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RESEARCH ARTICLE

# Phylotypes, Antibiotic Resistance and Biofilm Formation Determined in Enteropathogenic *Escherichia coli* Isolates in Diarrheic Cats

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**Abstract**

This study investigated the phylotypes, biofilm formation abilities, antibiotic resistance of enteropathogenic *Escherichia coli* (EPEC) isolates from cats with diarrhea. From 37 diarrheal cats, 28 *E. coli* isolates were obtained using conventional methods. Pathotypes and phylotypes were determined via PCR, biofilm formation potential via Congo Red Agar, and resistance profiles against eight antibiotics were examined using the disk diffusion method, evaluated according to the Clinical and Laboratory Standards Institute (CLSI). Chi-square tests assessed relationships between phylogenetic groups, biofilm formation, and MDR statuses. The results were considered statically significant at a 95% confidence interval and significance level of $P<0.05$. Pathotyping studies showed that 46% (13 isolates) of the isolates were EPEC. 93% of isolates were phylotyped. Seven phylotypes were detected: B2 (22%), C (18%), B1 (18%), D (14%), A (11%), E (7%) and F (3%). Of the isolates 39% formed biofilms and 86% were MDR. No significant association was found between pathotype and biofilm formation or MDR. However, a significant relationship was noted between pathotype EPEC and phylogenetic group B2. The correlation between EPEC pathotype and phylotype B2 in diarrheic cats suggests high pathogenic potential. Multidrug resistance, even in non-biofilm forming isolates, complicates treatment and poses public health risks, underscoring the need for detailed evaluation of *E. coli* diversity and zoonotic pathogens.

**Keywords:** Antibiotic resistance, Biofilm, Cats, Enteropathogenic *Escherichia coli*

## INTRODUCTION

*Escherichia coli*, a prominent cause of diarrhea, is classified into various groups based on virulence factors, including enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC/STEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), diffusely adherent *E. coli* (DAEC), and enteroinvasive *E. coli* (EIEC) [1,2]. EPEC, initially associated with childhood diarrhea epidemics, remains a significant cause of morbidity and mortality among infants and young children [3,4]. The distinction between typical (tEPEC) and atypical (aEPEC) strains is based on the presence of the *E. coli* adherence factor (EAF) plasmid [1,5], encoding bundle-forming pilus (BFP) and plasmid-encoded regulator (PER). tEPEC strains exhibit *eae*+ *bfpA*+ *stx*- genotype and attaching and effacing (A/E) phenotype, whereas aEPEC strains lack *bfpA*, with a genetic basis of *eae*+ *bfpA*- *stx*- [5,6]. Despite the positive impact of cats on human well-being, the potential for zoonotic disease transmission underscores the need for vigilance [7]. Türkiye, with an estimated 4 million cats and 2 million dogs as pets, witnesses significant pet ownership [8]. Cats, constituting 60% of identified pets, are associated with high levels of antimicrobial resistance in *E. coli* isolates, reflecting the challenges posed by indiscriminate antibiotic usage [9]. Environmental factors, including the presence of small children in cat-owning households, further contribute to *E. coli* colonization [7,9,10]. Cats represent potential reservoirs of antimicrobial resistance, increasing the risk of *E. coli* transmission to humans through close contact. Recent classification of *E. coli* into eight phylotypes based on genetic markers provides insights into their genetic diversity and pathogenic potential [11]. Phylotypes B2 and D are associated with severe extraintestinal infections, while A, B1, C, and E are mostly commensal or apathogenic [11-13]. Biofilm formation, facilitated by bacterial adhesion, contributes to virulence and recurrent infections [14-16]. Congo Red Agar (CRA) and other methods quantify biofilm formation capacities [17,18]. The shedding of *E. coli* in cat feces poses

a significant zoonotic risk, underscoring the importance of understanding EPEC prevalence and characteristics [19]. Given geographic variability, investigating cat-derived EPEC isolates' phylotypes, antibiotic resistance, and biofilm formation capacities is crucial for veterinary and public health.

This study aims to evaluate the phylotypes, antibiotic resistance, and biofilm formation potentials of EPEC isolates from diarrheic cats.

## Material and Methods

### Ethical Statement

This study was conducted with the approval of the Local Ethics Committee for Animal Experiments of Aydın Adnan Menderes University (ADÜ-HADYEK), dated 24.08.2023, numbered: 68583101/2023/130.

### Material

This study utilized rectal swab samples from 37 diarrheic cats, received from a private clinic at Aydın Adnan Menderes University University Faculty of Veterinary Medicine Department of Microbiology Laboratories between September and December 2023. The feces of these cats, brought with complaints of diarrhea, were evaluated by the veterinarian during clinical examinations. The color of the stool generally varied from light brown to yellow, and its consistency was watery to semi-solid. Mucus and/or blood has been detected in some stool samples. The general condition of cats is characterized by loss of appetite, weakness and, in some, signs of dehydration. These cats, mostly mixed-breed, ranged in age from 2 months to 6 years. Cats that had not received antibiotic treatment were included in the study. Owners provided informed consent for their pets to participate. Fecal samples were collected using rectal swabs by a veterinarian, inserted approximately one cm into the anal sphincter and rotated to obtain visible fecal material. Samples were immediately placed in Stuart transport medium (Remel, USA) and stored at 4-8°C until inoculation [20].

### Bacterial Isolation and Identification

Rectal swab samples were inoculated onto EMB agar (Merck, Germany) and aerobic conditions incubated at 37°C for 18-24 h. Metallic greenish shiny colonies were selected and subcultured onto MacConkey agar (Merck, Germany). After incubation, pink lactose-fermenting colonies were subcultured onto blood agar. Gram-negative rod morphology, lactose fermentation, negative oxidase, positive catalase and indole tests, and motility were considered presumptive for *E. coli* [21]. The isolates were preserved in Brain Heart Infusion Broth (Merck, Germany) with 20% glycerol at -20°C. Suspected *E. coli* isolates were molecularly identified by examining the *trpA* genes [11].

### Biofilm Formation

Biofilm formation was assessed using the CRA method [18]. *E. coli* isolates were streaked onto CRA and incubated at 37°C for 24 h. Black, dry consistency colonies indicated positive biofilm production [18]. *E. coli* ATCC 25922 and *S. aureus* ATCC 25932 served as positive and negative controls for CRA method, respectively.

### Antibiotic Susceptibility Tests

Antibiotic susceptibility tests were performed using the disk diffusion method according to CLSI (2020) [22]. Eight antibiotics from eight different families were tested: ampicillin (AMP, 10 µg), amoxicillin-clavulanic acid (AMC, 20 µg/10 µg), ciprofloxacin (CIP, 5 µg), tetracycline (TET, 30 µg), gentamicin (GEN, 10 µg), trimethoprim-sulfamethoxazole (SXT, 1.25 µg/23.75 µg), cefotaxime (CTX, 30 µg), meropenem (MEM, 10 µg) (Oxoid, United Kingdom) *(Table 1)*. *E. coli* ATCC 25922 served as the quality control strain. Isolates resistant to three or more antibiotic classes were classified as MDR [23].

### Polymerase Chain Reaction

DNA was extracted using the InstaGene™ Matrix kit

***Table 1.*** *Antibiotics used in the study, disk contents, evaluation criteria, and resistance statuses*

| Antimicrobial Family | Antibiotic | Disc Content (µg) | ≥S | ≤R | Number of Resistant Isolates (%) |
|---|---|---|---|---|---|
| Penicillin | Ampicillin | 10 | 17 | 13 | 24 (86) |
| Beta Lactam | Amoxicillin Clavulanic acid | 20/10 | 18 | 13 | 21 (75) |
| Quinolones | Ciprofloxacin | 5 | 26 | 21 | 20 (71) |
| Tetracycline | Tetracycline | 30 | 15 | 11 | 18 (64) |
| Aminoglycoside | Gentamicin | 10 | 15 | 12 | 17 (61) |
| Sulfonamide | Trimethoprim sulfamethoxazole | 1.25/23.75 | 16 | 10 | 15 (54) |
| Cephem | Cefotaxime | 30 | 26 | 22 | 14 (50) |
| Carbapenem | Meropenem | 10 | 23 | 19 | 0 (0) |

***S:*** *Susceptible,* ***R:*** *Resistant*

*Table 2. Primers used in the study*

| Primer | Target Gene | Sequence (5'-3') | Amplicon Size (bp) |
|---|---|---|---|
| EHEC | *stx*1 | CTGGATTTAATGTCGCATAGTG<br>AGAACGCCCACTGAGATCATC | 150 |
| EHEC | *stx*2 | GGCACTGTCTGAAACTGCTCC<br>TCGCCAGTTATCTGACATTCTG | 255 |
| EHEC/<br>EPEC | *eae*A | GACCCGGCACAAGCATAAGC<br>CCACCTGCAGCAACAAGAGG | 384 |
| EPEC | *bfp*A | GGAAGTCAAATTCATGGGGGTAT<br>GGAATCAGACGCAGACTGGTA GT | 300 |
| *chu*A.1b<br>*chu*A.2 | *chu*A | ATGGTACCGGACGAACCAAC<br>TGCCGCCAGTACCAAAGACA | 288 |
| *yja*A.1b<br>*yja*A.2b | *yja*A | CAAACGTGAAGTGTCAGGAG<br>AATGCGTTCCTCAACCTGTG | 211 |
| *TspE*4C2.1b<br>*TspE*4C2.2b | *TspE*4.C2 | CACTATTCGTAAGGTCATCC<br>AGTTTATCGCTGCGGGTCGC | 152 |
| *Ace*K F<br>*Arp*A1 R | *arp*A | AACGCTATTCGCCAGCTTGC<br>TCTCCCCATACCGTACGCTA | 400 |
| *Arp*AgpE F<br>*Arp*AgpE R | *arp*A | GATTCCATCTTGTCAAAATATGCC<br>GAAAAGAAAAAGAATTCCCAAGAG | 301 |
| *trp*AgpC.1<br>*trp*AgpC.2 | *trp*A | AGTTTTATGCCCAGTGCGAG<br>TCTGCGCCGGTCACGCCCC | 219 |
| *trp*BA.F<br>*trp*BA.R | *trp*A | CGGCGATAAAGACATCTTCAC<br>GCAACGCGGCCTGGCGGAAG | 489 |

(Biorad, Dubai) per the manufacturer's instructions. Purity and quantity were assessed via a nanodrop spectrophotometer (Maestrogen, Taiwan), with an OD260/OD280 ratio of 1.6-2.0 [24]. Primers targeting *stx1*, *stx2*, *eaeA* genes identified EHEC [25], while *eaeA* and *bfpA* genes identified EPEC [26] *(Table 2)*. Phylogenetic distribution was determined using the PCR method [11,27-29] *(Table 2)*.

PCRs were performed in 25 µL volumes with final concentrations: 1x Taq enzyme buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.4 ρmol primers, and 1.5 U Taq DNA polymerase (Fermentas, USA). PCR tubes were prepared with 22 µL of master mix and 3 µL of DNA for each sample. Amplification involved initial denaturation at 95°C for 5 min, 30 cycles of 95°C for 30 sec, annealing at 55°C *(stx1, stx2, eaeA, bfpA)* and 56°C *(chuA, yjaA, tspE4.C2, arpA, trpA)* for 30 sec, 72°C for 60 sec, and a final extension at 72°C for 10 min. *E. coli* ATCC 35150 (EHEC; *stx1, stx2, eaeA)* served as the positive control, and *S.* Typhimurium ATCC 14028 as the negative control. Target genes producing a single band of the expected size upon amplification were considered positive.

### Statistical Analysis

For statistical analysis, Statistical Package for Social Sciences (SPSS) version 23.0 (SPSS Inc., USA) was used. Pearson's chi-square ($\chi 2$) test (Fisher's Exact $\chi 2$ Test) compared frequency data. The relationship between isolate pathotypes and biofilm formation, phylotype, and MDR status was evaluated using the $\chi 2$ test. Results were evaluated at a 95% confidence interval and with $P<0.05$ were considered statistically significant.

## Results

### Isolation and Identification

*E. coli* was isolated in 75% (28 isolates) of rectal swab samples taken from 37 cats with diarrhea. Molecular confirmation via PCR targeting the *trpA* gene verified all isolates as *E. coli*. Subsequent analysis included evaluation of pathotypes, phylotypes, biofilm formation potentials, and antibiotic resistance profiles of the isolates.

### Pathotyping

Among the 28 *E. coli* isolates, 46% (13 isolates) were classified as EPEC isolates. The prevalence of tEPEC was found to be 18% (5 isolates) (both *eaeA* and *bfpA* positive), and the prevalance aEPEC was 28% (8 isolates) (*eaeA* gene positive). There was no EHEC pathotype among the isolates *(Fig. 1)*.

### Phylotyping

Using available primers, 93% (26 isolates) of *E. coli* isolates were phylotyped. Among these, 22% were phylogroup B2, 18% B1, 18% C, 14% D, 11% A, 7% E, and 3% F. The phylotype of two isolates (7%) could not be determined *(Fig. 2)*.

| ***Table 3.*** *Phylotypes of E. coli isolates* | |
|---|---|
| **Phylogroups** | **Isolate number (n=28) (%)** |
| A | 3 (11) |
| B1 | 5 (18) |
| C | 5 (18) |
| E | 2 (7) |
| B2 | 6 (22) |
| D | 4 (14) |
| F | 1 (3) |
| ? | 2 (7) |

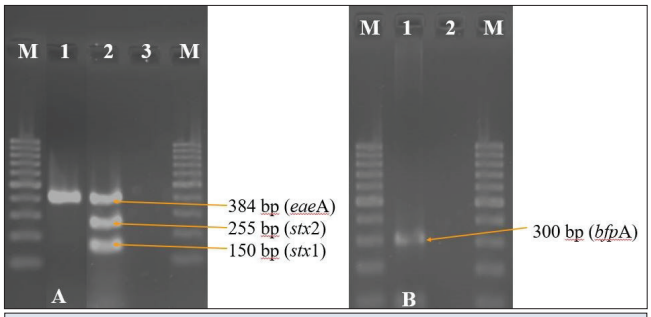


**Fig 1.** Agarose gel electrophoresis of virulence gene PCR products associated with pathotype. A.1. *eaeA* gene (384 bp) 2. Positive Control EHEC (*E. coli* ATCC 35150, *stx1*:150 bp, *stx2*: 255 bp, *eaeA*: 384 bp) 3. Negative Control (S. Typhimurium ATCC 14028) M: 100 bp DNA Ladder (Fermentas, USA). B.1. *bfpA* gene (300 bp) 2. Negative Control (S. Typhimurium ATCC 14028) M: 100 bp DNA Ladder (Fermentas, USA)

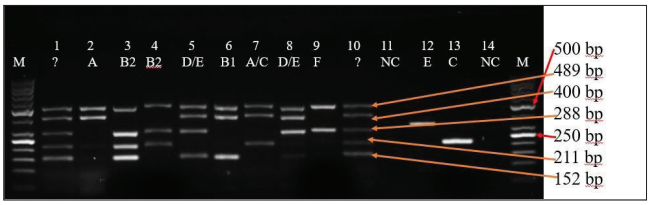


**Fig 2.** PCR profiles with the new Clermont phylotyping method. 1. Unknown phylogroup (+ + + + +) (152 bp, 211 bp, 288 bp, 400 bp, 489 bp), 2. Phylogroup A (– – – + +), 2. Phylogroup B2 (+ + + – + ), 3. Phylogroup B2 (– + + – +), 5. Phylogroup D/E (+ – + + +), 6. Phylogroup B1 (+ – – ++), 7. Phylogroup A/C (– + – + +), 8. Phylogroup D/E (– – + + +), Group F (– – + – +) 10. Unknown phylogroup (+ + + + +) (152 bp, 211 bp, 288 bp, 400 bp, 489 bp), 11. Negative Control (NC): DNA-free master mix 12. Phylogroup E (301 bp) 13. Phylogroup C (219 bp) 14. NC: S. Typhimurium ATCC 14028 M: Marker (50 bp) (Fermentas, USA), ?: Isolates whose phylotype could not be determined

In cat diarrhea cases, the most common phylogroup was B2 (22%), and the least observed was F (3%). Commensal phylogroups (A, B1, C, E) comprised 54% (15 isolates), while pathogenic phylogroups (B2, D, F) comprised 39% (11 isolates) *(Table 3, Fig. 3)*.

## Biofilm Formation

Of the 28 clinical *E. coli* isolates, 39% (11 isolates) were determined to form biofilm *(Fig. 4)*.

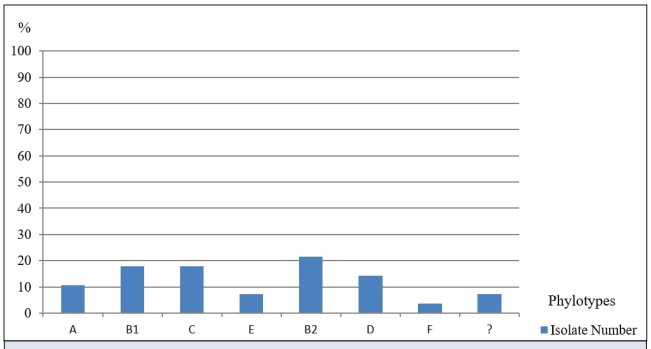


**Fig 3.** Distribution of *E.* coli phylotypes (n=28)

## Antimicrobial Resistance

The antimicrobial resistance profiles of *E. coli* isolates varied: most were resistant to ampicillin (86%), followed by amoxicillin-clavulanate (75%), ciprofloxacin (71%), tetracycline (64%), gentamicin (61%), trimethoprim-sulfamethoxazole (54%), and cefotaxime (50%). No isolates were resistant to meropenem *(Table 1, Fig. 5)*.

## Multiple Antibiotic Resistance

Eighty-six percent of *E. coli* isolates exhibited MDR, while 15% were non-multidrug resistant (NMDR). Multiple antibiotic resistance status and antibiotic resistance phenotypes of isolates are shown in *Table 4, Fig. 6.*

## Statistical Results

Our study found no significant relationship between the pathotype of *E. coli* isolates and their biofilm formation or MDR status. However, a significant relationship was identified between the pathotype and phylotype B2. No significant relationships were found between the other phylotypes (A, B1, C, D, E, F) and the pathotype *(Table 5)*.

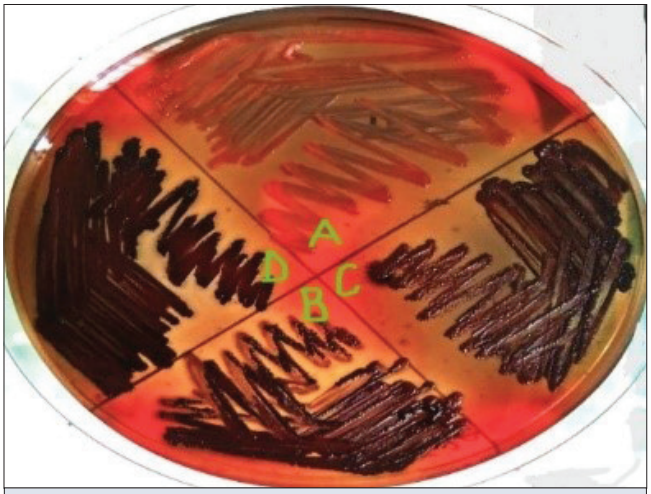


**Fig 4.** Biofilm-forming and non-biofilm-forming isolates on Congo Red Agar. A: Negative Control (*S. aureus* ATCC 25932), B: Positive Control (*E. coli* ATCC 25922), C, D: Biofilm-forming *E. coli* isolates

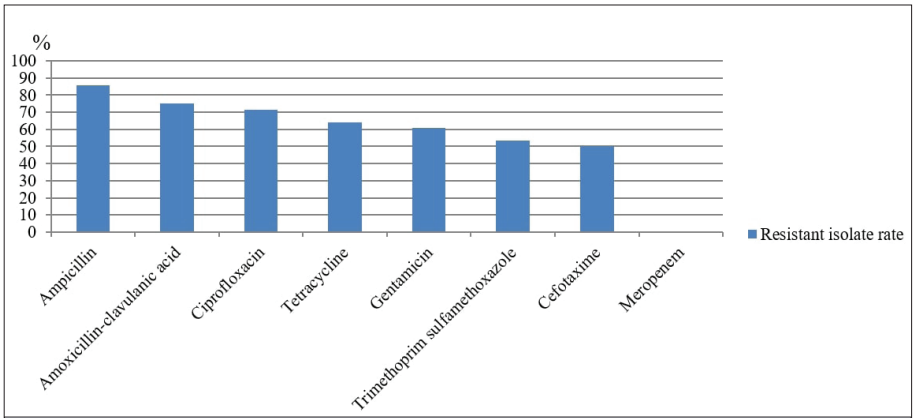


**Fig 5.** Resistance rates of *E. coli* isolates to different antibiotics

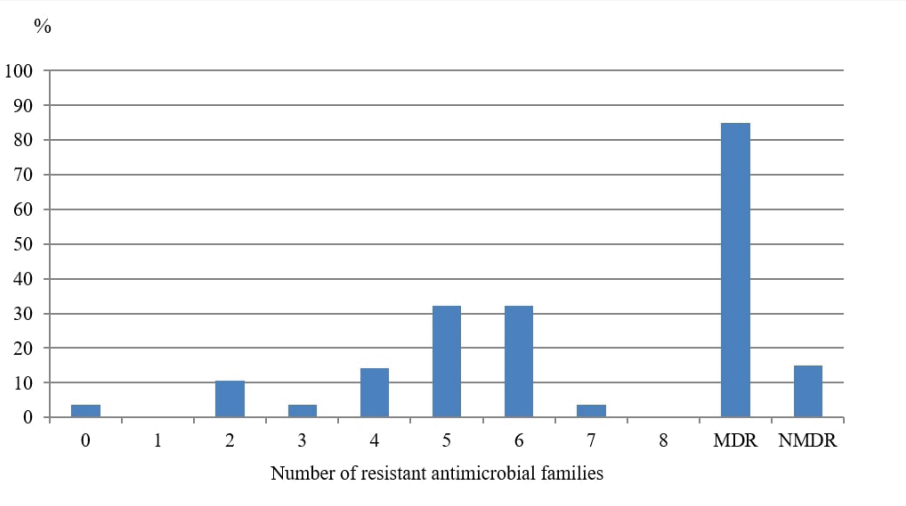


**Fig 6.** MDR/NMDR resistance rates of *E. coli* isolates (n=28).

***Table 4.*** *Multiple antibiotic resistance status of isolates*

| **Antibiotic Resistance Phenotype (Number of Isolates)** | **Number of Isolates (n=28) (%)** | **MDR/NMDR status** |
|---|---|---|
| Beta Lactam, Sulfonamid (1)<br>Tetracycline, Aminoglycoside (1)<br>Aminoglycoside, Cephem (1) | 3 (11) | NMDR<br>4 (15) |
| Penicillin, Beta Lactam, Quinolones (1) | 1 (4) | MDR<br>24 (85) |
| Penicillin, Quinolones, Aminoglycoside, Sulfonamide (1)<br>Penicillin, Beta Lactam, Tetracycline, Aminoglycoside (1)<br>Penicillin, Beta Lactam, Tetracycline, Sulfonamide (1)<br>Penicillin, Beta Lactam, Aminoglycoside, Sulfonamide (1) | 4 (15) | |
| Penicillin, Beta Lactam, Quinolones, Tetracycline, Sulfonamide (4)<br>Penicillin, Quinolones, Aminoglycoside, Sulfonamide, Cephem (1)<br>Penicillin, Quinolones, Tetracycline, Aminoglycoside, Cephem (1)<br>Penicillin, Beta Lactam, Quinolones, Sulfonamide, Cephem (2)<br>Penicillin, Beta Lactam, Quinolones, Tetracycline, Cephem (1)<br>Penicillin, Beta Lactam, Quinolones, Aminoglycoside, Cephem (1) | 10 (34) | |
| Penicillin, Beta Lactam, Quinolones, Tetracycline, Aminoglycoside, Cephem (5)<br>Penicillin, Beta Lactam, Quinolones, Tetracycline, Aminoglycoside, Sulfonamide (2)<br>Penicillin, Quinolones, Tetracycline, Aminoglycoside, Sulfonamide, Cephem (1) | 8 (28) | |
| Penicillin, Beta Lactam, Quinolones, Tetracycline, Aminoglycoside, Sulfonamide, Cephem (1) | 1 (4) | |

***MDR:*** *Multi-Drug Resistance,* ***NMDR:*** *Non Multi Drug Resistance*

***Table 5.*** *Relationship between biofilm formation, multiple antibiotic resistance and phylotypes of enteropathogenic and non-enteropathogenic E. coli isolates*

| Biofilm formation, MDR, Pylotypes Characteristics of Isolates | Pathotype | | χ2 | P Value |
|---|---|---|---|---|
| | EPEC (n=13) | NEPEC (n=15) | | |
| Biofilm (+) | 7 | 4 | 2.16 | 0.246 |
| Biofilm (-) | 6 | 11 | | |
| MDR (+) | 12 | 12 | 0.83 | 0.600 |
| MDR (-) | 1 | 3 | | |
| Phlotype A (+) | 1 | 2 | 0.22 | 1 |
| Phlotype A (-) | 12 | 13 | | |
| Phlotype B1 (+) | 2 | 3 | 0.09 | 1 |
| Phlotype B1 (-) | 11 | 12 | | |
| Phlotype B2 (+) | 6 | 0 | 8.50 | **0.005**** |
| Phlotype B2 (-) | 7 | 15 | | |
| Phlotype C (+) | 2 | 3 | 0.09 | 1 |
| Phlotype C (-) | 11 | 12 | | |
| Phlotype D (+) | 2 | 2 | 0.02 | 1 |
| Phlotype D (-) | 11 | 13 | | |
| Phlotype E (+) | 0 | 2 | 1.80 | 0.484 |
| Phlotype E (-) | 13 | 13 | | |
| Phlotype F (+) | 0 | 1 | 0.86 | 1 |
| Phlotype F (-) | 13 | 14 | | |

***MDR:*** *Multi-Drug Resistance,* ***EPEC:*** *Enteropathogenic Escherichia coli,* ***NEPEC:*** *Non-Enteropathogenic Escherichia coli*

## Discussion

Pet ownership is increasing, yet pets can harbor zoonotic diseases, posing public health risks [7,20,30]. Previous research suggests that *E. coli* is a cause of diarrhea in cats [19,30,31]. Characterizing EPEC isolates from diarrheic cats and assessing their antibiotic resistance are crucial for veterinary and public health. This study aims to determine the diversity and pathogenic properties of *E. coli* in cats, enhancing understanding of potential zoonotic risks from pets to humans.

EPEC has been documented in cats with diarrhea, with prevalence rates of 18% in the United States [32], 2.5% in Brazil [19]. In this study, a higher prevalence of EPEC (46%) was observed. The variability in EPEC prevalence cannot be ascribed to a singular factor. Environmental influences, climate, and living conditions of pets significantly impact the spread and prevalence of microorganisms. Regional prevalence differences may stem from these geographical disparities. Furthermore, variations in sample sizes and selection criteria across studies can influence prevalence rates. The diagnostic methods employed to detect EPEC also contribute to differing results. Additionally, the general health, feeding habits, and care conditions of cats are crucial factors, with infections being more prevalent in immunocompromised cats.

Our study found EPEC prevalence in diarrheic cats to be 46%, with 28% attributed to aEPEC and 18% to tEPEC. Previous research has reported EPEC cases in cats [19,20,33], some indicating the presence of the *bfpA* gene, associated with tEPEC [34,35]. Similarly, our study detected tEPEC, but aEPEC prevalence was higher, consistent with other studies [19]. However, EHEC pathotype detection was negative in our study, aligning with certain researchers' findings [19] but conflicting with others [30]. We speculate that other pathotypes like EHEC may exist in diarrheic cats, but our limited sample size may have precluded a definitive conclusion.

In this study, only the EHEC pathotype, aside from EPEC, was examined. The EHEC pathotype is crucial for assessing zoonotic risks that can be transmitted from cats to humans and poses a significant threat to human health. While other pathotypes are also important, the focus on EHEC was due to limited resources and time, prioritizing this pathotype for its high risk to human health. We used the CRA method to assess biofilm formation, which is cost-effective and rapid [17,18]. *E. coli* utilizes extracellular curli for adhesion and biofilm formation [36]. Biofilm

formation is a fundamental bacterial survival mechanism and their default lifestyle [14]. In a previous study, *E. coli* isolates obtained from calves with diarrhea were reported to be strong biofilm producers (62.5%) by the CRA method [37]. In this study, it was observed that EPEC isolates had a significant biofilm formation potential in the CRA method. Although the CRA method is widely used and provides useful information; may not capture the full complexity of biofilm formation compared to other methods (microplate, electron microscopy, etc.). Therefore, we acknowledge the limitations of using CRA alone and recommend using complementary methods to provide a more comprehensive assessment of biofilm formation. We found 39% of all isolates and 54% of EPEC isolates formed biofilms, suggesting a heightened biofilm production potential in EPEC isolates. Additionally, all seven biofilm-forming EPEC isolates were MDR, consistent with other studies [38], highlighting the importance of understanding the link between antimicrobial resistance and biofilm formation in EPEC isolates. However, when the data were analyzed in detail, we could not detect a significant relationship between the pathotype of the *E. coli* isolates in our study and their ability to form MDR or biofilm. This result may be due to the small sample size, which limits the statistical power to detect associations. Additionally, genetic variation and environmental factors such as prior antibiotic exposure and changing conditions that influence biofilm formation may also contribute to this lack of correlation.

The genomic structure of *E. coli* suggests that different phylogroups may correlate with disease status and isolation source [11]. It has been reported in previous studies that the B2 phylogroup can be found in both intestinal and extraintestinal pathogenic *E. coli* species [39]. In this study, phylogroup B2 was detected as the most common phylogroup in intestinal EPECs. These phylogroups are crucial in determining *E. coli*'s pathogenic characteristics and infection risks. Limited research exists on *E. coli* pathotypes in cats globally [19]. Prior studies on EPEC pathotypes in companion animals did not assess *E. coli*'s phylogenetic grouping [19,40]. A study in Brazil reported higher EPEC prevalence in pets with diarrhea, classified into phylogroups B1 and E [33]. Our study identified diverse phylotypes (B2, B1, C, D, A, E, F), highlighting genetic diversity in *E. coli* isolates from cats. It is important to consider that differences in phylogroup distribution may occur as a result of a combination of multiple factors (geographical factors, sampling methods, environmental conditions, diagnostic techniques used, etc.).

In this study, the phylogenetic group of two *E. coli* isolates that did not form biofilms could not be determined. Notably, one of these isolates exhibited MDR, while the other was susceptible to all tested antibiotics. This suggests significant genetic diversity among *E. coli* isolates associated with diarrheal cases, indicating potentially distinct virulence properties.

The rise of MDR *E. coli* is a global public health concern due to its opportunistic pathogenic nature [7]. Daily interactions between pets and their owners facilitate the sharing of *E. coli* strains, highlighting the need to monitor antimicrobial resistance in *E. coli* from domestic animals [7,20,30]. Our study found antibiotic-resistant EPEC strains in the intestines of cats, with resistance levels between 50% and 86% for all tested antibiotics except meropenem. Additionally, 86% of isolates exhibited multiple antibiotic resistance. However, the small sample size limits the generalizability of these findings.

In this study, we evaluated *E. coli* resistance profiles to eight antibiotics using the disc diffusion method. Ampicillin resistance was most prevalent at 86%, followed by 75% for amoxicillin-clavulanic acid, consistent with findings from Bangladesh [30]. These results are clinically significant as broad-spectrum antibiotics like penicillin and β-lactams are frequently used for gastrointestinal issues in pets [41]. Notably, antibiotic-resistant *E. coli* can persist in dogs' intestines for up to 21 days post-treatment, highlighting the gut as a reservoir for resistant bacteria [42]. Resistance genes can persist for years after antibiotic exposure [43]. While resistance was detected to seven antibiotics tested in this study, no resistance was detected to meropenem. Meropenem is an antibiotic generally used in human medicine as a last resort effective against Gram-negative bacteria. The lack of meropenem resistance can be explained by both clinical usage habits and the genetic and biological characteristics of the isolates. In a study from Bangladesh, cefotaxime resistance was 100%, compared to 50% in our study, likely due to less frequent use of third-generation cephalosporins in our sampled cats [30]. Resistance rates for trimethoprim-sulfamethoxazole, amoxicillin-clavulanic acid, gentamicin, and ciprofloxacin were also lower than those reported in Egypt [44]. Our findings showed that 86% of *E. coli* isolates were MDR, compared to 49% in a recent Chinese study [45]. These differences underscore how antibiotic usage and veterinary policies influence resistance rates in different regions.

Our study identified a significant correlation between *E. coli* pathotype and the B2 phylotype, which is commonly associated with ExPEC strains that cause various infections in animals and humans. The significant relationship between EPEC pathotype and B2 phylotype may indicate that these EPEC isolates may have high pathogenic potential. The B2 phylotype is generally associated with more virulent *E. coli* isolates [39]. This is important to highlight the seriousness of EPEC infections in cats and possible zoonotic risks. In contrast, no significant relationship was found between other phylotypes (A, B1,

C, D, E, F) and the EPEC pathotype, indicating that these phylotypes may not independently determine pathogenic properties.

Two isolates carrying all the genes examined in this study were identified. However, these two isolates could not be phylotyped with the primers available in the method used [11]. This indicates that these two isolates may be a new phylotype that cannot be detected with existing primers. To more precisely determine the genetic diversity and phylogenetic relationships of *E. coli* isolates, more comprehensive and detailed analysis methods such as Multi-Locus Sequence Typing (MLST) need to be applied.

In conclusion, our findings offer valuable insights into the prevalence, phylogroup distribution, biofilm formation potential, and antibiotic resistance of EPECs isolated from cats with diarrhea. The noteworthy association between the EPEC pathotype and the B2 phylotype suggests a potential risk posed by these isolates.

## Declarations

**Availability of Data and Materials:** The corresponding author (H. T. Yüksel Dolgun) can provide the datasets of this research upon reasonable request.

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**Author Contributions:** HTYD, YS, ST: Conceptualization, Methodology, Validation, Analysis, Investigation, Writing-Original Draft, Editing. All authors read and approved the final manuscript.

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