Acta Veterinaria-Beograd 2021, 71 (4), 435-450
UDK: 579.84:575
636.1/.9.09:616.98
DOI: 10.2478/acve-2021-0036

*Research article*

# CHARACTERISATION OF AmpC / ESBL GENES IN SOME PATHOGEN GRAM-NEGATIVES ISOLATED FROM CLINICAL CASES OF LIVESTOCK AND COMPANION ANIMALS

Faruk PEHLİVANOGLU*, Ezgi SABABOGLU

Department of Microbiology, Faculty of Veterinary Medicine, Mehmet Akif Ersoy University, Istiklal Campus, Burdur-TURKEY

(Received 02 March, Accepted 14 October 2021)

This study was aimed to search and characterize the AmpC and/or ESBL genes of *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* isolated from clinical cases of local livestock and companion animals between 2017 and 2019. A total of eight ceftiofur-resistant *E. coli* (n= 7) and ceftiofur-resistant *K. pneumoniae* (n= 1) and seven *P. aeruginosa* were isolated from different cases in local animals. By combination disc method, six *E. coli* isolates and one *K. pneumoniae* isolate were found to be ESBL producers. By combination of the disc method and double disc synergy test, no *P. aeruginosa* isolates were found as ESBL producers. In the agar disc diffusion test (ADDT) performed with cefoxitin and cefoxitin-boronic, only one *E. coli* was determined as AmpC producer. In ESBL-producing isolates, only the CTX-M class gene was detected by polymerase chain reaction (PCR) and subsequent sequence analysis revealed CTX-M-3 and CTX-M-15 variants. An AmpC positive *E. coli* isolate was found to carry plasmidic *ampC* gene in cmy-2 variant from CIT family. It was observed that *P. aeruginosa* isolates did not carry the plasmidic *ampC* gene. After the chromosomal *ampC* gene of one *P. aeruginosa* was amplified by PCR and sequenced, R79Q and T105A mutations in the chromosomal *ampC* gene was revealed. This showed that overproduction of the *ampC* enzyme is involved in the resistance to β-lactams in *P. aeruginosa* isolates in the study.

**Keywords:** β-lactamase, dog, Enterobacterales, *Pseudomonas aeruginosa*, ruminant

## INTRODUCTION

The β-lactamase enzymes produced by Gram-negative bacteria vary widely, and these variations usually develop due to the change in the active site of the enzyme after point mutations. As a result, β-lactamase groups with different substrate (antibiotic) specificity and inactivating activity emerge. Among the groups, AmpC and extended-spectrum β-lactamases (ESBLs) are the most common ones found in bacteria belong to Enterobacterales and other Gram-negative bacteria [1,2].

*Corresponding author: e-mail: pehlivanoglu@mehmetakif.edu.tr

AmpC-producing bacteria are resistant to most penicillins, cephalosporins (except cefepime and cefpirome), cephamycins and, although variable, monobactams [2,3]. ESBLs are known for their ability to hydrolyze oxyimino-cephalosporin (3rd and 4th generation) and monobactams but not cephamycin such as cefoxitin and carbapenems (meropenem, imipenem, ertapenem, and doripenem) [4]. ESBLs are generally susceptible to β-lactamase inhibitors (clavulanic acid, sulbactam, and tazobactam), but AmpC enzymes are not susceptible. Genes encoding ESBLs are generally located on plasmids but AmpC genes can be found embedded in the bacterial chromosome and/or plasmids [1,2]. The most common ESBL genes from Enterobacterales are identified as CTX-M, SHV and TEM classes [1]. As of plasmid origin, AmpC enzymes are divided into six families according to their amino acid similarity as ACC, CIT, DHA, EBC, FOX and MOX [5]. In *E. coli*, chromosomal *ampC* gene was constitutively expressed at low level, not inducible, while *K. pneumoniae* lack a chromosomal *ampC* gene [2]. Plasmid-derived AmpC enzymes are not inducible with some exception (such as DHA-1 and DHA-2) in Enterobacterales [2]. *P. aeruginosa* harbors the inducible chromosomal *ampC* gene. Also, AmpC is produced constituvely at low level and contributes to intrinsic resistance to β-lactams. The other main mechanism in intrinsic resistance of *P. aeruginosa* to β-lactams is an efflux pump system and an outer membrane with low permeability [6]. In addition, overproduction of AmpC following mutations in inducible *ampC* gene (derepressed mutants) creates increased resistance to cephalosporins (including 3rd and 4th generations of cephalosporins), monobactams and antipseudomonal penicillins (antipseudomonal penicillins and piperacillin) [7].

Penicillin G (narrow spectrum), aminopenicillins (amoxycillin and ampicillin, wide spectrum), penicillin-β lactamase inhibitor combinations (amoxicillin clavulanic acid and ampicillin sulbactam) and cephalosporins are the most preferred antibiotics in veterinary medicine for livestock and companion animals due to low side effects. AmpC and/or ESBL production by pathogen Gram-negatives create treatment difficulties in veterinary medicine. Additionally, AmpC-/ESBL-producing Gram-negative isolates often exhibit multiple antibiotic resistance phenotypes, further reducing treatment options [8]. Even though in human medicine, numerous reports on clinical infections due to AmpC- or ESBL-producing Gram-negatives exist in literature; a small number of reports is present on the clinical impact of AmpC- or ESBL-producing Gram-negatives in the veterinary field. AmpC-/ESBL-producing Gram-negatives have been reported mostly as *E. coli* and *K. pneumoniae* isolates in mastitis cases in cattle [9-15] and in UTI cases in dogs and cats [16-21], but more studies are needed in different animal populations to trace and control the development of such isolates. In addition, studies will increase our knowledge about the genetic characteristics of these isolates in terms of ampC/ESBL genes in livestock and companion animals. Similarly, in Turkey, no research data are available on the ampC-/ESBL genes of Gram-negatives isolated from diseased livestock and companion animals. Therefore, the present study was conducted to investigate the presence of ampC and/or ESBL genes in *E. coli*, *K.*

*pneuemoniae* and *P. aeruginosa* isolated from the specimens taken from diseased livestock and companion animals in Burdur city over a period of time between 2017 and 2019.

## MATERIALS AND METHODS

### The isolates

The Gram-negative isolates subject to this study were ceftiofur-resistant *E. coli* (n= 7), ceftiofur-resistant *K. pneumoniae* (n= 1) and *P. aeruginosa* (n= 7). All the isolates were from the specimens taken from diseased animals and sent to our laboratory for disease diagnosis and antibiogram (agar disc diffusion test) between 2017 and 2019. To meet the purpose of the study, we included only ceftiofur-resistant *E. coli* and cefiofur-resistant *K. pneumoniae* to the study because ceftiofur as a 3rd generation cephalosporin has been used widely for treatment in veterinary medicine. Ceftiofur resistance of *E. coli* and *K. pneumoniae* was determined by agar disc diffusion test (ADDT) (inhibition zone diameter narrower than 21 mm) [22]. Since *P. aeruginosa* is intrinsically resistant to ceftiofur, we included all *P. aeruginosa* isolates isolated from diseased animals in our laboratory during the same time period. The isolates were isolated from calf/lamb internal organs (liver, lung and spleen), cow mastitic milk, dog urine and dog ear swab samples by the following protocols.

### Microbiological isolation and identification of the isolates

Sudden death had occurred in the calves and lambs from which the organ samples (liver, lung and spleen) were taken. A congested and haemorrhagic picture prevailed in the lungs, liver and spleen. Approximately 10 gr of each of the organ samples were triturated in sterile physiological saline (10 ml) separately. Fifty µl of the obtained suspension was taken and planted on blood agar (BA)(Oxoid, UK) and MacConkey agar (MCA) (Oxoid, UK) by spreading method and incubated under aerobic conditions at 37 °C for 24-48 hrs.

The cows from which the milk samples were collected were suffering from clinical mastitis. There were coagulation and traces of blood, as well as a color change in the milk. After homogenisation, 50 µl of the incoming mastitic milk samples were planted on BA and MCA, and the plates were incubated at 37 °C for 24-48 hrs in aerobic atmosphere.

Urine samples of at least 10 ml sent from dogs with suspected urinary tract infection were first centrifuged at 5000 rpm for 5 min, then 50 µl was taken from the bottom of the tube and planted on BA and MCA by spreading method, and the plates were incubated at 37 ºC for 24-48 hrs in aerobic conditions.

Ear swabs from the dogs with suspicion of otitis externa were streaked directly on BA and MCA plates, and incubated at 37 ºC for 24-48 hrs in aerobic conditions.

Identification of *E. coli*, *K. pneumoniae* and *P. aeruginosa* was carried out by standard microbiological tests [23]. Since identification of one isolate (EC-35) in the study could not be concluded by phenotypic tests, 16S rRNA gene amplification by PCR (with primers 8F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R: 5'-GGTTACCTTGTTACGACTT-3') and sequence analysis of amplicon were performed for species-based identification [24].

### CLSI Initial Screening Test

Suspensions of ceftiofur-resistant *E. coli* (n= 7) and *K. pneumoniae* isolates (n= 1) (0.5 McFarland turbidity) were tested with aztreonam (monobactam, ATM 30 μg), cefotaxime (3rd generation cephalosporim; CTX 30 μg), ceftazidime (3rd generation cephalosporin, CAZ 30 μg), cefpodoxime (3rd generation cephalosporin; CPD 10 μg) and ceftriaxone (3 rd generation cephalosporin; CRO 30 μg) discs by the ADDT at Mueller Hinton Agar (MHA) (incubation at 37 °C for 18 hrs in aerobic condition) [22]. Isolates resistant to at least one of these antibiotics were reserved for further tests.

### Phenotypic detection of ESBL-producing isolates

The combination disc test was used as a CLSI-recommended phenotypic confirmation test to determine whether *E. coli* (n= 7) and *K. pneumoniae* isolates (n= 1) (ceftiofur resistant) have an ESBL phenotype [22]. In the test, *K. pneumoniae* ATCC 700603 was used as a positive control and *E. coli* ATCC 25922 was used as a negative control strain, as recommended by CLSI [22].

Similarly, combination disc test with ceftazidime (CAZ, 30 μg) and ceftazidime-clavulanate (CAZ-CLA, 30/10 μg) discs was used to determine ESBL-producing *P. aeruginosa* isolates (n= 7) [22]. As a second phenotypic test for the determination of ESBL-producing *P. aeruginosa,* double disc synergy test was applied with a disc containing imipenem (IPM 10 μg) as the ESBL inhibitor and with ceftazidime (CAZ 30 μg) and cefepim (FEP 30 μg) discs [25, 26]. Both disc diffusion tests were performed on MHA and with suspensions of *P. aeruginosa* isolates at 0.5 McFarland turbidity.

### Phenotypic determination of AmpC - producing isolates

For the detection of presumptive AmpC producers, suspensions of *E. coli* (n= 7) and *K. pneumoniae* isolates (n= 1) (ceftiofur resistant) with 0.5 McFarland turbidity were tested by ADDT using cefoxitin (FOX 30 μg) disc [27]. Isolates forming an inhibition zone diameter of 17 mm and narrower were recorded as resistant [28]. As a second test, ADDT was repeated with discs containing the combination of cefoxitin-boronic acid (FOX-BA 30/400 μg) to phenotypically reveal AmpC production in cefoxitin-resistant isolates [29]. The formation of an inhibition zone 5 mm and wider around the FOX-BA (430 μg) disc than the inhibition zone around the FOX (30 μg) disc was considered as evidence of AmpC production [29]. These phenotypic test were not

performed for *P. aeruginosa* isolates (n= 7) since *P. aeruginosa* is intrinsically resistant to cefoxitin.

## PCR for AmpC and ESBL genes

### DNA extraction

One ml suspension of AmpC or ESBL-producing *E. coli*, *K. pneumoniae* and *P. aeruginosa* at 5.0 McFarland turbidity were prepared in sterile double distilled water and frozen at -20 °C for one hr. Then the suspensions were boiled at 100 °C for 10 min. After cooling, the suspensions were centrifuged at 14,000 rpm for 10 min, and 50 µl of the supernatant from each isolate was stored at -20 ºC as template DNA.

### ESBL genes

CTX-M, TEM and SHV class β-lactamase genes were investigated in *E. coli* and *K. pneumoniae* isolates confirmed phenotypically as ESBL producers with the primer pairs taken from Heffernan et al. [30] and Jeong et al. [31], and with the cycling conditions that we established in our previous study (Table 1) [32].

**Table 1.** PCR cycling conditions for the detection of AmpC and ESBL genes [32]

| Target gene | | Initial denaturation | Cycle | | | | Final elangation |
|---|---|---|---|---|---|---|---|
| | | | X | Denaturation | Primer annealing | Elongation | |
| TEM | | 94ºC-5 min | 35 | 94ºC-1 min | 48ºC-1 min | 72ºC-1 min | 72ºC-10 min |
| SHV | | 94ºC-5 min | 35 | 94ºC-30 sec | 58ºC-30 sec | 72ºC-1 min | 72ºC-7 min |
| CTX-M | Gp1 | 94ºC-5 min | 35 | 94ºC-1 min | 55ºC-1 min | 72ºC-1 min | 72ºC-7 min |
| | Gp2 | 94ºC-5 min | 35 | 94ºC-1 min | 55ºC-1 min | 72ºC-1 min | 72ºC-7 min |
| | Gp8 | 94ºC-5 min | 35 | 94ºC-1 min | 55ºC-1 min | 72ºC-1 min | 72ºC-7 min |
| | Gp9 | 94ºC-5 min | 35 | 94ºC-1 min | 55ºC-1 min | 72ºC-1 min | 72ºC-7 min |
| | Gp25 | 94ºC-5 min | 35 | 94ºC-1 min | 55ºC-1 min | 72ºC-1 min | 72ºC-7 min |
| pAmpC | | 94ºC-5 min | 35 | 94ºC, 45 sec | 64ºC, 45 sec | 72ºC-1 min | 72 ºC-7 min |
| cAmpC | | 94ºC-5 min | 30 | 94ºC-1 min | 48ºC-1 min | 72ºC-1 min | 72ºC-7 min |

X: number of cycles, Gp: Group, min: minute, sec: second, pAmpC: plasmidic AmpC, cAmpC: chromosomal AmpC

### Plasmidic AmpC genes

The presence of plasmidic *ampC* genes in one *E. coli* isolate confirmed as AmpC producer by phenotypic tests and *P. aeruginosa* isolates (n= 7) was investigated based on standard multiplex PCR performed with six primer pairs, developed by Perez-Perez and Hanson [5]. The protocol was modified as two separate triplex PCR tests (A and B) for better separation and visualization of amplicon bands after electrophoresis. Master mix-A contained ACC, CIT and FOX specific primer pairs and master mix-B

contained DHA, EBC, MOX specific primer pairs. The PCR amplification mixes were in accordance with Perez-Perez and Hanson [5]. The content of triplex PCR-A was PCR 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 μM ACC primers, 0.6 μM CIT primers, 0.4 μM FOX primers and 1.25 U of Taq polymerase. The ingredients of triplex PCR-B was 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.6 μM DHA primers, 0.5 μM EBC primers, 0.6 μM MOX primers and 1.25 U of Taq polymerase. One μl template DNA of the isolates was added to 24 μL of the PCR master mixes [5]. PCR cycling condition is presented in Table 1.

### Determination of mutations in chromosomal AmpC gene in *P. aeruginosa* isolate

*P. aeruginosa* has the chromosomal *ampC* gene. AmpC production in *P. aeruginosa* is inducible and also is produced continuously at low level, but mutations in choromosomal *ampC* gene cause overexpression of chromosomal *ampC* gene leading to higher resistance to β-lactams. Therefore, in one *P. aeruginosa* isolate (PA-20), the chromosomal *ampC* gene as a whole (1243 bp) was amplified by using PreAmpC-PA1 (5'-ATGCAGCCAACGACAAAGG-3') and PostAmpC-PA2 (5'-CGCCCTCGCGAGCGCGCTTC-3') primers in PCR [33] with the PCR cycling conditions presented in Table 1.

### Sequence Analysis

In the present study, all PCR amplicons were detected in 1.0 % agarose gel containing nontoxic DNA dye (ABM Safeview) in 1X Tris-borate-EDTA (TBE) at 85 V for one hr; the bands were then visualized under UV ligth of gel imaging system. All amplicons were sequenced in both directions by an external service (BM labosis, Ankara, Turkey). The obtained sequences were analysed using FinchTV (Version 1.4.0)(Geospiza, USA) and BLAST (the basic local alignment search tool)(US National Library of Medicine, USA) searches were performed on the NCBI (National Center of Biotechnology Information) databases. The sequences of ESBL and plasmidic *ampC* gene specific amplicons detected in *E. coli* and *K. pneumoniae* isolates were analysed for variants of the genes. Chromosomal *ampC* gene of one *P. aeruginosa* isolate (PA-20) was compared with *ampC* gene of the control strain *P. aeruginosa* PAO1 (in the NCBI database) to determine the ampC variant. Additionally, it was checked whether the detected mutations were the same as those identified in *P. aeruginosa* by Rodriguez-Martinez et al. [33].

### Determination of susceptibility to other classes of antibiotics

The susceptibility of the isolates to aminoglycosides, folate pathway inhibitors, quinolones, phenicols and tetracyclines which are commonly used in veterinary medicine, were determined by ADDT by preparing suspensions of AmpC-/ESBL-

producing *E. coli* and *K. pneumoniae* isolates at a turbidity of 0.5 McFarland [28]. The following antibiotics were tested (Oxoid, UK): gentamicin (GEN 10 μg), kanamycin (KAN 30 μg), streptomycin (STR 10 μg), sulphamethoxasole-trimethoprim (SXT 25 μg), ciprofloxacin (CIP 5 μg), enrofloxacin (ENR 5 μg), nalidixic acid (NAL 30 μg), chloramphenicol (CHL 30 μg), florfenicol (FFC 30 μg), oxytetracyclin (OT 30 μg) and imipenem (IPM, 10 μg). In the evaluation of the inhibition zone diameters, the updated documents of CLSI presented in Table 2 were used and the isolates were recorded as susceptible, intermediate or resistant [22, 28, 34]. Isolates resistant to antibiotics from three or more classes were recorded as multidrug-resistant (MDR) isolates. In identifying isolates as MDR, an isolate with intermediate resistance to an antibiotic was counted as resistant. Since *P. aeruginosa* is mostly resistant intrinsically to the classes of antibiotics above, we did not perform ADDT for *P. aeruginosa* isolates.

**Table 2.** CLSI documents used for the evaluation of Enterobacterales isolates in ADDT

| Disc content | CLSI document for Enterobacterales |
|---|---|
| GEN | Vet01S ED5:2020 |
| STR | Vet01S ED5:2020 |
| KAN | Vet01S ED5:2020 |
| CIP | CLSI M100-ED30:2020 |
| ENR | Vet01S ED5:2020 |
| NAL | CLSI M100-ED30:2020 |
| TET | Vet01S ED5:2020 |
| SXT | Vet01S ED5:2020 |
| FFC | Vet01-S2 |
| CHL | Vet01S ED5:2020 |
| IPM | Vet01S ED5:2020 |

## RESULTS

Bacteriological cultivation of lung, liver and spleen of each of four animals (three calves and one lamb) resulted in *E. coli* isolation. Antibiotic susceptibility profiles of a total of three *E. coli* (one from each of the liver, lung and spleen) from an animal were the same. Therefore, one *E. coli* strain was considered isolated from lung, liver and spleen tissue of each calf and lamb, and thus the study was continued with a total of four *E. coli* strains from the internal organs (lung, liver and spleen) of three calves and one lamb. From the cow milk samples, *E. coli* was isolated from one sample and *K. pneumoniae* from the other. Cultures of remaining four cow milk samples resulted in *P. aeruginosa* (n= 4) isolation. Microorganisms isolated from two dog urine samples were identified as *E. coli* (n= 2). From dog ear swab samples (n=3), three *P. aeruginosa* were isolated. All bacteria (7 *E. coli*, 1 *K. pneumoniae* and 7 *P. aeruginosa*) in the study showed growth as pure cultures in the first bacteriological cultivation made directly from

the specimens on BA and MCA. Enterobacterales isolates in the study were found resistant to ceftiofur in the routine antibiotic susceptibility test (ADDT) performed in the laboratory.

In CLSI initial screening test, all *E. coli* and *K. pneumoniae* isolates (n= 8) were found to be resistant to ATM, CPD, CRO and CTX. For CAZ, two isolates (EC-2 and EC-43) were susceptible, and the remaining six isolates were resistant (Table 3).

**Table 3.** Enterobacterales isolates

| Isolate Code | Gram (-) isolate | Specimen | Multidrug resistance pattern* | AmpC / ESBL gene |
|---|---|---|---|---|
| EC-2 | *E. coli* | Calf internal organs, 3.5 months of age | AMP, CF, CXM, ATM, CTX, CRO, CPD, GEN, STR, KAN, CIP, ENR, NAL, OT, SXT | CTX-M-15** |
| KP-6 | *K. pneumoniae* | Milk (mastitis), Cow | CF, CXM, ATM, CTX, CAZ, CRO, CPD, GEN, STR, KAN, CIP, ENR, NAL, OT, CHL, FFC, SXT | CTX-M-15** |
| EC-11 | *E. coli* | Lamb internal organs, 20 days of age | AMP, CF, CXM, ATM, CTX, CAZ, CRO, CPD, STR, ENR, OT, SXT | CTX-M-15** |
| EC-14 | *E. coli* | Calf internal organs, 4 days of age | AMP, CF, CXM, ATM, CTX, CAZ, CRO, CPD, GEN, STR, KAN, CIP, ENR, NAL, OT, CHL, FFC, SXT | CTX-M-15** |
| EC-25 | *E. coli* | Dog urine (UTI) | AMP, CF, CXM, ATM, CTX, CAZ, CRO, CPD, GEN, STR, KAN, CIP, ENR, NAL, OT | CTX-M-15 |
| EC-35 | *E. coli* | Calf internal organs, 2 days of age | AMP, CF, CXM, ATM, CTX, CAZ, CRO, CPD, GEN, KAN, NAL, OT, SXT | CTX-M-15 |
| EC-43 | *E. coli* | Milk (mastitis), cow | AMP, CF, CXM, ATM, CTX, CRO, CPD, GEN, STR, KAN, CIP, ENR, NAL, OT, CHL, FFC, SXT | CTX-M-3** |
| EC-3 | *E. coli* | Dog urine (UTI) | AMP, CF, CXM, ATM, CTX, CAZ, CRO, CPD, GEN, STR, KAN, CIP, ENR, NAL, OT, CHL, FFC, SXT | CIT (cmy-2)** |

*Multidrug resistant isolate (aminoglicosids, beta lactams, folate pathway inhibitors, phenicols, quinolones and tetracyclines). ATM (aztreonam), CTX (cefotaxime), CAZ (ceftazidime), CPD (cefpodoxime), CRO (ceftriaxone), CHL (chloramphenicol), CIP (ciprofloxacin), ENR (enrofloxacin), FFC (florfenicol), GEN (gentamicin), KAN (kanamycin), NAL (nalidixic acid), STR (streptomycin), SXT (sulfamethoxazole-trimethoprim), OT (oxytetracycline), UTI (urinary tract infection).
**In addtion to AmpC/ESBL genes, TEM-1 beta lactamase (non-ESBL) genes were detected.

According to combined disc test carried out for the confirmation of ESBL production in seven ceftiofur-resistant *E. coli* and one ceftiofur-resistant *K. pneumoniae*, six *E. coli* and *K. pneumoniae* were found to be ESBL producers. One *E. coli* (EC-3) isolated from dog urine sample was negative for ESBL production but it was confirmed as an AmpC producer according to the ADDT performed with FOX and FOX/BA. Other seven isolates which were ESBL producers were FOX sensitive (negative for AmpC

production). In two phenotypic ESBL confirmation tests performed for *P. aeruginosa* isolates, all isolates were found to be negative for ESBL production.

The ESBL gene belonging to the CTX-M class (group 1) was detected in all of the seven ESBL-producing isolates (6 *E. coli* and *K. pneumoniae*). Five out of seven isolates carrying the CTX-M gene were also found to carry the β-lactamase gene belonging to the TEM class. β-lactamase gene from the SHV class was not found in any of the isolates. *E. coli* (EC-3), which was the AmpC-producing isolate, carried the plasmidic *ampC* gene belonging to the CIT family and TEM β-lactamase gene (Table 3). All of the remaining *E. coli*, *K. pneumoniae* and all *P. aeruginosa* isolates were determined to have no plasmidic *ampC* gene.

According to the result of the sequence analysis of PCR amplicons, it was seen that one (14.3 %) of six CTX-M genes were CTX-M-3 variant and other five (85.7 %) were CTX-M-15 variant. It was determined that all of the TEM genes in the ESBL-producing isolates were TEM-1 variant (encoding none ESBL) and thus, it was determined that the ESBL phenotype in the isolates originated only from the CTX-M genes (CTX-M-3 or CTX-M-15). According to the result of the sequence analysis, CIT family β-lactamase gene of *E. coli* (EC-3) isolated from the dog urine sample was encoding cmy-2 variant and this isolate had also TEM-1 variant β-lactamase (Table 3).

All Enterobacterales isolates in the study were found to be resistant to three or more classes of antibiotics, and therefore they had the MDR phenotype, according to the results of aminoglycosides, folate pathway inhibitors quinolones, phenicols and tetracyclines resistance in addition to β-lactams resistance. Three different MDR phenotypes were determined in the isolates. One of the isolates was resistant to at least one antibiotic from each of aminoglicosides, β-lactams, quinolones and tetracyclines classes, three of them were resistant to at least one antibiotic from each of aminoglicosides, β-lactams, folate pathway inhibitors, quinolones and tetracyclines classes and five of them were resistant to at least one antibiotic from each of aminoglicosides, β-lactams, folate pathway inhibitors, phenicols and quinolones classes (Table 3).

Amplification and sequencing of chromosomal *ampC* gene in one *P. aeruginosa* isolate (PA-20) revealed the changes in the 79'th and 105'th amino acids. These mutations were the substitution of glutamine (Q) for arginine (R) at position 79 and alanine (A) for threonine (T) at position 105.

## DISCUSSION

World Health Organization (WHO) announced that ESBL-producing Enterobacterales are creating a life threatening problem for human health due to increase in isolation rates from clinical cases [35]. Similarly in veterinary medicine, the number of reports is increasing on these bacteria from diseased livestock and companion animals.

As first-line treatment options ampicillin and amoxicillin and few 1'st and 2'nd generation cephalosporins are used in the treatment of animal infections, but an increasing trend in resistance to them has been observed. To overcome this problem, extended-spectrum cephalosporins, such as ceftiofur (3rd geneation cephalosporin) and cefquinome (4th generaton cephalosporin) have been approved to be used for the treatment of animal infections in Turkey and other countries, but unfortunately the increasing trend of incidence of AmpC-/ESBL-producing Gram-negative isolates have been making those antibiotics inefficient for the treatments in the veterinary field. In the present study, one CTX-M-15 variant ESBL-producing *K. pneumoniae* (KP-6) and one CTX-M-3 variant ESBL-producing *E. coli* (EC-43) (both MDR phenotype) were isolated from two cow mastitic milk samples. This low incidence has been found to be consistent with other research results around the world. In a study conducted in South Korea (covering the years 2012 and 2015), 15 *E. coli* isolates were found to be resistant to ceftiofur from bovine mastitic milk and CTX-M gene (5 CTX-M-15, 4 CTX-M-3 and 3 CTX-M-1 genes) were identified in 12 of them [36]. In a study conducted in China (covering the years 2015-2016), 46 ESBL-producing *E. coli* were isolated from 1440 milk samples (69 farms across China) collected from cows with mastitis. CTX-M-15 was detected in 29 isolates, and CTX-M-3 was detected in two isolates [37].

In the present study, we isolated the multidrug resistant *E. coli* producing CTX-M-15 variant ESBL (EC-2, EC-11, EC-14 and EC-35) from the internal organs (liver, lung and spleen) of three calves and one lamb. In these cases, we did not fully confirm that *E. coli* strains isolated from the internal organs of three calves and one lamb were the primary cause of the clinical problem in the animals because we did not exclude other pathogens which could be responsible for the cases. However, obtaining the same isolate from all internal organs of an animal gave clear evidence that *E. coli* septicemia had been formed in the animals. Therefore, it was valuable to include these isolates into the present study. As a matter of fact, the fact that the age of the animals was less than 4 months shown us that *E. coli* septicemia could have been developed in the animals. When considered the MDR profiles of the *E. coli* isolates, it was clear that they had caused health problems with little treatment options for 3 calves and one lamb. In the same way, Singh et al. [38] reported the isolation of ESBL-producing *E. coli* from septicemic sheep and goat lung tissues in India.

In our study, we isolated two *E. coli* from two dogs that had UTI, one producing CTX-M-15 variant ESBL (EC-25) and the other producing cmy-2 variant AmpC β-lactamase (EC-3). We can say that this result is consistent with the results of the studies conducted in Asian and European countries. Indeed, Wagner et al. [16] detected 15 CIT family plasmidic AmpC-producing *E. coli* with MDR phenotypes as a result of screening urine samples taken from canine UTIs between 2002-2011 in the veterinary hospital, University of Edinburg. Huber et al. [19] detected five ESBL-producing *E. coli* from urine samples of dogs admitted to the clinics at the University of Zurich in Switzerland between March 2010 and December 2011. Valat et al. [17] analyzed 403

uropathogenic *E. coli* collected from all over France (January-November 2017) and detected 12 AmpC-producing *E. coli* (all cmy-2 carriers) and 13 ESBL-producing *E. coli* (9 CTX-M-15 and 1 CTX-M-3). Marques et al. [21], in their retrospective study (a 16-year study covering the years 1999-2014), isolated a total of eight cmy-2 variant plasmidic AmpC-producing *E. coli* (MDR phenotype) and six CTX-M-15 variant β-lactamase-producing *E. coli* from canine UTIs. Kuan et al. [20] isolated two ESBL-producing (CTX-M-15 producer) and multi-antibiotic-resistant *E. coli* out of 52 *E. coli* isolates from dogs with UTI, in Taipei (Taiwan) (between December 2011-March 2013). In the present study, *E. coli* isolates EC-25 and EC-3 carrying CTX-M-15 and cmy-2 β-lactamase genes respectively demonstrated the presence of clinically significant *E. coli* causing canine UTIs in the dog population in our region. MDR phenotypes of these isolates show that treatment options for the cases were limited since isolate EC-25 resistant to aminoglicosides, quinoles and oxytetracyclines, and isolate EC-3 resistant to aminoglicosides, quinolones, oxytetracycline, folate pathway inhibitors and fenicols. Taking into account the increased incidence of MDR and AmpC-/ESBL-producing *E. coli* isolates shown by other studies [16,21], we can say that veterinarians will need to use more the antibiotics developed for human use in treatment of UTIs in companion animals.

There are studies showing that the prevalence of *E. coli* producing cmy-2 variant β-lactamase is increasing in clinical cases of animals [21]. It has been known that cmy-2 β-lactamase production can lead to weak carbapenem resistance along with porin deficiency [21,39]. Therefore, the fact that *E. coli*, which produces cmy-2 β-lactamase in our study, caused UTI is also of clinical importance in this respect.

Resistance to extended-spectrum cephalosporins in *P. aeruginosa* isolates is caused by either ESBL production or overproduction of AmpC β-lactamases and excessive operation of the efflux pump system [7,40]. In this study, it was determined that there was no ESBL production in seven *P. aeruginosa* isolated from four cows with mastitis and three dogs with otitis externa. In our study, the whole sequence of the chromosomal *ampC* gene of one *P. aeruginosa* isolate (PA-20), which was isolated from a dog with otitis externa, was analysed and it was observed that there were two mutations causing changes in amino acid sequence at the 79th and 105th position of the protein. The mutations were the substitution of glutamine (Q) for arginine (R) at position 79 and alanine (A) for threonine (T) at position 105. Rodriguez-Martinez et al. [33] initiated the classification of chromosomal ampC variants (Pseudomonas-derived cephalosporinase, PDC) and numbered the ampC variant containing R79Q and T105A changes as PDC-5 variant. Up to date, 466 variants have been characterized in ampC protein of *P. aeruginosa* [41]**.** Rodriguez-Martinez et al. [33] stated that when the *ampC* gene containing the R79Q and T105A mutations was transferred experimentally to ampC-deficient *P. aeruginosa* mutant strain, the MIC values of the *P aeruginosa* isolate to ceftazidime, imipenem and meropenem increased due to ampC enzyme overproduction. Therefore, the presence of these mutations in the PA-20 isolate in our study may indicate the sligth overproduction of ampC enzyme and may indicate

that the enzyme's catalytic activity is increased against β-lactams. However, to be able to make the same judgment for other six *P. aeruginosa* isolates in the study, sequencing of their chromosomal *ampc* genes are needed.

In an overview, studies conducted in different parts of the world show that the prevalence of Enterobacterales, which is resistant to multiple antibiotics and produces CTX-M β-lactamase, is increasing. Similarly, in the present study, we identified the CTX-M gene in all ESBL-producing Enterobacterales isolates (n=7). SHV and TEM class ESBL genes were not determined in any of the isolates.

When examined the distribution of CTX-M gene variants in Enterobacterales strains isolated from clinical cases of livestock and companion animals, it is seen that CTX-M-15 is the most common CTX-M variant identified in all continents [42]. Similarly, among the CTX-M genes in this study, the CTX-M-15 variant was the most detected; CTX-M-3 variant was detected in one isolate (EC-43) only. The presence of CTX-M-3 β-lactamase has also been demonstrated in Enterobacterales isolates that infect domestic animals in European and Asian countries [42]. However, its prevalence is much lower than CTX-M-15, as in our results [36,37].

The clinical effect of the infections caused by AmpC-/ESBL-producing Enterobacterales strains in domestic animals is not yet fully known. There are large-scale studies on this subject on humans, and it is seen that the mortality rate increases due to the delay of the necessary treatment due to empirical antibiotic applications [43]. It is not difficult to predict that the same scenario occurs in animals, but there is no research data on this subject. As a matter of fact, *E. coli* isolates from one lamb and three calves in our study were isolated from deceased animal necropsy material and no response had been obtained from antibiotic administrations in the cases in the field. Likewise, one of the dogs whose urine samples were taken died shortly after isolation of ESBL-producing *E. coli*. The mastitic milk samples of two cows from which ESBL-producing *E. coli* and *K. pneumoniae* were isolated were submitted to our laboratory by veterinarians, due to the ineffectiveness of different antibiotic applications in the field. Therefore, it is important to investigate the presence of AmpC-/ESBL in Gram-negative pathogens that cause diseases in livestock, to control these pathogens and to apply appropriate treatment without delay. Doing so will prevent/slow the spread of these pathogens, as well as reduce the economic losses from animal deaths and ineffective empirical antibiotic treatments.

We continue routine screening in our laboratory to identify Gram-negative isolates producing AmpC/ESBL from clinical cases of local animals. The low local incidence in the study may be due to the low number of samples sent to the laboratory and not representing the whole population. Actual incidence may be much higher. However, in general, we can say that this article points to the potential dangers of Enterobacterales and *P. aeruginosa* infections. Infections from such bacteria will continue to be an increasingly clinically important and therapeutically challenging problem in veterinary medicine.

Our next goal will be to test if ceftiofur resistance can be an efficient indicator for selective isolation of ESBL-producing Enterobacterales from animals because ceftiofur is available only for veterinary use and it has been used in antibiogram tests in routin laboratories in the veterinary field.

**Authors' contributions**

FP designed the study, isolated the microorganisms from clinical cases, performed the all antibiogram tests - polymerase chain reaction tests, performed the evaluation of the results of the tests and writing the manuscript. ES participated in the isolation of microorganisms, participated in the antibiogram tests and polymerase chain reaction tests.

**Declaration of conflicting interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## REFERENCES

1. Bradford PA: Extended-spectrum β-lactamases in the 21th century: Characterisation, epidemiology, and detection of this important resistance threat. Clin Microbiol Rev 2001, 14: 933-951.
2. Jacoby GA: AmpC β-lactamases. Clin Microbiol Rev 2009, 22 (1): 161-182.
3. Philippon A, Arlet G, Jacoby GA: Plasmid-determined AmpC-type β-lactamases. Antimicrob Agents Chemother 2002, 46(1): 1-11.
4. Sadeeq R, Tariq A, Ijaz A, Nazir AK, Bo H, Jian G: The growing genetic and functional diversity of extended spectrum beta lactamases. Biomed Res Int 2018, March 26: 9519718.
5. Perez-Perez FJ, Hanson ND: Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. J Clin Microbiol 2002, 40: 2153-2162.
6. Poole K: Pseudomonas aeruginosa: Resistance to the max. Front Microbiol 2011 (April 5), 2: 65.
7. Berrazeg M, Jeannot K, Ntsogo Enguéné VY, Broutin I, Loeffert S, Fournier D, Plésiat P: Mutations in β-lactamase AmpC increase resistance of *Pseudomonas aeruginosa* isolates to antipseudomonal cephalosporins. Antimicrob Agents Chemother 2015, 59: 6248-6255.
8. Paterson DL: Recommendation for treatment of severe infections caused by Enterobacteriaceae producing extended-spectrum β-lactamases (ESBLs). Clin Microbiol Infect 2000, 6: 460-463.
9. Ali T, ur Rahman S, Zhang L, Shahid M, Zhang S, Liu G, Gao J, Han B: ESBL-producing Escherichia coli from cows suffering mastitis in China contain clinical class 1 integrons with CTX-M linked to ISCR1. Front Microbiol 2016, 7: 1931.
10. Saidani M, Messadi L, Soudani A, Daaloul-Jedidi M, Chatre P, Chehida FB, Mamlouk A, Mahjoub W, Madec J-Y, Haenni M: Epidemiology, antimicrobial resistance, and entended-

spectrum β-lactamase-producing Enterobacterales in clinical bovine mastitis in Tunisa. Microb Drug Resist 2018, 24 (8): 1242-1248.

11. Ghatak S, Singha A, Sen A, Guha C, Ahuja A, Bhattacharjee U, Das S, Pradhan NR, Puro K, Jana C, Dey TK, Prashantkumar KL, Das A, Shakuntala I, Biswas U, Jana PS: Detection of New Delhi Metallo-β-lactamase and extended-spectrum β-lactamase genes in Escherichia coli isolated from mastitic milk samples. Transbound Emerg Dis 2013, 60: 385-389.
12. Timofte D, Maciuca IE, Evans NJ, Williams H, Wattret A, Fick JC, Willams NJ: Detection and molecular characterisation of Escherichia coli CTX-M-15 and Klebsiella pneumoniae SHV-12 β lactamases from bovine mastitis isolates in the United Kingdom. Antimicrob Agents Chemother 2014, 58 (2): 789-794.
13. Eisenberger D, Carl A, Balsliemke J, Kampf P, Nickel S, Schulze G, Valenza G: Molecular characterisation of extended-spectrum β-lactamase-producing Escherichia coli isolates from milk samples of dairy cows with mastitis in Bavaria, Germany. Microb Drug Resist 2018, 24 (4): 505-510.
14. Pehlivanoglu F, Turutoglu H, Ozturk D: CTX-M-15-type extended-spectrum β-lactamase-producing Escherichia coli as causative agent of bovine mastitis. Foodborne Pathog Dis 2016, 13 (9): 477-482.
15. Locatelli C, Scaccabarozzi L, Pisoni G, Moroni P: CTX-M1 ESBL-producing *Klebsiella pneumoniae* subsp. *pneumoniae* isolated from cases of bovine mastitis. J Clin Microbiol 2010, 48: 3822-3823.
16. Wagner S, Gally DL, Argyle SA: Multidrug-resistant Escherichia coli from canine urinary tract infections tend to have commensal phylotypes, lower prevalence of virulence determinants and ampC-replicons. Vet Microbiol 2014, 169 (3-4) :171-178.
17. Valat C, Drapeau A, Beurlet S, Bachy V, Boulouis H-J, Pin R, Cazeau G, Madec J-Y, Haenni M: Pathogenic Escherichia coli in dogs reveals the predominance of ST372 and the human-associated ST73 extra-intestinal lineages. Front in Microbiol 2020, 11: 580.
18. Pehlivanoglu F, Sababoglu E, Ozturk D, Turutoglu H: Characterisation of extended-spectrum β-lactamase genes of Klebsiella pneumoniae isolated from urinary tract infection of a dog. In: Proceedings of the 12th National Congress of Veterinary Microbiology; Kapadokya, Nevşehir, Türkiye; 2016. Pp. 19-20.
19. Huber H, Zweifel C, Wittenbrink WW, Stephan R: ESBL-producing uropathogenic Escherichia coli from dog and cats in Switzerland. Vet Microbiol 2013, 162 (2-4): 992-996.
20. Kuan NL, Chang CW, Lee CA, Yeh KS: Extended-spectrum β-lactamase producing Escherichia coli and Klebsiella pneumoniae isolates from the urine of dogs and cats suspected of urinary tract infection in a Veterinary teaching hospital. Taiwan Vet J 2016, 42 (3): 143-148.
21. Marques C, Belas A, Franco A, Aboim C, Gama LT, Pomba C: Increase in antimicrobial resistance and emergence of major international high-risk clonal lineages in dogs and cats with urinary tract infection: 16 year retrospective study. J Antimicrob Chemother 2018, 73 (2): 377-384.
22. CLSI: Performans standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. 5th edition. CLSI document Vet01S. Wayne, PA: Clinical and Laboratory Standards Institute; 2020.
23. Winn W, Allen S, Janda W, Koneman E, Procop G, Schreckenberger P, Woods G: *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*. 6th edition, Philadelphia, USA: Lippincott Williams and Wilkins; 2006.

24. Weisburg WG, Barns SM, Pelletier DA, Lane DJ: 16S ribosoma DNA amplification for phylogenetic study. J Bacteriol 1991, 173 (2): 697-703.
25. Laudy AE, Róg P, Smolińska-Król K, Ćmiel M, Słoczyńska A, Patzer J, Dzieerzanowska D, Wolinowska R, Starosciak B, Tyski S: Prevalence of ESBL-producing *Pseudomonas aeruginosa* isolates in Warsaw, Poland, detected by various phenotypic and genotypic methods. Plos One 2017, 12 (6): e0180121.
26. Weldhagen GF, Poirel L, Nordmann P: Ambler class A extended-spectrum β-lactamases in Pseudomonas aeruginosa: novel development and clinical impact. Antimicrob Agents Chemother 2003, 47 (8): 2385-2392.
27. Polsfuss S, Bloemberg GV, Giger J, Meyer V, Böttger EC, Hombach M: Practical approach for reliable detection of AmpC β-lactamase producing *Enterobactericeae*. J Clin Microbiol 2011, 49 (8): 2798-2803.
28. CLSI: Performance standards for antimicrobial susceptibility testing, 30th edition. CLSI Supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2020.
29. Coudron PE: Inhibitor based methods for detection of plasmid mediated AmpC β-lactamases in Klebsiella spp., Escherichia coli, and Proteus mirabilis J Clin Microbiol 2005, 43 (8): 4163-4167.
30. Heffernan HM, Woodhouse RE, Pope CE, Blackmore TK: Prevalence and types of extended-spectrum β-lactamases among urinary *Escherichia coli* and Klebsiella spp. in New Zealand. Int J Antimicrob Agents 2009, 34: 544-549.
31. Jeong SH, Bae IK, Kwon SB, Lee JH, Song JS, Jung HI, Sung KH, Jang SJ, Lee SH: Dissemination of transferable CTX-M-type extended-spectrum β-lactamase-producing *E. coli* in Korea. J Appl Microbiol 2005, 98: 921–927.
32. Pehlivanoglu F, Turutoglu H, Ozturk D, Yardimci H: Molecular characterisation of ESBL-producing Escherichia coli isolated from healthy cattle and sheep. Acta Vet- Beograd 2016, 66 (4): 520-533.
33. Rodrigues-Martines JM, Poirel L, Nordmann P: Extended-spectrum cephalosporinases in P. aeruginosa. Antimicrob Agents Chemother 2009; 53 (5): 1766-1771.
34. CLSI: Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. 4th edition. CLSI document VET01-A4 and VET01-S2, Wayne, PA: Clinical and Laboratory Standards Institute; 2013.
35. WHO (World Health Organisation): Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. https://www.who.int/publications
36. Tark D-S, Moon DC, Kang HY, Kim S-R, Nam H-M, Lee H-S, Jung S-C, Lim S-K: Antimicrobial susceptibility and characterization of extended-spectrum β-lactamases in Escherichia coli isolated from bovine mastitic milk in South Korea from 2012 to 2015. J Dairy Sci 2017, 100 (5): 3463-3469.
37. Ali T, ur Rahman S, Zhang L, Shahid M, Han D, Gao J, Zhang S, Ruegg PL, Saddique U, Han B: Characteristics and genetic diversity of multi-drug resistant extended-spectrum β-lactamase (ESBL)-producing Escherichia coli isolated from bovine mastitis. Oncotarget 2017, 8 (52): 90144-90163.
38. Singh F, Sonawane GG, Kumar J, Dixit SK, Meena RK, Tripathi BN: Antimicrobial resistance and phenotypic and molecular detection of extended-spectrum ß-lactamases among extraintestinal *Escherichia coli* isolated from pneumonic and septicemic sheep and goats in Rajasthan, India. Turk J Vet Anim Sci 2019, 43: 754-760.

39. Chia JH, Siu LK, Su LH, Lin HS, Kuo AJ, Lee MH, Wu TL: Emergence of carbapenem-resistant *Escherichia coli* in Taiwan: Resistance due to combined CMY-2 production and porin deficiency. J Chemother 2009, 21(6): 621-626.
40. Lister PD, Wolter DJ, Hanson ND: Antibacterial-resistant *Pseudomonas aeruginosa*: Clinical impact and complex regulation of chromosomally encoded resistance mechanismis. Clin Microbiol Rev 2009, 22: 582-610.
41. Naas T, Oueslati S, Bonnin RA, Dabos ML, Zavala A, Dortet L, Retailleau P, Iorga BI: B-lactamase database (BLDB)-structure and function. J Enzyme Inhib Med Chem 2017, 32 (1): 917-919.
42. Palmeira JD, Ferreira HMN: Extended-spectrum β-lactamase (ESBL)- producing Enterobactericeae in cattle production- a threat around the world. Heliyon 2020, 6 (1): e03206.
43. Schwaber MJ, Carmeli Y: Mortality and delay in effective therapy associated with extended-spectrum β-lactamase production in Enterobacteriaceae bacteraemia: a systematic review and meta-analysis. J Antimicrob Chemother 2007, 60 (5): 913-920.

## KARAKTERIZACIJA AmpC /ESBL GENA KOD NEKIH PATOGENIH GRAM NEGATIVNIH BAKTERIJSKIH IZOLATA IZ KLINIČKIH SLUČAJEVA KOD DOMAĆIH I SOCIJALNIH VRSTA ŽIVOTINJA

Faruk PEHLİVANOGLU, Ezgi SABABOGLU

Cilj studije je bio da se ispitaju i karakterizuju AmpC i/ili ESBL geni *Escherichia coli*, *Klabsiella pneumoniae* i *Pseudomonas aeruginosa*, izolovanih iz lokalnih kliničkih slučajeva oboljevanja domaćih i socijalnih vrsta životinja u periodu od 2017. do 2019. godine. Iz uzoraka poreklom od lokalnih životinja, ukupno je izolovano osam ceftiofur-rezistentnih sojeva *E. coli* (n=7), jedan ceftiofur-rezistentan soj *K. pneumoniae* i sedam izolata *P. aeruginosa*. Kombinacijom disk metode, šest izolata *E. coli* i jedan *K. pneumoniae* izolati su bili proizvođači ESBL. Kombinacijom disk metode i dvostrukog sinergijskog disk testa, ni jedan izolaz *P. aeruginosa* nije proizvodio ESBL. Upotrebom agar disk difuzionog testa (ADDT), uz korišćenje cefoxitin-a i cefoxitin-boronik-a, samo je jedan izolat *E. coli* pronađen kao AmpC-proizvođač. Kod izolata koji su proizvodili ESBL, primenom lančane reakcije polimeraze (PCR) detektovana je samo CTX-M klasa gena, a kasnijom analizom sekvence, ustanovljene su CTX-M-3 i CTC-M-15 varijante. Ustanovljeno je da AmpC pozitivan izolat *E. coli* poseduje ampC gen u formi plazmida u cmy-2 varijanti CIT familije. Uočeno je da izolati *P. aeruginosa* ne nose ampC gen u formi plazmida. Posle amplifikacije hromozomskog ampC gena iz jednog izolata *P. aeruginosa* (primenom PCR i sekvencioniranjem), pronađene su R79Q i T105A mutacije u hromozomskom ampC genu. Ovi rezultati pokazuju da je preterana proizvodnja ampC enzima kod izolata *P. aeruginosa* u studiji, uključena u rezistenciju na β-laktamske antibiotike.