Egyptian Journal of Veterinary Sciences

https://ejvs.journals.ekb.eg/

Phenotypic and Genotypic Determination of ESBL Producing *E. coli* and *K. pneumoniae* Strains Isolated From Dogs and Cats

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Abstract

THE emergence and rapid spread of extended-spectrum beta-lactamase (ESBL)-producing bacteria of animal origin pose a threat to public health. Pathogens with zoonotic potentials, such as ESBL/AmpC-producing *Escherichia coli* and *Klebsiella pneumoniae*, can be transmitted from animals to humans through direct contact or contaminated environmental materials. This study was carried out to determine by phenotypic and genotypic methods ESBL/AmpC production of *E. coli* and *K. pneumoniae* strains isolated from healthy and diarrheic cats/dogs and determine multi-drug resistance via Kirby-Bauer Disc Diffusion Methods. Thirty-three *K. pneumoniae* and 167 *E. coli* were isolated and identified from stool samples. In the screening test, (38.5%) 77 isolates were evaluated as suspicious for ESBL production, and through confirmation tests performed with the double-disc synergy method, it was determined that 63 isolates produced ESBL phenotypically. The bla_{SHV}, bla_{TEM}, bla_{CTX-M}, and bla_{OXA} genes which were found to be 2, 33, 22, and 8, respectively responsible for ESBL production were determined by PCR. A total of 35 isolates were suspected of phenotypic AmpC production, and (94.2%) 33 were confirmed as AmpC positive by a three-dimensional test. The AmpC gene groups in *E. coli* strains have as follows: 14 strains CIT (AmpC origin-*Citrobacter freundii*), 13 strains FOX (cefoxitin), 11 strains ACC (Ambler class C), 3 strains DHA (Dhahran Hospital, Saudi Arabia), and 2 strains EBC (AmpC origin-*Enterobacter cloacae*). AmpC gene groups in *K. pneumoniae* strains have as follows: 4 strains CIT, 4 strains FOX, 3 strains ACC, and 2 strains EBC. Among *E. coli* strains canine origin was 69.7% (60/86), while 32 strains (39.5%) from feline origin showed Multi-drug resistance (MDR) profile. MDR among the *K. pneumoniae* strains from isolated dog origin was 28.5% (4/14) and six (31.5%) strains from feline origin were found to be MDR. Screening of phenotypic ESBL production of isolates was performed using antibiotic discs reported by CLSI. According to the results of our study, it was determined that ESBL/AmpC-producing bacteria were found at a higher rate in dogs. It was concluded that companion animals may pose a public health threat because they can contaminate the environment with their feces, and these bacteria may pose a zoonotic risk.

Keywords: AmpC, Cat, Dog, ESBL, MDR.

Introduction

Antibiotics are biologically or synthetically derived bioactive substances that inhibit growth or kill bacteria. Resistance against beta-lactam group antibiotics was known to be developed by various mechanisms. The most common of these mechanisms is the hydrolysis of the beta-lactam ring in the structure by enzymes called "beta-lactamase" [1]. Beta-lactamases produced by Gram-negative microorganisms are generally secreted when antibiotics are present in the environment. As a result of continuous exposure of Gram-negative bacteria to beta-lactam antibiotics, the dynamic and uninterrupted production of beta-lactamase enzymes in these bacteria is mutated. These enzymes are being identified as extended-spectrum beta-lactamase (ESBL) [2]. Beta-lactamases generally interrupt the beta-lactam ring and inactivate the antibiotic. The first found TEM-1 and TEM-2 enzymes are the most common plasmid-mediated beta-lactamases in Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Neisseria gonorrhoeae* and, *Enterobacteriaceae* [3].

Beta-lactamases are classified according to their basic structure and functional characteristics [4]. The most valid classification is based on protein sequences [5]. According to the Ambler molecular classification scheme, beta-lactamases are grouped

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(Received 10 January 2025, accepted 23 May 2025)
DOI: 10.21608/ejvs.2025.351647.2595

into four groups, A, B, C, and D, as they have different amino acid sequences [6]. Functional classification is based on the ability of enzymes to hydrolyse specific beta-lactam classes and their ability to inactivate beta-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam. These systems include 3 main groups and many subgroups [7,8]. Extended-spectrum beta-lactamases (ESBLs) are beta-lactamases that hydrolyse most beta-lactam antibiotics, including penicillins, cephalosporins, and aztreonam. They do not hydrolyse cephamycins and carbapenems, and are inhibited by beta-lactamase inhibitors, specifically clavulanate. The global emergence and spread of members of the ESBL-producing *Enterobacteriaceae* threaten the ability to treat an infection. ESBLs are the enzymes most commonly found in *E. coli* and *K. pneumoniae* and are becoming more predominant in *Enterobacterales* [9,10]. AmpC beta-lactamases are chromosomally encoded cephalosporinases resistant to penicillin, cefoxitin, cefazolin, cephalothin, and combined beta-lactams with beta-lactamase inhibitors [11,12]. AmpC beta-lactamases are generally found in Enterobacteriaceae, they have also been rarely reported in bacteria other than *Enterobacteriaceae* [11,12,13].

Enterobacterales that produce extended-spectrum β-lactamases (ESBL) are a common cause of antimicrobial resistance (AMR) in both people and animals [14]. The third and fourth generation cephalosporins and aztreonam, two of the last antibiotics still in use to prevent infections caused by enterobacteria like *E. coli* and *K. pneumoniae*, are resistant to ESBL [7]. Therefore, a rise in ESBL-producing *Escherichia coli* (ESBL-*E. coli*) in companion animals is frequently linked to the use of broad-spectrum cephalosporins in small animal management [15]. Likewise, ESBL-producing bacteria have been detected in the urinary tract, wound lesions, preputial discharge, and feces of healthy cats and dogs. In addition, the presence of ESBL/AmpC-producing *Enterobacteriaceae* has been reported in clinical specimens from companion animals [16,17]. However, it has been reported that ESBL/AmpC producing bacteria could be a part of bacterial flora in healthy pets [18]. This study was carried out to determine the presence of *E. coli* and *K. pneumoniae* producing ESBL/AmpC beta-lactamase and multi-drug resistance in the feces of healthy and diarrheic companion animals.

Material and Methods

Samples

Animals brought to Ondokuz Mayıs University Veterinary Hospital between 2018-2019 were grouped as cats and dogs. Faecal samples were collected from fifty healthy and fifty diarrheal animals. A total of 200 faecal samples were examined.

Isolation and Phenotypic Identification

The swabs were directly inoculated on MacConkey agar and incubated at 37ºC for 24 hours. After incubation, lactose-fermenting (pink) colonies were subcultured on Eosin-Methylene Blue (EMB) and Simmons Citrate agar. E. coli isolates that showed green metallic sheen on EMB agar but did not use citrate were identified as *E. coli*, and isolates that used citrate and did not produce green metallic sheen were identified as *K. pneumoniae*.

All isolates were subcultured on TSA for DNA extraction. DNA extraction was made by boiling method. For genotypic confirmation of the isolates, Polymerase Chain Reaction (PCR) analyses were performed by modifying the methods reported previously [19,20]. The protocol used by Abd El-Razik et al. [19] for the identification of *E. coli* isolates by PCR was modified and optimized. All reactions were performed in a final volume of 50 µl. A PCR mixture containing 200 ng extracted target DNA, 1X PCR buffer, 1 µM primer, 0.2 mM dNTP and 2 U Taq polymerase was prepared. Amplification conditions were set as 2’ initial denaturation at 95°C, 35 cycles of denaturation at 94°C for 45 secs, annealing at 57°C for 45 secs, extension at 72°C for 45 secs and a final extension step of 10 min at 72°C after the last cycle.

The protocol used by Chander et al. [20] was applied for the identification of *K. pneumoniae* isolates. Accordingly, PCR mixture was prepared by adding 25 µl master mix, 200 nM forward and reverse primers, 100 ng target DNA and DEPC-treated water to make a final volume of 50 µl. Amplification conditions were set as initial denaturation at 95ºC for 15 minutes, 35 cycles at 95ºC for 1 minute, primer annealing at 55ºC for 1 minute, extension at 72ºC for 2 minutes and final extension was set at 72ºC for 10 minutes.

Phenotypic Screening of Isolates for ESBL Production

Kirby-Bauer disk diffusion method was used for phenotypic screening of ESBL production of isolates. For this purpose, discs containing aztreonam (ATM; 30 µg), cefpodoxime (CPD; 10 µg), cefotaxime (CTX; 30 µg), ceftazidime (CAZ; 30 µg), and ceftriaxone (CRO; 30 µg) were used. Test results were evaluated according to CLSI (2019) criteria. Isolates resistant to one or more antibiotics were considered suspicious for ESBL production, and phenotypic confirmation tests were performed [21]. Briefly, 100 µl of the bacterial suspension with a density of 0.5 McFarland was inoculated onto Mueller-Hinton Agar. Antibiotic disks were placed on the agar surface and incubated. After incubation, inhibition zone diameters were measured and evaluated.

Phenotypic Confirmation of ESBL Producing Isolates

Brun-Buisson [22] described a disk diffusion test in which synergy between cefotaxime and clavulanate was detected by placing a disk of amoxicillin/clavulanate (20 µg/10 µg, respectively) and a disk of cefotaxime (30 µg), 30 mm apart (center to center) on an inoculated agar plate. After the incubation period, the expansion of the inhibition zone towards amoxicillin/clavulanic acid (keyhole image) was accepted as an indication that the isolate was positive for phenotypic ESBL production.

Phenotypic Screening of Isolates for AmpC Production

The phenotypic screening of AmpC beta-lactamase production of isolates was performed by the Kirby-Bauer disk diffusion method using cefoxitin disc (FOX; 30 µg). Isolates with a zone diameter <18 mm were considered suspicious, and a confirmatory test was performed according to CLSI (2019) [21].

Phenotypic Confirmation of AmpC Producing Isolates

The modified three-dimensional test method was carried out by extracting the AmpC enzymes of the isolates that are suspected for AmpC production. Tests were performed and evaluated according to the method as described by Singhal et al. [21].

Genotypic Determination of ESBL Production

Detection of genes responsible for the production of ESBL enzymes was performed by PCR according to the method as reported previously [22-26]. The presence of bla_{SHV}, bla_{TEM}, bla_{OXA}, $bla_{CTX\text{-}M\text{-}Group\ 1,2,9,8,25}$ genes responsible for ESBL production were investigated by PCR in all isolates that were evaluated as positive or suspicious for ESBL production. All primers, sequences and annealing temperatures used for PCR are given in Table 1.

Genotypic Determination of AmpC Production

DHA, ACC, CIT, FOX, MOX, CMY, and EBC gene groups responsible for AmpC production were investigated by multiplex PCR. The amplification conditions described by Pérez-Pérez and Hanson [27] were used.

Determination of Antibiotic Susceptibilities and Multi-Drug Resistance Profiles

The susceptibilities of the isolates among 17 different antibiotics belonging to 6 different classes of antibiotics were investigated. For this purpose, beta-lactam class [amoxicillin/clavulanic acid (20 µg+10 µg), imipenem (10 µg), ampicillin (10 µg), penicillin G (6 µg/10 IU), cefaperazone (75 µg), meropenem (10 µg), cefquinom (10 µg), cefoxitin (30 µg), cefepime (30 µg)], aminoglycoside class [gentamicin (10 µg), amikacin (30 µg)], quinolone class [ciprofloxacin (5 µg), enrofloxacin (5 µg)], tetracycline class [nitrofurantoin (100 µg), oxytetracycline (30 µg)], phenicol class [chloramphenicol (30 µg)], and antimetabolic class [trimethoprim/sulfamethoxazole (1,25/23.75 µg)] were chosen. Kirby-Bauer disk diffusion method was used, and test results were evaluated according to CLSI [19]. Isolates resistant to three or more antibiotic classes were considered as multi-drug resistance (MDR) isolates [28].

Results

Isolation and Identification

While ten *K. pneumoniae* (20%) and forty *E. coli* (80%) were isolated and identified from dogs with diarrhoea, forty-six *E. coli* (92%) and four *K. pneumoniae* (8%) were isolated from healthy dogs. Moreover, fourteen *K. pneumoniae* (%28) and 36 *E. coli* (%72) were identified in cats with diarrhoea, forty-five *E. coli* (%90) and five *K. pneumoniae* (%10) were obtained from healthy cats. In total, 167 *E. coli* and 33 *K. pneumoniae* were identified by PCR.

Phenotypic Screening and Confirmation of ESBL Producing Isolates

Twenty-four (48%) isolates from dogs with diarrhoea were suspected of ESBL production. Out of these isolates, 5 were *K. pneumoniae* and 19 were *E. coli*. Two *K. pneumoniae* and 18 *E. coli* isolates from healthy dogs were suspected of ESBL production. Sixteen (32%) isolates from cats with diarrhoea were considered suspicious for ESBL production. Out of these isolates, 5 were *K. pneumoniae* and 11 were *E. coli*. Four *K. pneumoniae* and 13 *E. coli* isolates from healthy cats were suspected of ESBL production.

All 24 isolates isolated from dogs with diarrhoea and suspected of ESBL production were positive in the phenotypic confirmatory test. Among fifteen isolates from healthy dogs evaluated as suspicious for ESBL production, two *K. pneumoniae* and thirteen *E. coli* strains were determined as ESBL positive in the phenotypic confirmatory test. A total of 14 isolates, nine *E. coli* and five *K. pneumoniae*, isolated from cats with diarrhoea and suspected ESBL, were phenotypically confirmed to be ESBL positive. Of the healthy cat isolates which were suspicious for ESBL production, ten isolates, including one *K. pneumoniae* and nine *E. coli*, were confirmed as ESBL positive. It was confirmed that 63 out of 77 isolates determined as suspicious in the phenotypic screening test produced ESBL according to the double-disc diffusion method (Fig. 1.).

Phenotypic Screening and Confirmation of AmpC Producing Isolates

It was determined that a total of fourteen isolates (four *K. pneumoniae*, ten *E. coli*) from dogs with

diarrhoea and three isolates of healthy dog origin (one *K. pneumoniae*, two *E. coli*) were suspicious for AmpC production. In addition, it was detected that a total of 13 isolates (six K. *pneumoniae* and seven *E. coli*) from cats with diarrhoea and a total of five isolates of healthy cat origin (two *K. pneumoniae* and three *E. coli*) were suspicious for phenotypic AmpC production. A total of 35 isolates were suspected for phenotypic AmpC production from 200 isolates, and a phenotypic confirmatory test (three-dimensional test) was made. From all 35 isolates from dogs and cats, 33 (94,2%) were confirmed as AmpC positive by a three-dimensional test (Figure 2.).

Among these AmpC positive isolates, 12 *E. coli* isolates from the canine origin (diarrheic and healthy) and nine *E. coli* strains from the feline origin (diarrheic and healthy) were AmpC positive, four and eight *K. pneumoniae* isolates from canine and feline origins were AmpC positive, respectively.

Detection of genes responsible for ESBL production

It was determined that two (8.3%) of the diarrhoea dog isolates carried the bla_{SHV} gene. No bla_{SHV} gene was found in sixteen isolates of feline origin with diarrhoea. In addition, the bla_{SHV} gene was not found in healthy cat and dog isolates. The PCR result to determine the presence of the bla_{SHV} gene is shown in Figure 3.

The bla_{TEM} gene was found in 66.7% (16 isolates) of dogs with diarrhoea, 56.25% (9 isolates) of cats with diarrhoea, four healthy cat isolates (23.53%), and 6% (4 isolates) healthy of dogs (Figure 4).

The bla_{CTX-M} gene was found in 11 isolates of dogs with diarrhoea (45.8%), five cat isolates with diarrhoea (31.25%), five healthy cat isolates (29.41%), and 10% (2 isolates) of healthy dog (Figure 5.)

The bla_{OXA-10} gene was detected in 12.5% (3), two (11.76%), and three (15%) isolates of dogs with diarrhoea, healthy cats, and dogs, respectively. The bla_{OXA-10} gene was not found in isolates of feline origin with diarrhoea.

PCR results are given in Figure 3,4,5,6. Table 2 shows the ESBL genes and origins of the isolates.

Detection of genes responsible for AmpC production

It was determined that nine *E. coli* and one *K. pneumoniae* isolate from dogs with diarrhoea had CIT and FOX group genes. Eight *E. coli* and one *K. pneumoniae* isolates had ACC group genes. It was detected that three isolates had DHA group genes, and one isolate had EBC genes. It was determined that ten isolates of dog origins with diarrhoea had CIT and FOX, and nine, three and one isolates had ACC, DHA, and EBC gene groups, respectively. It was determined that 38.46% (five isolates) of the 13 isolates originating from cats with diarrhoea carried both CIT and FOX group genes, 23.08% (3 isolates) had ACC group genes and 15.38% (two isolates) had EBC group genes. In addition, it was revealed that none of the isolates had DHA and MOX genes. It was determined that two of the five isolates isolated from healthy cats had CIT group genes and one had FOX, ACC, and EBC group genes. One of the healthy canine origin isolates had both CIT and FOX group genes, while the other isolate had only ACC genes. Multiplex PCR results are given in Figure 4. The AmpC genes of *E. coli* and *K. pneumoniae* isolates from different origins are shown in Tables 2 and 3, respectively.

Antibiotic Susceptibility Testing and Multi-Drug Resistance

Fourteen *K. pneumoniae* isolates of dog origin that showed the resistance against antibiotics were as follows: amoxicillin/clavulonic acid (71.4%), ampicillin (57.1%), penicillin (78.5%), gentamicin (14.2%), nitrofurantoin (21.4%), trimethoprim/sulfamethoxazole (35.7%), oxytetracycline (21.4%), cefquinome (35.7%), cefepime (42.8%), cefoxitin (21.4%), enrofloxacin (7.1%), ciprofloxacin (7.1%), cefoperazone (7.1%), chloramphenicol (14.2%). However, all isolates were susceptible to imipenem, meropenem, and amikacin. Multi-drug resistance (MDR) among the *K. pneumoniae* strains from isolated dog origin was 28.5%. The antibiotic resistance rates for 19 *K. pneumoniae* isolates from cats were as follows: amoxicillin+ clavulanic acid (47.1%), ampicillin (78.9%), penicillin (100%), meropenem (15.7%) gentamicin (21%), amikacin (15.7%) nitrofurantoin (42.1%), trimethoprim/sulfamethoxazole (42.1%), oxytetracycline (42.1%), cefquinome (57.8%), cefepime (52.6%), cefoxitin (47.3%), enrofloxacin (31.5%), ciprofloxacin (15.7%), cefoperazone (31.5%), chloramphenicol (31.5%). However, all isolates were susceptible to imipenem. Six (31.5%) *K. pneumoniae* strains were found to be MDR. The resistance rates of 86 *E. coli* isolates of dog origin were as follows: amoxicillin/clavulonic acid (60.4%), ampicillin (58.1%), penicillin (43%), meropenem (2.3%), gentamicin (36%), amikacin (34.8%), nitrofurantoin (4.6%), trimethoprim/sulfamethoxazole (75.5%), oxytetracycline (45.3%), cefquinome (34.8%), cefepime (30.2%), cefoxitin (15.1%), enrofloxacin (20.9%), ciprofloxacin (22%), cefoperazone (22%), chloramphenicol (27.9%). In addition, all isolates were susceptible to imipenem, and the MDR rate among canine *E. coli* strains was 69.7%. The antibiotic resistance rates for the 81 strains of *E. coli* isolated from cats were as follows: amoxicillin/clavulonic acid (60.4%), imipenem (4.9%), ampicillin (40.7%), penicillin (90.1%), meropenem (1.2%) gentamicin (18.5%), nitrofurantoin (24.6%), trimethoprim/sulfamethoxazole (35.8%), oxytetracycline (40.7%), cefquinome (80.2%),

cefepime (29.6%), cefoxitin (12.3%), enrofloxacin (14.8%), ciprofloxacin (16%), cefoperazone (13.5%), chloramphenicol (20.9%). However, all isolates were susceptible to amikacin and 32 strains (39.5%) showed MDR profile.

Multi-Drug Resistance among ESBL and AmpC beta-lactamase Producing Strains

When the multi-antibiotic resistance was evaluated in the isolates determined to be both ESBL and AmpC positive in the study, it was determined that a total of 14 (13 were *E. coli* and one was *K. pneumoniae*) isolates had multi-drug resistance. The origin of the isolates and how many antibiotic classes they are resistant to are shown in Table 4.

In the study, it was determined that a total of three *E. coli* isolates that were negative for ESBL production but positive for AmpC had multiple antibiotic resistance. The origin of these isolates and how many antibiotic classes they show resistance to are given in Table 5.

Discussion

The unconscious use of antibiotics is one of the most important causes of antibiotic resistance in bacteria. Antibiotic resistance not only develops against a single antibiotic or a class, but also multi-drug-resistant bacterial infections are on the rise. MDR, which is frequently observed in bacterial strains belonging to the *Enterobacteriaceae* family, has emerged as a significant threat to human and animal health [31]. In the last decade, there has been a rapid increase in infections due to ESBL-producing bacteria, and attention has been drawn to animal reservoirs that are thought to be related to the issue [32]. Various sources of ESBL have been reported in veterinary medicine [33]. Companion animals are seen as potential reservoirs for antimicrobial-resistant bacteria. The presence of ESBL producing *Enterobacteriaceae* has been reported in clinical specimens from companion animals, but reports have been limited to the microbiota of ESBL-producing bacteria from healthy pets [16]. In a study, 65 *E. coli* and 37 *Klebsiella* spp. isolates were found to be suspicious for ESBL production from total 110 isolates (70 *E. coli* and 40 *K. pneumoniae*), and 36 of the *E. coli* strains (55%) and 22 of *Klebsiella* spp. (59.4%) have been found to be positive for ESBL production according to the double-disc synergy test [34].

In the study conducted by Dalela, isolated from different origins which phenotypically ESBL suspicious 117 *E. coli* and 43 *K. pneumoniae* strains. ESBL production of 22 *K. pneumoniae* (51.1%) and 82 *E. coli* (70%) strains was detected by double disc synergy method [34]. Giriyapur et al. [35] reported the rates of 71.87% and 62.19% in the confirmatory test performed with the same method in 83 *K. pneumoniae* and 51 *E. coli* strains, respectively. In this study, it was determined that 77 (38.5%) of 200 isolates (167 *E. coli* and 33 *K. pneumoniae*) were suspicious for ESBL production, and 63 (81.8%) of them were positive in the confirmatory test performed with the double-disc synergy method. The confirmatory test revealed that 48.5% of *K. pneumoniae* isolates and 35.5% of *E. coli* isolates were ESBL positive. The presence of the $bla_{\text{OXA-10}}$ gene was 41.6% in 178 isolates of animal origin, and it was greater in isolates of dog origin than in isolates of other species, according to Schmiedel et al. [36]. In addition, they found that the blaOXA-10 gene was 23.6% in isolates from hospitalized animals and 32.8% in isolates from outpatients. In a study conducted in Europe, 22 isolates obtained from samples taken from sick animals were found to be suspicious for ESBL production. The $bla_{\text{OXA-10}}$ gene was found in three of these isolates (13.6%) [37]. In this study, the $bla_{\text{OXA-10}}$ gene was found in three (12.5%) isolates of dogs with diarrhoea and in three (15%) and two (11.76%) healthy dog and cat isolates, respectively. Upon comparison with other studies, the $bla_{\text{OXA-10}}$ gene was found to be less common in this study. O'Keefe et al. [38] reported ESBL production in 60 of 150 *E. coli* strains from cats and dogs, but they found that only one isolate (1.6%) carried the bla_{SHV} gene. In another study, the presence of the bla_{SHV} gene in isolates of companion animals has been investigated and, nine isolates (16.6%) from 54 *E. coli* were found to have the bla_{SHV} gene [33]. In Turkey, Gümüş detected the bla_{SHV} gene in four (7.2%) *E. coli* isolates from 39 healthy dogs and 16 cats [39]. In this study, among all *E. coli* isolates, only 2 (1.1%) E. coli strains had the bla_{SHV} gene. It was seen that there were differences between strains in terms of carrying the bla_{SHV} gene. Tamang et al. [40] have detected the bla_{TEM} gene in 17 (36.1%) *E. coli* strains isolated from 47 healthy dogs. Rzewuska et al. [41] have reported that four of 119 *E. coli* strains isolated from sick dogs were ESBL positive both phenotypically and genotypically; and all four (3.36%) were found to have the bla_{TEM} gene. In this study the bla_{TEM} gene was found at a rate of 42% among all isolates. So et al. [42] found that 21 (33.3%) of 63 dog origin E. coli isolates had the $bla_{\text{CTX-M}}$ gene. In Europe, Bogaerts et al. [37] reported that 10 *E. coli* isolated from dogs and cats produced ESBL and 9 of them had the $bla_{\text{CTX-M}}$ gene. Shimizu et al. [43] detected the $bla_{\text{CTX-M}}$ group gene in 83 (92.2%) of 90 ESBL-producing *E. coli* isolates. In Japan, Umeda et al. [44] reported the prevalence of ESBL producing *E. coli* by taking faecal samples from 151 shelter dogs and 182 cats. According to the results, it was determined that 22 strains produced ESBL. Among these isolates, 8 isolates of canine origin were identified as *E. coli*, 13 isolates of cat origin were identified as *E. coli*, and 1 was identified as *K. pneumoniae*. All strains had the $bla_{\text{CTX-M}}$ group gene. In our study, unlike other studies, $bla_{\text{CTX-M}}$ group

gene was found in 23 (36.5%) of 63 ESBL-producing isolates. The findings from this study, along with other study findings, reveal that the prevalence of the bla_{CTX-M} gene varies significantly between regions. In a study from South Korea by Tamang et al. 29 *E. coli* isolates from dogs were found to be phenotypically suspicious for AmpC production and the result of the confirmatory test showed the AmpC production in 23 (79.3%) isolates. In the study conducted in Brazil, faecal samples from 74 patients and 113 of healthy cat/dog origin were examined [45]. It was determined that 22 (11.7%) isolates produced ESBL, 18 (9.6%) isolates produced AmpC, and 4 (2.1%) isolates produced both beta-lactamases. Umeda et al. [43] isolated and examined *E. coli* isolates from the feces of 151 dogs and 182 cats. It was revealed that 21 (6.3%) of the isolates were ESBL, 20 (6%) of them were AmpC, and 1 (0.3%) of them had production of both beta-lactamases. In another study, out of 40 *E. coli* isolates isolated from faecal samples of cats, 9 (22.5%) were positive for both ESBL and AmpC positive and 8 (20%) of them were determined to have genes responsible for ESBL and AmpC beta-lactamase production [46]. In this study, both ESBL and AmpC production was determined phenotypically in 20 (10%) from healthy and diarrheic pet animal's origin isolates. This study revealed that both ESBL and AmpC beta-lactamase production rates from healthy or domestic animals with diarrhoea were similar in Turkey compared to other countries. It is noteworthy that the gene groups identified in the studies differ, but there is an increase in the prevalence of resistant bacteria. The rapid increase in ESBL/AmpC-producing bacteria worldwide has been a concern for both animal and human health, as the genes responsible for ESBL/AmpC production are transferable between Gram-negative bacteria. Timofte et al. [47] revealed *E. coli* strains of dog origin resistance to ampicillin, amoxicillin+clavulonic acid, cefotaxime, cefpodoxime, ceftazidime, and tetracycline. In addition, 65% cefoxitin, 71% ciprofloxacin, and 59% trimethoprim/sulfamethoxazole resistance rate were found. In New Zealand, antibiotic resistance and MDR has examined in healthy pets 74 dogs and 29 cats [48]. Resistance to amoxicillin+clavulonic acid, cefoxitin, cefoxitin, cefovecin, cefotaxime, ceftazidime, aztreonam, gentamicin, enrofloxacin, trimethoprim/sulfamethoxazole, and tetracycline were found as 82%, 91%, 52%, 70%, 33%, 25%, 35%, 12%, 26%, 42% and 64%, respectively. Furthermore, 42 isolates (35%) were found MDR. Zogg et al. [49] isolated 58 *E. coli*, 11 *K. pneumoniae*, and 3 *E. cloacae* from dogs and cats. Among the isolates, ESBL-producing and MDR rates were 20.8% and 73.6%. In letters 361 faecal samples were examined from dogs with healthy and diarrhoea by Carvalho et al. [50] 3 *K. pneumoniae* and 13 *K. pneumoniae* were isolated from healthy and diarrheic dogs, respectively. The resistance rates of the isolates to tetracycline were 88%, to cefotaxime, ceftazidime, aztreonam, and ciprofloxacin were %100. It has been reported that ESBL-producing *Enterobacteriaceae* often show resistance to aminoglycosides, trimethoprim/sulfamethoxazole, and tetracyclines [51]. In this study, it was determined that out of 15 isolates carrying the gene(s) responsible for the production of both beta-lactamases. Thirteen of them were resistant to tetracyclines, 14 of them to trimethoprim/sulfamethoxazole, and 8 isolates to aminoglycosides. It has been reported that fluoroquinolone resistance in *E. coli* and *K. pneumoniae* strains carrying the gene(s) responsible for ESBL and AmpC beta-lactamase production may result from point mutations in topoisomerase genes and increased activation of efflux pumps [52]. Similarly, resistance to fluoroquinolone was determined in 13 of 14 ESBL and AmpC beta-lactamase-producing isolates in this study. This situation limits the use of fluoroquinolone drug groups, which are frequently used treatment options in Veterinary Medicine. When antibiotic resistance profiles are examined over the years among cats and dogs, it is obvious that antibiotic resistance rises in both healthy and diarrheic cats and dogs. This is assumed to be attributable to an increase in the unconscious and excessive use of antibiotics. Also, multi-drug resistance can often be found in ESBL/AmpC-producing bacteria. Considering the faecal shedding potential of bacteria producing these enzymes, its zoonotic risk should not be ignored.

Conclusion

In this study, high levels of ESBL/AmpC-producing *E. coli* and *K. pneumoniae* were detected in the enteric microbiota of healthy animals as well as diarrheic cats/dogs. In conclusion, it is thought that this carrier state in healthy animals may create a risk of transmission to humans by contaminating the environment or by direct contact.

Acknowledgments

This study summarizes from the PhD thesis of the first author.

Funding statement

This study was supported by the Scientific Research Projects Commission of Ondokuz Mayıs University with the project number PYO.VET.1904.19.010

Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

This study does not require ethical approval.

TABLE 1. Oligonucleotide primers to be used in PCR

Target	Primers	Gene(s) Sequence	Amplicon Size (bp)	Annealing Temperature (ºC)	References
E. coli	Eco 2083	GCTTGACACTGAACATTGAG	662	57	19
	Eco 2745	GCACTTATCTCTTCCGCATT			
K. pneumoniae	KO(F)	CAACGGTGTGGTTACTGACG	108	55	20
	KO(R)	TCTACGAAGTGGCCGTTTTC			
CTX-M	MA1	SCSATGTGCAGYACCAGTAA	550	57	27
	MA2	CCGCRATATGRTTGGTGGTG			
TEM	TEM-F	ATGAGTATTCAACATTTCCG	858	55	24
	TEM-R	CCAATGCTTAATCAGTGAGC			
SHV	SHV-F	AAGATCCACTATCGCCAGCAG	213	52	26
	SHV-R	ATTCAGTTCCGTTTCCCACCGG			
OXA-10	OPR1	GTCTTTCGAGTACGGCATTA	720	55	25
	OPR2	ATTTTCTTAGCGGCAACTTAC			
DHA-1, DHA-2	DHAM-F	AACTTTCACAGGTGTGCTGGGT	405		
	DHAM-R	CCGTACGCATACTGGCTTTGC			
ACC	ACCM-F	AACAGCCTCAGCAGCCGGTTA	346		
	ACCM-R	TTCGCCGCAATCATCCCTAGC			
MIR-1T, ACT-1	EBCM-F	TCGGTAAAGCCGATGTTGCGC	302		
	EBCM-R	CTTCCACTGCGGCTGCCAGTT			
FOX-1-3-4-5b	FOXM-F	AACATGGGGTATCAGGGAGATG	190	64	29
	FOXM-R	CAAAGCGCGTAACCGGATTGG			
MOX-1, MOX-2, CMY-1, CMY-8-9-10-11	MOXM-F	GCTGCTCAAGGAGCACAGGAT	520		
	MOXM-R	CACATTGACATAGGTGTGGTGC			
LAT-1,LAT-4, CMY-2,3,4,5,6,7 BIL-1	CITMF	TGGCCAGAACTGACAGGCAAA	462		
	CITMR	TTTCTCCTGAACGTGGCTGGC			

TABLE 2. ESBL genes in *E. coli* and *K. pneumoniae* isolates from different origins

Isolate (n)		Gene	Origin
E.coli	***K. pneumoniae***		
2	-	bla_{SHV}	diarrheic dogs
15	1	bla_{TEM}	diarrheic dogs
8	1	bla_{TEM}	diarrheic cats
3	1	bla_{TEM}	healthy cats
4	-	bla_{TEM}	healthy dogs
10	1	bla_{CTX-M}	diarrheic dogs
3	2	bla_{CTX-M}	diarrheic cats
3	1	bla_{CTX-M}	healthy cats
2	-	bla_{CTX-M}	healthy dogs
3	-	bla_{OXA-10}	diarrheic dogs
2	-	bla_{OXA-10}	healthy cats
3	-	bla_{OXA-10}	healthy dogs

TABLE 3. AmpC genes of *E. coli* isolates isolated from different origins

Origin	*E. coli* isolates					
	CIT	FOX	ACC	DHA	EBC	MOX
DD (n=10)	9	9	7	3	0	0
DC (n=7)	3	3	2	0	2	0
HC (n=3)	1	0	1	0	0	0
HD (n=2)	1	1	1	0	0	0

DD: Diarrheic dogs; DC: Diarrheic cats; HC: Healthy Cats, HD: Healthy Dogs

TABLE 4. AmpC genes of *K. pneumoniae* isolates isolated from different origins

Origin	*K. pneumoniae* isolates					
	CIT	FOX	ACC	DHA	EBC	MOX
DD (n=4)	1	1	2	0	1	0
DC (n=6)	2	2	1	0	0	0
HC (n=2)	1	1	0	0	1	0
HD (n=1)	0	0	0	0	0	0

DD: Diarrheic dogs; DC: Diarrheic cats; HC: Healthy Cats, HD: Healthy Dog

TABLE 5. MDR strains producing both ESBL and AmpC

Origin	Strain	BL	AG	FL	T	S	P	RAC
DD	*E. coli*	R	R	R	R	R	R	6
DD	*E. coli*	R	R	R	R	R	R	6
DD	*E. coli*	R	R	R	R	R	R	6
DD	*E. coli*	R	R	R	R	R	R	6
DD	*E. coli*	R	S	R	R	R	R	5
DD	*E. coli*	R	R	S	R	R	R	5
DD	*E. coli*	R	R	R	R	R	R	6
DD	*E. coli*	R	R	R	R	R	R	6
DD	*E. coli*	R	R	R	R	R	R	6
DC	*E. coli*	R	R	R	R	R	R	6
DC	*E. coli*	R	S	R	R	R	S	4
DC	*E. coli*	R	S	R	R	R	S	4
DC	*K. pneumoniae*	R	R	R	S	R	R	5
HD	*E. coli*	R	S	R	R	R	R	5

DD: Diarrheic dogs; DC: Diarrheic cats; HD: Healthy Dog, R: resistant, S: susceptible, BL: Beta-lactamases, AG: Aminoglycosides; FL: Fluoroquinolones, T: Tetracyclines, S: Sulfonamides, P: Phenicols, RAC: Resistance to antibiotic class

TABLE 6. MDR strains that produce only AmpC but no ESBL

Origin	Strain	BL	AG	FL	T	S	P	RAC
HD	*E. coli*	R	S	S	R	R	R	4
HC	*E. coli*	R	R	S	R	R	S	4
DC	*E. coli*	R	R	R	R	R	R	6

DC: Diarrheic cats; HC: Healthy Cats, HD: Healthy Dog; R: resistant, S: susceptible, BL: Beta-lactamases, AG: Aminoglycosides; FL: Fluoroquinolones, T: Tetracyclines, S: Sulfonamides, P: Phenicols, RAC: Resistance to antibiotic class

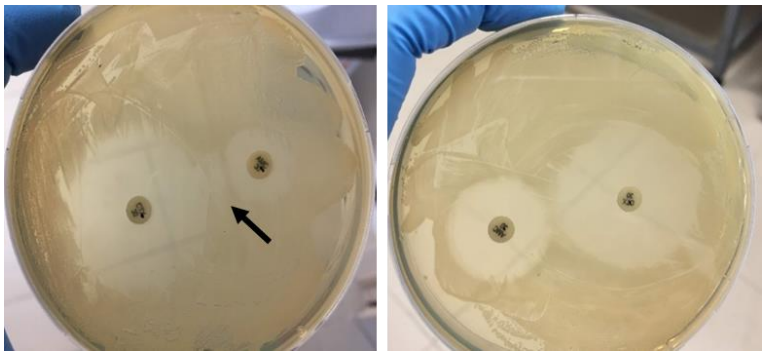

Fig. 1. Determination of ESBL production by the double disk diffusion method
ESBL producing isolate (left), non-ESBL producing isolate (right); black arrow; Keyhole view)

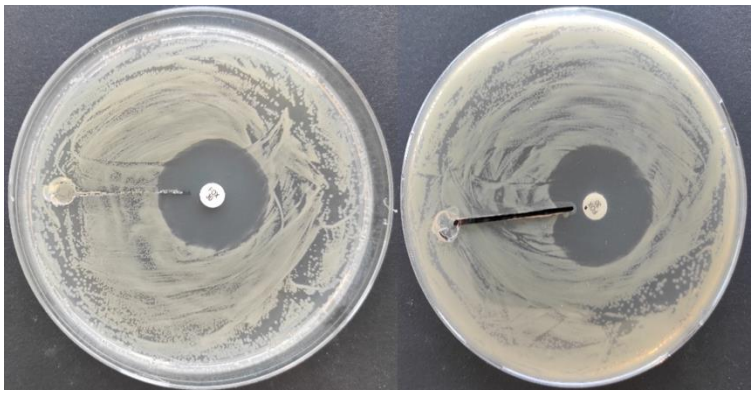

Fig. 2. Phenotypic confirmation of AmpC production by three-dimensional assay (3D)

Positive AmpC production (right), negative AmpC production (left)

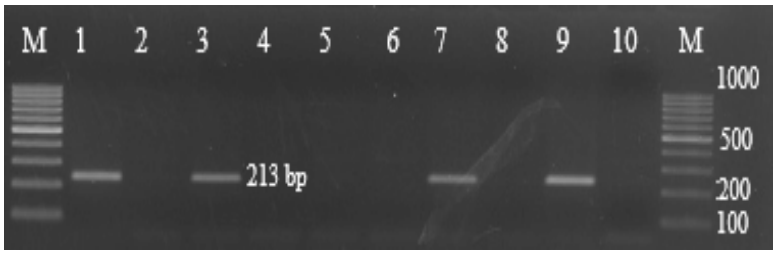

Fig. 3. *bla*$_{SHV}$ gene-specific PCR screening

M: Marker (100-1000 bp), 1: positive control, 2: negative control, 3,7,9: positive isolates, 4,5,6,8,10: negative isolates

Fig. 4. *bla*$_{TEM}$ gene-specific PCR screening

M: Marker (100-1000 bp), 1: positive control, 4: negative control, 2,3,6,7: positive isolates, 5,8,9,10: negative isolates

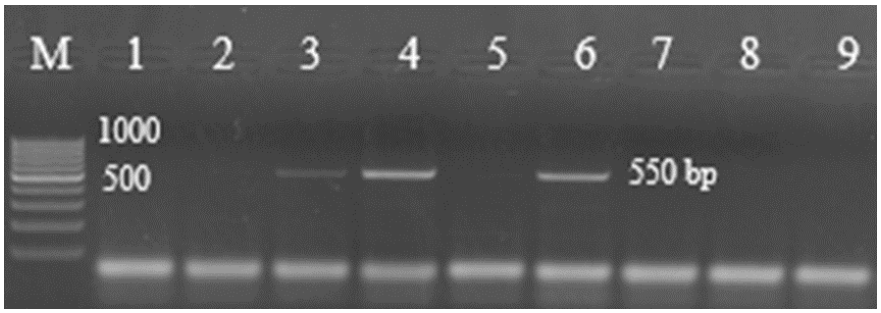

Fig. 5. *bla*$_{\text{CTX-M}}$ gene-specific PCR screening

M: Marker (100-1000 bp), 3: positive control, 1: negative control, 4,6: positive isolates, 2,5,7,8,9: negative isolates

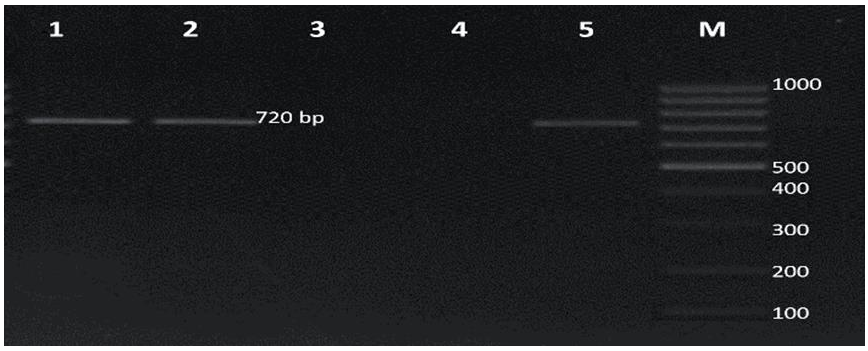

Fig. 6. *bla*$_{\text{OXA-10}}$ gene-specific PCR screening

M: Marker (100-1000 bp), 1: positive control, 2,5: positive isolates, 3: negative control, 4: negative isolate

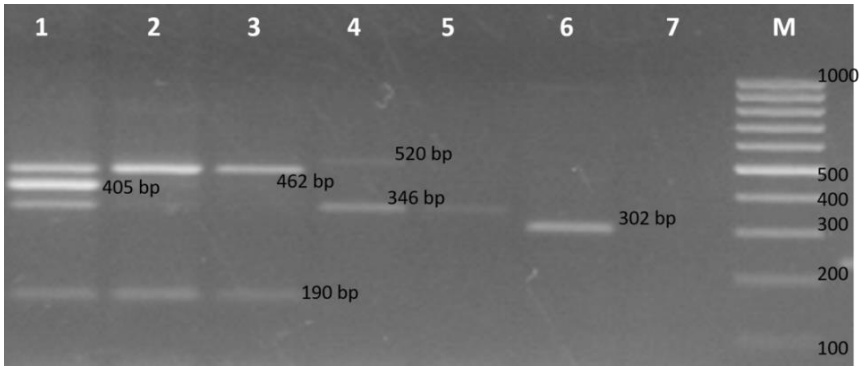

Fig. 7. Specific appearance of the gene group(s) responsible for AmpC production (CIT; 462 bp, FOX; 190

M: Marker, 1: positive control, 2,3: FOX and CIT genes positive isolates, 4: ACC and MOX genes positive isolates, 5: ACC gene positive isolate, 6: EBC gene positive isolate, 7: negative control
bp, ACC; 346 bp, DHA; 405 bp, EBC; 302 bp, MOX; 520 bp)

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