea cases (72.5%; 29/40); 26 controls (65.0%; 26/40)). A total of 200 isolates were passaged, with 100 colonies from diarrhea and control group samples each. The identification of isolates was performed both phenotypically using the BD Phoenix 100 automated microbiology system and genotypically using PCR.

Isolates identified as *E. coli* were first determined for their pathotypes using PCR. The phylogroups of isolates with identified pathotypes were examined, and antibiotic susceptibility tests were conducted.

PCR

Pathotyping

To determine the presence of target virulence genes, all *E. coli* isolates were examined using PCR for pathotyping purposes. The results showed that 47.0% (47/100) of isolates from

Table 2. Pathotypes of *E. coli* isolates obtained from diarrheic and healthy dogs.

Virulence Gene/Pathotype	Clinical Status		Total (n=55) (%)
	Diarrhea (n=47) (%)	Healthy (n=8) (%)	
EHEC	24 (51.0)	2 (25.0)	26 (47.2)
***stx*1**	3 (6.4)	0 (0.0)	3 (5.5)
***stx*2**	5 (10.6)	1 (12.5)	6 (10.9)
***stx*1+*stx*2**	3 (6.4)	0 (0.0)	3 (5.5)
***stx*1+*eae*A**	5 (10.6)	1 (12.5)	6 (10.9)
***stx*2+*eae*A**	7 (14.5)	0 (0.0)	7 (12.7)
***stx*1+*stx*2+*eae*A**	1 (2.1)	0 (0.0)	1 (1.8)
EPEC	16 (34.0)	3 (37.5)	19 (34.5)
Atipik EPEC (*eae*A)	12 (25.5)	3 (37.5)	15 (27.3)
Tipik EPEC (*eae*A+*bfp*A)	4 (8.5)	0 (0.0)	4 (7.2)
ETEC	5 (10.6)	2 (25.0)	7 (12.8)
st	1 (2.1)	2 (25.0)	3 (5.5)
lt	3 (6.4)	0 (0.0)	3 (5.5)
st+lt	1 (2.1)	0 (0.0)	1 (1.8)
EIEC	2 (4.3)	1 (12.5)	3 (5.5)
iaI	2 (4.3)	1 (12.5)	3 (5.5)

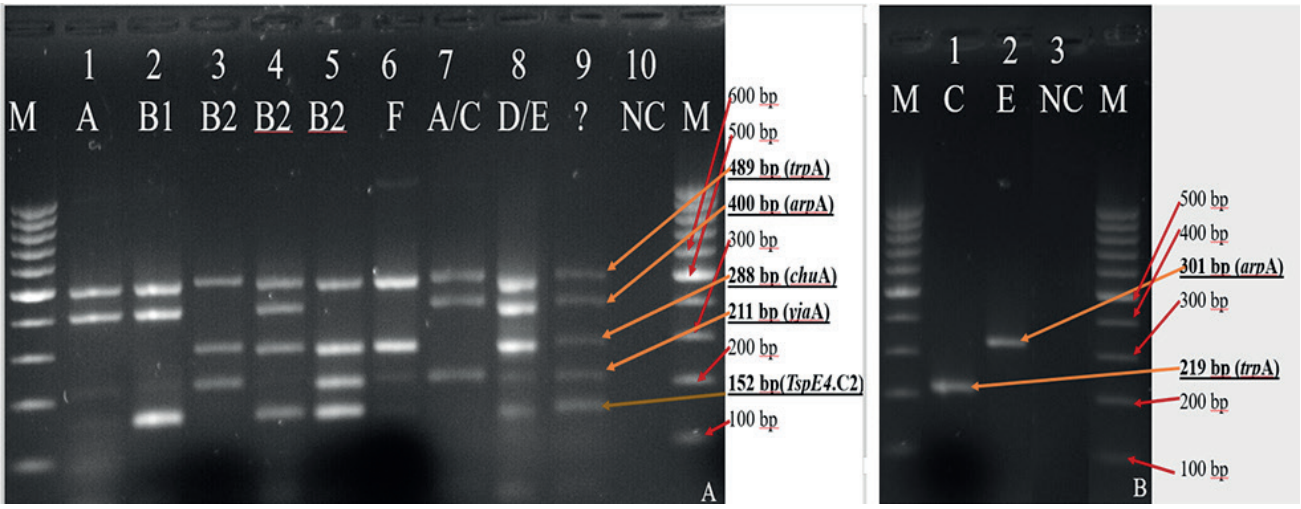

Figure 2. A. Quadruplex PCR profiles using the new Clermont phylogenetic method. **1.** Group A (– – – + +), **2.** Group B1 (+ – – + +), **3.** Group B2 (– + + – +), **4.** Group B2 (+ – + + +), **5.** Group B2 (+ + + – +), **6.** Group F (– – + – +), **7.** Group A/C (– + – + +), **8.** Group D/E (– – + + +), **9.** Unknown Group (+ + + + +) (152 bp, 211 bp, 288 bp, 400 bp, 489 bp), **NC:** DNA-free master mix, **M:** Marker (100 bp, Fermentas). **B. 1.** Group C (219 bp), **2.** Group E (301 bp), **NC:** DNA-free master mix, **M:** Marker (100 bp, Fermentas).

diarrheic dogs and 8.0% (8/100) of isolates from healthy dogs carried at least one gene associated with diarrhea. Out of 55 isolates, 60.0% (33/55) amplified only one gene (*stx*1, *stx*2, *eae*A, *st*, *lt*, *iaI*), while 40.0% (22/55) had combinations of virulence genes (*stx*1+*stx*2, *stx*1+*eae*A, *stx*2+*eae*A, *eae*A+*bfp*A, *st+lt*) (Table 2, Figure 2).

Among diarrheic dogs, 51.0% (24/47) were identified as EHEC, 34.0% (16/47) as EPEC, 10.6% (5/47) as ETEC, and 4.3% (2/47) as EIEC. In contrast, healthy dogs were identified as 25.0% (2/8) EHEC, 37.5% (3/8) EPEC, 25.0% (2/8) ETEC, and 12.5% (1/8) EIEC. Among all isolates, 47.2% (26/55) were classified as EHEC, 34.5%

Table 3. Phylogroups of *E. coli* isolates obtained from diarrheic and healthy dogs.

Phylogroup	Clinical Status		Total (n=55) (%)
	Diarrhea (n=47) (%)	Healthy (n=8) (%)	
A	0 (0.0)	1 (12.5)	1 (1.8)
B1	5 (10.6)	2 (25.0)	7 (12.7)
B2	13 (27.6)	0 (0.0)	13 (23.7)
C	10 (21.3)	3 (37.5)	13 (23.7)
D	10 (21.3)	1 (12.5)	11 (20.0)
E	4 (8.5)	1 (12.5)	5 (9.0)
F	2 (4.3)	0 (0.0)	2 (3.6)
?	3 (6.4)	0 (0.0)	3 (5.5)

?: The number of isolates for which the phylogroup could not be determined and required MLST.

Table 4. Virulence genes and phylotypes of *E. coli* isolates.

Virulence Gene/Genes	Clinical Status																Total (n=55)
	Diarrhea (n=47)								Healthy (n=8)								
	A	B1	B2	C	D	E	F	?	A	B1	B2	C	D	E	F	?	
*stx*1		1			2												3
*stx*2			2		3					1							6
*stx*1+*stx*2			3														3
*stx*1+*eae*A			2		3								1				6
*stx*2+*eae*A			5		2												7
*stx*1+*stx*2+*eae*A			1														1
Atipik EPEC (*eae*A)				6		2	2	2				3					15
Tipik EPEC (*eae*A+*bfp*A)				4													4
st		1							1	1							3
lt		2						1									3
st+*lt*		1															1
iaI						2								1			3
Total	0	5	13	10	10	4	2	3	1	2	0	3	1	1	0	0	55

(19/55) as EPEC, 12.8% (7/55) as ETEC, and 5.5% (3/55) as EIEC.

Phylotyping

For phylotyping purposes, the presence of target genes in *E. coli* isolates with known pathotypes were examined using PCR. Among isolates obtained from diarrheic dogs, 10.6% (5/47) were classified as phylogroup B1, 27.8% (13/47) as phylogroup B2, 21.3% (10/47) as phylogroup C and phylogroup D, 8.5% (4/47) as phylogroup E, and 4.3% (2/47) as phylogroup F. In contrast, isolates from healthy dogs were found to be 12.5% (1/8) phylogroup A, phylogroup D, and phylogroup E, 25.0% (2/8) phylogroup B, and 37.5% (3/8) phylogroup C. The phylogroup of 6.4% (3/47) of isolates obtained from diarrheic dogs could not be determined. Among all isolates, 1.8% (1/55) were phylogroup A, 12.7% (7/55) phylogroup B1, 23.7% (13/55) phylogroup B2 and phylogroup C, 20.0% (11/55) phylogroup D, 9.0% (5/55) phylogroup E, 3.6% (2/55) phylogroup F, while the phylogroup of 5.5% (3/55) of isolates could not be determined using the available primers (Table 3, Figure 2).

Table 5. Antimicrobial resistance of *E. coli* isolates.

Antimicrobial Family-Antibiotic Name	Clinical Status		Total (n=55) (%)
	Diarrhea (n=47) (%)	Healthy (n=8) (%)	
Aminoglycoside			
Amikacin	1 (2.1)	0 (0.0)	1 (1.8)
Gentamicin	25 (53.2)	5 (62.5)	30 (54.5)
Netilmicin	0 (0.0)	0 (0.0)	0 (0.0)
Carbapenem			
Ertapenem	6 (12.8)	0 (0.0)	6 (10.9)
Imipenem	7 (14.9)	0 (0.0)	7 (12.7)
Meropenem	1 (2.1)	0 (0.0)	1 (1.8)
Cephem			
Cefuroxime	30 (63.8)	3 (37.5)	33 (60.0)
Ceftazidime	26 (55.3)	2 (25.0)	28 (50.9)
Ceftriaxone	28 (59.6)	2 (25.0)	30 (54.5)
Cefepime	27 (57.4)	2 (25.0)	29 (52.7)
Monobactam			
Aztreonam	27 (57.4)	2 (25.0)	29 (52.7)
Penicillin			
Ampicillin	42 (89.4)	4 (50.0)	46 (83.6)
Piperacillin	36 (76.6)	4 (50.0)	40 (72.7)
Beta Lactam			
Amoxicillin Clavulanate	41 (87.2)	4 (50.0)	45 (81.8)
Piperacillin Tazobactam	20 (42.6)	1 (12.5)	21 (38.2)
Lipopeptide			
Colistin	4 (8.5)	0 (0.0)	4 (7.3)
Folate			
Trimethoprim Sulfamethoxazole	27 (57.4)	3 (37.5)	30 (54.4)
Quinolone			
Ciprofloxacin	25 (53.2)	1 (12.5)	26 (47.3)
Tetracycline			
Tigecycline	41 (87.2)	4 (50.0)	45 (81.8)

According to Clermont's phylogenetic method, the most prevalent phylogroup among diarrheic dogs was B2 (27.6%; 13/47), whereas in healthy dogs, the dominant phylogenetic group was C (37.5%; 3/8). In all isolates, phylogroups B2 and C (23.7%; 13/55) were identified (Table 3).

Virulence genes and phylotypes of *E. coli* isolates obtained from diarrheal and healthy dogs are shown in Table 4.

Antimicrobial Resistance

In this study, the antibiotic resistance profiles of a total of 55 *E. coli* isolates, comprising 47 obtained from 40 dogs with diarrhea and 8 from 40 healthy dogs, were investigated for 19 antibiotics belonging to ten antimicrobial families using an automated system (Table 5).

E. coli isolates were resistant to some antibiotics at low levels (1.8%-12.7%) (amikacin, imipenem, ertapenem, meropenem, colistin), at moderate levels to some antibiotics (38.2%, 47.3%) (pipercillin-tazobactam, ciprofloxacin), and at high levels to some antibiotics (50.9%-83.6%) (gentamicin, cefuroxime, ceftazidime, ceftriaxone, cefepime, aztreonam, ampicillin, pipercillin, amoxicillin-clavulanate, trimethoprim-

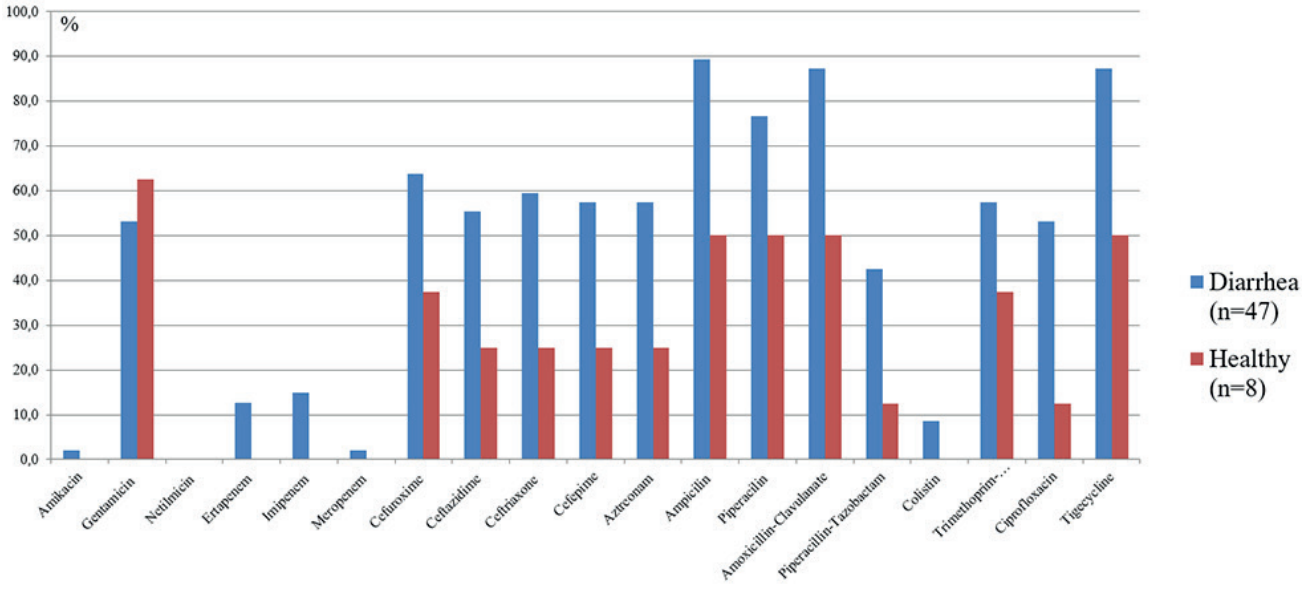

Figure 3. Antibiotic resistance profiles of *E. coli* isolates obtained from diarrheic and healthy dogs.

sulfamethoxazole, tigecycline). All isolates were susceptible to netilmicin (Table 5, Figure 3).

Multiple Antibiotic Resistance

Among the *E. coli* isolates obtained from diarrheic dogs, 83.0% (39/47), 50.0% (4/8) from apparently healthy dogs, and 78.2% (43/55) of all isolates were found to be MDR. The resistance rates of all isolates to 10, 9, 8, 7, 6, 5, 4, 3, 2, and 1 antimicrobial families were determined as follows: 5.4% (3/55), 12.7% (7/55), 21.8% (12/55), 3.6% (2/55), 5.4% (3/55), 7.3% (4/55), 1.8% (1/55), 20.0% (11/55), 14.5% (9/55), and 5.4% (3/55), respectively (Table 6, Figure 4).

Statistical Analysis

The relationship between the clinical condition of the sampled dogs and the resistance status of *E. coli* isolates to antibiotics is presented in Table 7.

The significant statistical relationship was detected between the clinical status of the dogs and resistance to ampicillin (p=0.019), amoxicillin clavulanate (p=0.029), ciprofloxacin (p=0.054), and tigecycline (p=0.019) (Table 7) ($p<0.05$).

The relationship between the clinical status of the dogs and the MDR status of *E. coli* isolates is shown in Table 8.

A significant statistical relationship between the clinical status of the dogs and their multi-antibiotic resistance status could not be detected (Table 8).

The relationship between the clinical status of the dogs and the pathotypes and phylotypes of *E. coli* isolates is shown in Table 9 and Table 10.

A significant statistical relationship between the clinical status of the dogs and the phylotypes and pathotypes of *E. coli* isolates could not be detected.

DISCUSSION

Pets are an important part of our lives and frequently come into close contact with humans. Through this close contact, pet animals can transmit *E. coli* pathotypes that can cause diarrhea in humans (26). Therefore, the health of our pets is of critical importance not only for their own well-being but also for public health. In this study, we determined the frequency of virulence factors associated with DEC pathotypes in *E. coli* isolates obtained from diarrheic and apparently healthy dogs. Subsequently, we conducted phylotyping studies to better understand the genetic origins and characteristics of the isolates. Finally, we attempted to determine the resistance status of our isolates to commonly used antibiotics in both human and veterinary medicine after identifying their pathotypes and phylotypes. In this context, by examining DEC pathotypes, phylogroups, and antibiotic resistance profiles originating

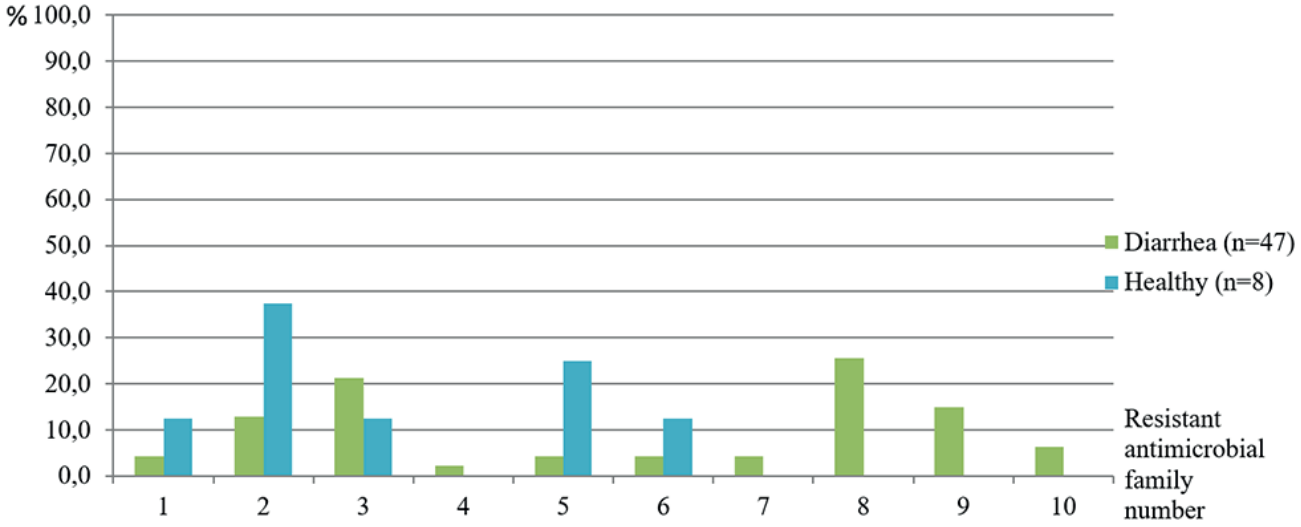

Figure 4. Distribution of MDR status of *E. coli* isolates obtained from diarrheic and healthy dogs.

Table 6. Multiple antibiotic resistance profiles of *E. coli* isolates obtained from diarrheic and healthy dogs.

Number of Resistant Antimicrobial Families	Clinical Status		Total (n=55) (%)	MDR
	Diarrhea (n=47) (%)	Healthy (n=8) (%)		
1	2 (4.3)	1 (12.5)	3 (5.4)	12 (21.8)
2	6 (12.8)	3 (37.5)	9 (14.5)	
3	10 (21.3)	1 (20.0)	11 (20.0)	43 (78.2)
4	1 (2.1)	0 (0.0)	1 (1.8)	
5	2 (4.3)	2 (20.0)	4 (7.3)	
6	2 (4.3)	1 (12.5)	3 (5.4)	
7	2 (4.3)	0 (0.0)	2 (3.6)	
8	12 (25.5)	0 (0.0)	12 (21.8)	
9	7 (14.9)	0 (0.0)	7 (12.7)	
10	3 (6.4)	0 (0.0)	3 (5.4)	

from dogs, we aimed to better understand potential risks for both our dogs and human health.

The prevalence of *E. coli* in dogs with diarrhea varies according to studies. In recent studies, the rate of *E. coli* isolation from dogs showing diarrhea symptoms was reported as 67.7% in Egypt (27) and 73.5% in Nigeria (28). In this study, the isolation rate of *E. coli* was similarly high (72.5%) as in other studies. The sampled dogs were puppies aged 1 to 6 months. This is consistent with studies that have reported a high incidence of *E. coli* isolation in young diarrheic dogs (27). Differences in isolation rates in various studies could be attributed to a combination of factors such as sample selection, isolation methods used, the ages of sampled animals, geographical factors and population variations.

Antimicrobial resistance in bacteria has been continuously evolving since the discovery of antibiotics. There are several factors contributing to bacterial resistance, including the prophylactic or incorrect use of antibiotics and the transmission of resistant bacteria from animals to humans (or vice versa) (29). However, taking into account local antibiotic usage, animal populations, and environmental factors, different resistance patterns can be observed in various regions and at different times. This variability in resistance patterns is one of the primary reasons for the emergence of differing antibiotic resistance profiles, particularly in studies conducted in different countries, and even within different regions of the same country. Therefore, our results are in line with findings from studies conducted in Egypt regard-

Table 7. Relationship between clinical status and resistance status of isolates to antibiotics.

Antibiotic	Clinical Status		χ2	P
	Diarrhea (n=47) (%)	Healthy (n=47) (%)		
Amikacin, Meropenem R+	1	0	0.170	1
Amikasin. Meropenem R-	46	8		
Gentamicin R+	25	5	0.235	0.715
Gentamicin R-	22	3		
Ertapenem R+	6	0	1.125	0.577
Ertapenem R-	41	8		
Imipenem R+	7	0	1.340	0.577
Imipenem R-	40	8		
Cefuroxime R+	30	3	1.939	0.244
Cefuroxime R-	17	5		
Ceftazidime R+	26	2	2.469	0.143
Ceftazidime R-	21	6		
Ceftriaxone R+	28	2	3.236	0.123
Ceftriaxone R-	19	6		
Cefepim. Aztreonam R+	27	2	2.835	0.131
Cefepim. Aztreonam R-	20	6		
Ampicillin R+	42	4	7.599	0.019*
Ampicillin R-	5	4		
Piperacillin R+	36	4	2.394	0.193
Piperacillin R-	11	4		
Amoxicillin Clavulanate, Tigecycline R+	41	4	6.255	0.029*
Amoxicillin Clavulanate, Tigecycline R-	6	4		
Piperacillin Tazobactam R+	20	1	2.568	0.136
Piperacillin Tazobactam R-	27	7		
Colistin R+	4	0	0.721	1
Colistin R-	43	8		
Trimethoprim Sulfamethoxazole R+	27	3	1.077	0.446
Trimethoprim Sulfamethoxazole R-	20	5		
Ciprofloxacin R+	25	1	4.459	0.054*
Ciprofloxacin R-	22	7		

* Degree of statistical significance.

ing high susceptibility to amikacin (26,27). However, there are proportional variations in resistance patterns to other antibiotics compared to the results of studies conducted in Brazil and Egypt (13,26,27).

In our study, antimicrobial susceptibility test results have shown that isolates obtained from both healthy and diarrheic dogs were resistant to β-lactams, aminoglycosides, tetracyclines and folate inhibitors. This finding is consistent with the results of many studies worldwide on antimicrobial resistance in bacteria isolated from dogs (26,27,28,30).

E. coli isolates obtained from dogs exhibited high levels of resistance to gentamicin, cefuroxime, ceftazidime, ceftriaxone, cefepime, aztreonam, ampicillin, piperacillin, amoxicillin-clavulanate, trimethoprim-sulfamethoxazole and tigecycline. Ceftazidime, ceftriaxone, aztreonam, tigecycline, and piperacillin are important drugs used in the treat-

ment of various infections in humans. The development of resistance to these antibiotics in dogs is significant in terms of the risk of spreading infections between dogs and humans.

Similarly, *E. coli* isolates exhibited low resistance (1.8%-12.0%) to imipenem, ertapenem, meropenem and colistin, which are used in human medicine, while they showed intermediate resistance (38.2%) to piperacillin-tazobactam. The fact that these antibiotics are not used in veterinary medicine may have reduced the likelihood of resistance developing in bacteria treated with these drugs in veterinary practice. However, resistance to these drugs can limit important treatment options for serious infections in humans. Especially carbapenem antibiotics like meropenem are used as a last resort in humans against highly resistant bacteria. The presence of bacteria resistant to these antibiotics in pet dogs can have implications for public health, as pet owners may come into contact with these bacteria, which have the potential to be transmitted to humans.

In the study, despite tigecycline not having been used in the sampled dogs, a high level of resistance (81.8%) was observed. There could be two possible reasons for this phenomenon. Firstly, these bacteria may have acquired the tigecycline resistance gene from the environment they came into contact with or from another source. Secondly, changes in ribosomal targets or transport systems in tetracycline-resistant bacteria may trigger tigecycline resistance, even though tigecycline has never been used. This is because tetracycline was commonly used where the samples were collected. Tetracycline and tigecycline share the same mechanism of action, binding to the same ribosomal targets to inhibit bacterial protein synthesis. Therefore, the use of tetracycline antibiotics may have contributed to the development of tigecycline resistance (31). Resistance genes can be transferred through plasmids or other genetic elements. The presence and transfer of resistance genes can contribute to the spread of resistance to different antibiotics (31).

One of the noteworthy findings of our study was that the isolates developed resistance to colistin, albeit at low levels (7.3%). Colistin resistance in *E. coli* is reported to result from mutations in chromosomal genes or the presence of plasmid-borne *mcr* genes (32). To the best of our knowledge, colistin resistance in *E. coli* isolates from diarrheic dogs in Türkiye has not been reported. Colistin is considered a last-resort antibiotic for the treatment of severe infections. Therefore, colistin resistance should be a serious concern for both veterinary and human health.

Similarly, we observed resistance to carbapenem group antibiotics, which are important for the treatment of infections in human medicine, albeit at low levels (1.8%-12.7%). The development of low-level resistance to carbapenems may be attributed to horizontal gene transfer between bacteria. This occurrence could be a result of bacteria adapting to environmental conditions. Additionally, in areas where antibiotic usage is prevalent, the likelihood of bacteria developing carbapenem resistance in the environment may be higher.

In the study, a significant statistical relationship was identified between the clinical condition of dogs and resistance to ampicillin, amoxicillin-clavulanate, ciprofloxacin, and tigecycline (Table 7). This result suggests that the likelihood of developing resistance to antibiotics is associated with the diarrheal condition of dogs. This finding may have several important clinical implications, such as limitations in the treatment options for cases of diarrhea in dogs that involve the use of certain antibiotics. Over time, antibiotics like ampicillin, amoxicillin-clavulanate, and ciprofloxacin may become ineffective. This could mean that veterinarians may need to reevaluate their treatment choices. When selecting appropriate antibiotics for the treatment of dogs, veterinarians should consider these resistance profiles. Moreover, the antibiotic resistance acquired by *E. coli* isolates is a concern not only for animal health but also for human health. Antibiotic resistance observed in dogs can be transmitted to humans through contact, consumption of contaminated food, and other means. Therefore, the proper disposal of dog feces in a hygienic manner and the elimination of the risk of contamination in the home environment are important for individuals in contact with dogs.

In the study, while the MDR rates of our isolates were not as high as those reported in studies conducted in Egypt (26) and Nigeria (28), they were still at a considerable level (78.2%). The high prevalence of multi-drug resistance in diarrheic dogs can complicate their treatment because the options for effective antibiotics against bacteria with multiple resistance are limited. In addition, no significant statistical relationship was found between the clinical condition of dogs and their multi-drug resistance profiles (Table 8). The causes of diarrhea can be quite diverse and may not only be related

Table 8. The relationship between the clinical status and the multi-antibiotic resistance.

Antibiotic Resistance Phenotype	Clinical Status		χ2	P
	Diarrhea (n=47) (%)	Healthy (n=8) (%)		
MDR+	39	4	4.280	0.059
MDR-	8	4		

Table 9. Relationship between the clinical status and the pathotypes of isolates.

Pathotypes	Clinical Status		χ2	P
	Diarrhea (n=47) (%)	Healthy (n=47) (%)		
EHEC+	24	2	1.829	0.257
EHEC-	23	6		
EPEC+	16	3	0.035	1
EPEC-	31	5		
ETEC+	5	2	1.246	0.267
ETEC-	42	6		
EIEC+	2	1	0.885	0.382
EIEC-	45	7		

to bacterial infections but also to viruses, parasites, dietary changes and other factors. These factors can influence the formation and spread of resistant strains. The antibiotic usage habits in the environments where both diarrheic and healthy-looking dogs live may be similar. Therefore, there may not have been a significant difference observed in the likelihood of acquiring multi-drug resistance between diarrheic and healthy dogs.

Enteric *E. coli* pathotypes, which are a significant concern for public health and food safety, exhibit a high diversity of pathogenic mechanisms and virulence factors. In this study, EHEC was the most frequently detected pathovar, comprising 47.2% of the isolates. This pathovar was isolated from 51.0% of diarrheic dogs and 25.0% of healthy dogs. The prevalence of EHEC seemed to be lower in samples obtained from apparently healthy animals. In studies conducted on healthy pet dogs, the prevalence of EHEC was reported to be 4.08% in Iran (33) and 5.9% in Turkey (34); whereas in diarrheic dogs, it was reported to be 2.7% in Brazil (35). Overall, these studies indicate that the prevalence of EHEC in diarrheic dogs can vary by region and may also be present in some healthy dogs. The higher prevalence of EHEC found in our study could be attributed to regional differences. The region where our study was conducted may have a different pattern of EHEC spread compared to other studies. Additionally, the laboratory methods used, population of dogs examined may differ from other studies.

In the study, EPEC was the second most frequently detected pathovar. EPEC is characterized by the production of intimin and can be classified as typical or atypical based on its presence or absence (1). Similar to studies conducted in Brazil (10,35), we found that atypical EPEC had a higher distribution than typical EPEC in our study. Our results are consistent with other studies, demonstrating that EPEC is an important diarrheagenic pathotype in dogs (10,35).

In the study, ETEC was the third most commonly detected pathovar, with a prevalence of 10.6% in clinical cases and 25.0% in dogs without diarrhea. The prevalence of ETEC in diarrheic dogs can vary according to different studies. Sancak *et al.* (2004) found that ETEC was more common in dogs with acute and chronic diarrhea compared to healthy dogs in households and kennels (34). In a study conducted in Iran, the prevalence of ETEC in healthy dogs was reported to be 2.1% (33).

Among our isolates, the lowest detected pathovar was EIEC, which was found in both diarrheic (4.3%) and non-diarrheic dogs (12.5%). In previous studies, EIEC was reported to be absent in both healthy and diarrheic dogs in

Table 10. Relationship between the clinical status and the phylotypes of istolates.

Phylotypes	Clinical Status		χ^2	P
	Diarrhea (n=47)(%)	Healthy (n=8)(%)		
A+	0	1	5.875	0.145
A-	47	7		
B1+	5	2	1.246	0.267
B1-	42	6		
B2+	13	0	2.845	0.176
B2-	34	8		
C+	10	3	0.979	0.376
C-	37	5		
D+	10	1	0.323	1
D-	37	7		
E+	4	1	0.129	0.559
E-	43	7		
F+	2	0	0.347	1
F-	45	8		

Mexico (13), in diarrheic dogs in Brazil (10), while it was detected in healthy dogs in Iran (6.1%) (33).

In this study, the prevalence of EPEC, ETEC, and EIEC was higher in healthy dogs (37.5%, 25.0%, 12.5%) compared to diarrheic dogs (37.5%, 10.6%, 4.3%). This suggests that healthy dogs may harbor virulence genes as carriers but may not exhibit symptoms. The EPEC/ETEC/EIEC strains present in healthy dogs may be less pathogenic than those in diarrheic dogs, which could explain the absence of symptoms. Alternatively, the healthy dogs sampled in this study may have had stronger immune systems, which could have prevented the development of diarrhea symptoms. Considering that these differences may be influenced by multiple factors, further research with a larger sample size is needed to elucidate the exact reasons for these variations.

In this study, no significant statistical relationship was found between the clinical status of dogs and the *E. coli* pathotypes isolated (Table 9). *E. coli* can be categorized into different pathotypes, each having the potential to cause different types of infections. However, while these pathotypes can sometimes be associated with different clinical conditions, there may also be instances where different pathotypes are found in the same clinical condition (1). Therefore, the relationship between clinical status and pathotype can be complex. This complexity can make it challenging to detect a statistical relationship.

The Clermont phylotyping method was developed to classify *E. coli* isolates into phylogroups based on their genetic backgrounds (6). The original method assigned isolates to one of four phylogroups (A, B1, B2, or D) (36), but an updated method now recognizes eight phylogroups (A, B1, B2, C, D, E, F, and clade I). The updated method has been shown to correctly assign over 95% of *E. coli* isolates to a phylogroup (6). In the *E. coli* phylogroup analysis developed by Clermont and colleagues, commensal groups are typically referred to as A and B1 phylogroups. These phylogroups encompass strains of *E. coli* that are commensal or naturally found in the intestines of humans and animals. Virulent groups, on the other hand, include B2 and D phylogroups. There is not much information available about the pathogenicity of other phylogroups. Phylogroups are used to classify *E. coli* strains based on their genetic similarities and can help interpret different characteristics and infection potential of the bacterium. However, these phylogroups are just a classification method and do not alone provide a complete picture of pathogenicity (6).

It has been documented that the distribution of phylogenetic groups among dog isolates varies concerning extraintestinal, commensal and clinical samples. When it comes to extraintestinal isolates, B2 and D groups would be expected to dominate, although Gibson *et al.* (2010) reported no isolates belonging to the B2 group (37). However, Maynard *et al.*

(2004) reported an 88.0% frequency of the B2 phylogenetic group in extraintestinal samples (38). In a study by Harada *et al.* (2012), a high prevalence of the B2 group was observed in fecal strains of dogs (39). Davis *et al.* (2011) also observed the dominance of the B2 and D groups in strains isolated from various anatomical regions, including the rectal area, in healthy dogs (40).

In this study, phylogenetic group analysis revealed that commensal phylotypes A and B1 comprised 37.5% (3/8) of the *E. coli* isolates obtained from healthy dogs and 10.6% (5/47) of isolates from diarrheic dogs, while virulent B2 and D phylogroups constituted 12.5% (1/8) of isolates from healthy dogs and 48.9% (23/47) of isolates from diarrheic dogs. In a study conducted in Mexico, when *E. coli* isolates were analyzed based on phylogenetic characterization, commensal phylogroups A and B1 were found to account for 57.0% in healthy dogs and virulent phylogroups B2 and D for 43.0%, while in diarrheic dogs, commensal phylogroups were 31.0%, and virulent phylogroups were 69.0% (13). These findings seemingly indicate that healthy dogs can be colonized by both commensal and virulent strains. Similar to the study conducted in Mexico, this study also suggests that domestic dogs could serve as a reservoir for virulent phylogroups and potentially transmit these pathogens to their owners and individuals with indirect contact (12).

There were no significant statistical differences observed between the clinical status of the sampled dogs and the *E. coli* isolates' phylotypes (Table 10). *E. coli* can be divided into eight phylogroups, each with distinct characteristics. While these phylogroups can sometimes be associated with different clinical conditions, there can also be different phylogroups within the same clinical condition. Therefore, the relationship between clinical status and phylogroup can be complex. Diarrhea can have various causes, including bacterial, viral, parasitic, dietary changes, or other factors. These factors can influence clinical status, making it challenging to establish a clear relationship with phylogroups. Similarly, the sample sizes can affect the results of statistical analysis. The much larger size of the diarrhea group may have led to an imbalance in the analysis, making it more challenging to establish a statistically significant relationship. The results of this study indicate that not only diarrheal dogs but also seemingly healthy pet dogs can carry multidrug-resistant, pathogenic *E. coli* pathotypes and phylogroups that can potentially be transmitted to humans. Additionally, the presence of various phylogroups obtained from both diarrheal and healthy dogs may demonstrate the diversity within the *E. coli* population. This suggests that *E. coli* can naturally possess different phylogroups or adapt to different environmental conditions. The resistance of *E. coli* isolates obtained from dogs to antibiotics commonly used in human medicine (imipenem, ertapenem, meropenem, colistin, piperacillin-tazobactam, tigecycline) can pose a significant public health concern by limiting treatment options. It is crucial for dog owners and veterinarians to adhere to hygiene measures and use antibiotics based on antibiotic susceptibility test results. This is necessary to protect both the health of dogs and to avoid endangering human health.

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CONFLICT OF INTERESTS STATEMENT

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript. This original work and is not under review at any other publication.

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