First detection of carbapenem resistance in *Enterobacteriaceae* isolates isolated from dairy cows’ mastitis infection in Türkiye

Orkun BABACAN

Department of Veterinary, Kepsut Vocational School, Balıkesir University, Kepsut, Balıkesir, Türkiye

ORCID: 000-0003-0258-1825

**ABSTRACT**

With this study, carbapenem resistance genes were declared for the first time in *Enterobacteriaceae* isolates isolated from dairy cows’ mastitis infection in Türkiye. In the bacteriological examination of 212 milk samples, 14 (6.60%) *E. coli*, three (1.41%) *Klebsiella oxytoca*, and two (0.94%) *Klebsiella pneumonia* were isolated. At least two *E. coli* isolates were found to be resistant to all of the antibiotics used in the antibiogram test. The highest resistance was found against cefotaxime and amoxicillin in *K. oxytoca* isolates. According to the results of PCR targeting *blaCTX-M*, *blaTEM*, and *blaSHV* genes, the *blaCTX-M* gene was detected in one *K. oxytoca* and four *E. coli* isolates, which were found ESBL positive. According to the results of PCR targeting carbapenem and colistin resistance genes, the IMP gene was detected in four *E. coli*, one *K. oxytoca*, and one *K. pneumonia* isolates. OXA-48-like gene was detected in two *E. coli* isolates. This two *E. coli* isolates were also IMP gene positive. While NDM gene was detected in two *E. coli*, KPC gene was detected in one *E. coli* isolate. One of the colistin resistance genes, *mcr-1* was detected in two *E. coli* strains with PCR. This study showed that ESBL production and carbapenem resistance in *Enterobacteriaceae* family strains to become prevalent and increasing, especially among *E. coli* isolates. Furthermore, identification of multiple antibiotic resistance in the isolates indicated that antibiotic resistance also spread rapidly and increased.

**Keywords**

Antimicrobial resistance
Carbapenem resistance
*E. coli*
Esbl
Mastitis

**Corresponding author**

orkun_babacan@hotmail.com

How to cite this article: Babacan O (2023): First detection of carbapenem resistance in *Enterobacteriaceae* isolates isolated from dairy cows’ mastitis infection in Türkiye. Ankara Univ Vet Fak Derg, 70 (1), 65-74. DOI: 10.33988/auvfd.828306.

Introduction

Multidrug resistance in *E. coli* has been known worldwide. Because mastitis is one of the most frequent infections that cause several economic losses particularly due to milk and milk quality loss and treatment expenses in dairies (30, 33, 54), antibiotics are the most common important treatment choice in mastitis infections of dairy cows (20, 34, 52, 54). However, as antibiotic use in animals produced to obtain human food, the presence of antibiotic residue in the milk and/or multiple antibiotic resistance (MDR) developing in bacteria may pass to drinking milk and dairy products, has great importance for food hygiene, safety, and public health (31, 34). In recent years, as a global problem in livestock and public health fields, it has been reported that the existence of *Enterobacteriaceae* strains, producing ESBL, especially *E. coli*, increases in the mastitis infections of dairy cows (35, 52). In agriculture and livestock breeding, the resistance developing against antibiotics which are also used in the treatment of humans has become a global problem for public health (34). It has been reported that the resistance developing rapidly against a new antibiotic active substance is one of the severe problems threatening public health (3, 57). A good antibiotic treatment is performed by administering an effective antibiotic active substance selected after determining the antibiotic susceptibility of the agents isolated from udder tissue and/or milk. However, as antibiotic treatment is started mostly without determining mastitis pathogens, it has been reported that the development of antibiotic resistance should be necessarily monitored. Although mastitis treatment is similar in all the countries across the world,
penicillin, aminopenicillins and their clavulanic acid combinations, and third and fourth-generation cephalosporins are mostly used in Europe (20, 54).

Antibiotic-resistant E. coli strains increase due to the spread of carbapenem-resistant Enterobacteriaceae (CRE) isolates, which have multiple drug resistance, these are causing treatment difficulties (50). Third and fourth-generation cephalosporins are stated as critical antimicrobials by the World Health Organization. In Enterobacteriaceae, resistance to cephalosporins is often associated with the production of extended-spectrum β-lactamases (ESBLs). Among others, ESBL producing E. coli strains spreads around the world in humans as well as livestock, and it’s easily spread is associated with several factors such as high virulence gene content, transfer of plasmids carrying ESBL gene, or exchange of genes encoding ESBL on mobile elements (24).

Extended-spectrum beta-lactamase (ESBL) enzymes are responsible for the hydrolysis of oxyimino-beta-lactam antibiotics used in the treatment of human and animal infections. ESBL was first declared in Enterobacteriaceae (Enterobacterales in the new taxonomy) in 1983 and since then ESBL-producing Enterobacteriaceae (E-ESBL) has become a great risk to human health. These bacteria were responsible for 1700 deaths in the USA due to treatment errors in most infections in 2013 (49). ESBL is one of the most known resistance mechanisms frequently observed in E. coli, Klebsiella, and Enterobacter spp. species included in the Enterobacteriaceae family (22). The major ESBL types determined in the species included in the Enterobacteriaceae family are TEM, SHV, and CTX-M. CTX-M, TEM, and SHV type β-lactamases are considered to be plasmid-related (7, 22).

Carbapenemases are beta-lactamases that hydrolyze penicillins, most often cephalosporins and varying degrees of carbapenems and monocobactams. Monobactams are not degraded by metallo-beta-lactamases. Carbapenemases are a source of concern as they cause resistance to all beta-lactams and can easily spread. Detection of these isolates is very important for infection control and public health (26).

Carbapenem-resistant genes such as blaKPC, blaNDM, blaIMP, blaOXA-48, and a plasmid-mediated mcr-1 gene that conferred colistin resistance in Enterobacteriaceae, specialize in more information worldwide. The spread of mcr-1 encoding plasmids among carbapenem-resistant Enterobacteriaceae raises concerns about the emergence of incurable bacterium and becomes a serious risk for public health worldwide. IMP carbapenemase is also common in some countries in the world. (26). In Türkiye, this is the first research study for these carbapenemase genes in animal bacterium isolates.

The aim of this study is to perform phenotypic and genotypic characterization of Enterobacteriaceae strains isolated from mastitis infections in dairy cows in terms of ESBL production, carbapenem, and colistin resistance.

Materials and Methods

Materials and Sampling: In the study, 212 cow milk samples collected from the 52 different private family type dairy farms, which have 10 and more Holstein and/or Simmental cows, in Bileksir city during the period of June 2018 and June 2020 were examined. In these farms there were a total of 639 dairy cows. The milk samples were taken by veterinarians for microbiological examinations about 5 ml into the sterile sample containers, after teats were cleaned with antiseptic and first milk discarded. All samples were collected before administering antibiotic treatment. One hundred twenty milk samples were taken from the udder lobes from cows which with clinical mastitis symptoms such as inflammation, pain and reduced milk yield. Ninety two milk samples were collected from the udder lobe from dairy cows with subclinical mastitis, which were positive as a result of the California Mastitis Test. These samples were delivered to the laboratory under cold chain conditions (8, 42, 58). The samples not to be included in the analysis immediately were frozen at (-20) °C and stored.

Isolation and Identification of bacterial strains from milk samples: At first, milk samples were slowly shaken for homogenization and then were inoculated onto 5% sheep blood agar (Merck, Germany), MacConkey Agar (Merck, Germany), Tryptone Bile X-Glucuronide (TBX) agar, Bile Aesculin Azide Agar (Merck, Germany), and RPF-Baird Parker (RPF-BP) (Merck, Germany) agar. While 5% sheep blood agar, Bile Aesculin Azide agar, RPF-Baird Parker (RPF-BP), and MacConkey Agar were incubated at 37°C for 24 hours; TBX agar was incubated at 44°C for 18-24 hours as recommended by the manufacturers. At the end of the incubation, the pink colonies in MacConkey agar and blue-green colonies in TBX agar were selected for the identification of Klebsiella spp. and Escherichia coli (E. coli). Firstly, Gram staining, microscopic and colony morphologies, hemolysis characteristics, coagulase activity on RPF-BP agar, black or colorless colonies on Bile Aesculine Azide agar were evaluated, and then biochemical tests (indole, oxidase, catalase, TSI agar, metil red Voges Proskauer, etc.) were performed to all different colonies (53). As a result of the biochemical tests, the isolates identified as Klebsiella spp., K. pneumonia, K. oxytoxa, E. coli, S. aureus, Staphylococcus spp., S. uberis, Streptococcus spp., Micrococcus spp., Pseudomonas spp., Macor, Corynebacterium spp., Enterococcus spp.. A few other colonies were arranged in the tubes with 3 ml sterile saline and adjusted to McFarland 0.5-0.63 turbidity and they were identified and verified in Vitek 2 Compact device (Biomerieux, France)
with Gram negative and Gram positive identification cards (Biomerieux, Vitek 2 GN Card, 2019; Biomerieux, Vitek 2 GP Card, 2019). All identified isolates were taken into cryotubes and kept at -20°C.

**Determination of Antibiotic Susceptibilities of Enterobacteriaceae isolates:** The antibiogram tests of Klebsiella spp. and E. coli isolates were performed based on disc diffusion method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) standards (6, 12, 18, 19, 27). The antibiotic discs used in this study were selected not only for which they are used in the treatment of runnants (21, 29, 38, 42) but also observe for ESBL production and carbapenem resistance. For this purpose, a member of penicillins, cephalosporins, carbapenems quinolones, sulphonamides, aminoglycosides, macrolides, tetracyclines were used in disc diffusion tests. Used antibiotic discs are shown in Table 2.

The isolates were thawed and inoculated onto Brain Heart Infusion Broth (Biomerieux, France) from cryotubes to review and incubated at 37 °C for 24 hours. At the end of the incubation, all isolates were confirmed with Gram staining then antibiogram tests were performed (27).

After susceptibility tests, inhibition zones were recorded as resistant (R), intermediate (I), and susceptible (S) based on the breakpoints recommended by EUCAST in terms of Enterobacteriaceae for ampicillin, amoxicillin, amoxicillin-clavulanic acid, ofloxacin, doxycycline, cephalaxin, cefotaxime, ceftazidime, ertapenem, meropenem, gentamycin, chloramphenicol, ciprofloxacin, sulfamethoxazole-trimethoprim, erythromycin, and tetracycline (27). Cephoperazone, penicillin G, and streptomycin were interpreted based on the breakpoints suggested by CLSI for Enterobacteriaceae (16, 17, 18). The susceptibility of neomycin was interpreted by criteria reported by Fouad et al. (29). Marbofloxacin and enrofloxacin were evaluated based on the breakpoints suggested by CLSI VET08 ED4:2018 (19).

**Phenotypic Detection of ESBL and Carbapenemase in Enterobacteriaceae isolates:** In order to investigate the extended-spectrum beta-lactamase (ESBL) activities of the isolates, the combination disc diffusion tests were performed and evaluated according to EUCAST criteria (16). Cefotaxime (Liofilchem, 5µg) and cefotaxime-clavulanic acid (Liofilchem, 30+10µg) combination discs were used in this test.

According to the EUCAST procedure (26), isolates, whose zone diameters were found to be <28 and <25 mm against meropenem and ertapenem in the screening test, respectively, were subjected to the confirmatory combination disc tests (KPC, AmpC, metallo-beta lactamase, OXA-48, ESBL/loss of porin). In this test, meropenem (Liofilchem, 10µg), ertapenem (Liofilchem, 10µg), meropenem+diplotelic acid (Liofilchem, Italy), meropenem+EDTA (Liofilchem, Italy), meropenem+phenylboronic acid (Liofilchem, Italy), meropenem+cloxacillin (Liofilchem, Italy), ticarcillin (Liofilchem, Italy) combined and single discs were used. Tests were evaluated by the EUCAST procedure (26).

**Detection of ESBL, carbapenemase, and colistin resistance by PCR:** All E. coli and Klebsiella spp. isolates were subcultured into Nutrient Broth (NB, Oxoid, UK) to obtain pure cultures and incubated at 37°C for 18 hours. After incubation, 1 mL NB broth culture of the isolates was centrifuged at 5000 g for 10 min. After the centrifugation process, the supernatant was removed and DNA extraction was performed using the pellet through GeneJET Genomic DNA Purification kit (Thermo Scientific, US) and DNA Purification Protocol for Gram-negative bacteria.

PCR was performed to examine ESBL, carbapenemase, and colistin resistance gene regions. Specific primer pairs were used to amplify the sequences of these genes, which were synthesized commercially from the selected genes described previously by Bektas et al. (7) and Hatrongjit et al. (32) (Table 1).

The PCR reaction mix for all ESBL genes was carried out in a total volume of 25 µl, containing 5 µl of DNA extract (template DNA) and 20 µl of PCR mix. PCR mix contained 12.5 µL DreamTaq PCR Master Mix (2X) Kit (Thermo Scientific, US), 7.3 µL DEPC water, 0.1 µL Primer F (100 pmol/μL), and 0.1 µL Primer R (100 pmol/µL). Amplification conditions were performed for these genes according to as described previously by Bektas et al. (7).

Isolates were tested for the presence of carbapenemase and colistin resistance genes by multiplex PCR as described previously by Hatrongjit et al. (32). The PCR reaction mix was performed in a total volume of 15 µl, containing 2 µl of DNA extract (template DNA) and 13 µl of PCR mix. PCR mix consisted of 8.8 µL DreamTaq PCR Master Mix (2X) Kit (Thermo Scientific, US), 2.2 µl DEPC water, 0.2 µl each Primer F (100 pmol/μL), 0.2 µl each Primer R (100 pmol/µL). Amplification conditions were performed for these genes according to as described previously by Hatrongjit et al. (32).

All PCR amplicons (10 µl ampon and 2 µl Bluejuice gel loading buffer 10X, Thermo Scientific, US) were electrophoresed on 1.5% agarose (Prona) gel prepared into 200 ml Tris-borate-EDTA (TBE) buffer (Thermo Scientific, US) and visualized on a bio-visualizing system (EBOX CX5 TS EDGE, Vilber). DNA molecular weight marker (Gene Ruler 100bp DNA Ladder plus, Thermo Scientific, US) was used.
Table 1. Primer sequences, target ESBL, and carbapenemase genes, base pairs and references.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Target ESBL genes</th>
<th>Base pairs</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTX-M-F</td>
<td>TCTTCCAGAAATAAGGAAATCCC</td>
<td>blaCTX-M</td>
<td>909 bp</td>
<td>Bektaş et al. (7)</td>
</tr>
<tr>
<td>CTX-M-R</td>
<td>CCGTTTGGCTATTACCAAAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEM-F</td>
<td>TCCGCTCATGAGACAAATTACC</td>
<td>blaTEM</td>
<td>931 bp</td>
<td>Bektaş et al. (7)</td>
</tr>
<tr>
<td>TEM-R</td>
<td>TTGGTGCAGTTACCAATGGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHV-F</td>
<td>TGGTTATCCGTTATTCGCC</td>
<td>blaSHV</td>
<td>868 bp</td>
<td>Bektaş et al. (7)</td>
</tr>
<tr>
<td>SHV-R</td>
<td>GGTATAGCCGTTACGTTCT</td>
<td>IMP</td>
<td>232 bp</td>
<td>Hatrongjit et al. (32)</td>
</tr>
<tr>
<td>IMP-F</td>
<td>GGAATAGAGTGGCTTAAATCTC</td>
<td>OXA-48-like</td>
<td>438 bp</td>
<td>Hatrongjit et al. (32)</td>
</tr>
<tr>
<td>IMP-R</td>
<td>GGTATAGAGTGGCTTAAATCTC</td>
<td>NDM</td>
<td>621 bp</td>
<td>Hatrongjit et al. (32)</td>
</tr>
<tr>
<td>OXA-48-like-F</td>
<td>GCGTGTGTAAGGATGAAAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXA-48-like-R</td>
<td>CATCAAGTTCAACCCACC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDM-F</td>
<td>GGTGTTGACGGTTCCTGTTTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDM-R</td>
<td>CGGTATGACGGTTCCTGTTTC</td>
<td>KPC</td>
<td>798 bp</td>
<td>Hatrongjit et al. (32)</td>
</tr>
<tr>
<td>KPC-F</td>
<td>CGTCTAGGTCGAGTCTGCTGCTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KPC-R</td>
<td>CTGTGTCATCCCTGTTAGGCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCR1-F</td>
<td>GGGTGGTACCAAGGTGTTGC</td>
<td>mcr-1</td>
<td>1126 bp</td>
<td>Hatrongjit et al. (32)</td>
</tr>
<tr>
<td>MCR1-R</td>
<td>CATTGGGCGTGATGCGG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Primer sequences, target ESBL, and carbapenemase genes, base pairs and references.

Table 2. Isolates and milk sample numbers.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Number of isolates</th>
<th>Number of milk samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>K. oxytoca</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>K. pneumonia</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Staphylococcus spp.</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Enterococcus spp.</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Micrococcus spp.</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Streptococcus spp.</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Streptococcus uberis</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Pseudomonas spp.</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Corynebacterium spp.</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Kocuria spp.</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mucor spp.</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>No bacterial growth</td>
<td>7</td>
<td>148</td>
</tr>
<tr>
<td>Total</td>
<td>64</td>
<td>212</td>
</tr>
</tbody>
</table>

E. coli (ATCC ® 25922™), K. pneumoniae (ATCC ® 700603™), E. coli (CCUG 62975; CTX-M positive), E. coli (NCTC 13846; colistin resistant mcr-1 positive), E. coli (NCTC 13476; IMP positive), K. pneumonia (NCTC 13438; KPC positive) and E. coli (NCTC 14320; IMP, KPC and OXA-48-like positive) were used as control strains in the isolation, identification, disc diffusion and PCR tests.

Results

On bacteriological examination, 14 (6.60%) E. coli, three (1.41%) Klebsiella oxytoca, and two (0.94%) Klebsiella pneumonia were isolated from 212 milk samples. Two of 14 E. coli were isolated in the milk samples of the cows with clinical mastitis and 12 E. coli were isolated from milk samples of cows with subclinical mastitis. Klebsiella oxytoca (n:3) and Klebsiella pneumonia isolates (n:2) were isolated from subclinical mastitis cases. Table 2 shows the isolation data of all bacterium from milk samples.

Table 3 shows the results of disc diffusion tests. At least two E. coli isolates were found to be resistant to all of the antibiotics used in the antibiogram test. The highest resistance was found against ceftotaxime and amoxicillin in K. oxytoca isolates, whereas, the highest resistance was found in K. pneumonia isolates against sulphamethoxazole/trimethoprim.

According to the results of the disc diffusion test, ESBL was detected positive in four E. coli and one K. oxytoca isolates. K. pneumonia isolates were showed no ESBL production. In combined disc diffusion tests, these ESBL positive strains also were found positive, which were much more 5 mm broad zone diameter with ceftotaxime/clavulanic acid compared with ceftotaxime disc.

According to the PCR results of blaCTX-M, blaTEM, and blaSHV genes, the blaCTX-M gene was detected in one K. oxytoca and four E. coli isolates, which were found ESBL positive. (Figure 1A).

In antibiogram tests, seven E. coli, one K. oxytoca, and one K. pneumonia isolates were found resistant against meropenem and ertapenem. Also, these isolates were confirmed in terms of carbapenem resistance according to the EUCAST procedure (26). In phenotypic mechanism determination tests, the presence of the OXA-48-like gene was confirmed by the resistance of Temocillin. Two E. coli strains were shown Temocillin resistant. The presence of IMP and NDM gene to determine the mechanism of metallo-beta-lactamase (MBL), the presence of increased synergy with dipicolinic acid was detected in six E. coli (two of also have OXA-48 type), one K. oxytoca, and one K. pneumonia strains by combined disk diffusion tests. The presence of the KPC gene was confirmed by synergie with boronic acid and one E. coli was found positive.
Table 3. Disc diffusion test results of Enterobacteriaceae isolates.

<table>
<thead>
<tr>
<th>Antibiotic discs</th>
<th>S</th>
<th>I</th>
<th>R</th>
<th>S</th>
<th>I</th>
<th>R</th>
<th>S</th>
<th>I</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin (Oxoid, 10µg)</td>
<td>4</td>
<td>-</td>
<td>10</td>
<td>1</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Amoxicillin-clavulanic acid (Oxoid, 30µg)</td>
<td>4</td>
<td>1</td>
<td>9</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ampicillin (Oxoid, 10µg)</td>
<td>6</td>
<td>1</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Cephalaxin (Oxoid, 30µg)</td>
<td>8</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cefoperazone (Oxoid, 75µg)</td>
<td>3</td>
<td>-</td>
<td>11</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Cefotaxime (Liofilchem, 5µg)</td>
<td>7</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Cefazidime (Liofilchem, 30µg)</td>
<td>8</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Chloramphenicol (Oxoid, 30µg)</td>
<td>4</td>
<td>-</td>
<td>10</td>
<td>3</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ciprofloxacin (Oxoid, 5µg)</td>
<td>6</td>
<td>-</td>
<td>8</td>
<td>3</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Doxycyline (Oxoid,</td>
<td>4</td>
<td>3</td>
<td>7</td>
<td>3</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Enrofloxacin (Oxoid, 5µg)</td>
<td>2</td>
<td>1</td>
<td>11</td>
<td>3</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Erithromycin (Oxoid, 15µg)</td>
<td>3</td>
<td>-</td>
<td>11</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Gentamicin (Oxoid, 10µg)</td>
<td>9</td>
<td>5</td>
<td>-</td>
<td>3</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Marbofloxacin (5µg)</td>
<td>2</td>
<td>1</td>
<td>11</td>
<td>3</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Neomycin (Oxoid, 10µg)</td>
<td>3</td>
<td>-</td>
<td>11</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Ofloxoxine (Oxoid, 5µg)</td>
<td>12</td>
<td>-</td>
<td>2</td>
<td>3</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Penicillin G (Oxoid, 10U)</td>
<td>4</td>
<td>-</td>
<td>10</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Spiramycin (Oxoid, 100µg)</td>
<td>12</td>
<td>-</td>
<td>2</td>
<td>3</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Streptomycin (Oxoid, 10µg)</td>
<td>12</td>
<td>-</td>
<td>2</td>
<td>3</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tetracyclin (Oxoid, 30µg)</td>
<td>10</td>
<td>-</td>
<td>4</td>
<td>3</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trimethoprim-Sulfahamethoxazole (Oxoid, 25µg)</td>
<td>-</td>
<td>-</td>
<td>14</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
</tbody>
</table>

S: Susceptable, I: Intermediate, R: Resistant.

Figure 1. A) PCR results of CTX-M gene for Enterobacteriaceae isolates (M: Marker, PC: Positive control, NC: Negative control: Line 2-3: positive isolates Line 3-9: Negative isolates) B) Multiplex PCR results of carbapenemase and colistin resistance genes for Enterobacteriaceae isolates with 100 bp plus ladder (PC: Positive control, NC: Negative control, Marked Lines: positive Enterobacteriaceae strains with marked gene).
According to the results of PCR targeting carbapenem and colistin resistance genes, the IMP gene was detected in four *E. coli*, one *K. oxytoca* and one *K. pneumonia* isolates, respectively. OXA-48-like genes were detected in two *E. coli* isolates. Also in these OXA-48-like positive two *E. coli* isolates have an IMP gene. While NDm gene was detected in two *E. coli*, KPC gene was detected in one *E. coli* isolate. One of the colistin resistance genes, *mer*-1 was detected in two *E. coli* strains with PCR (Figure 1B).

**Discussion and Conclusions**

In dairy cows, *E. coli* is a foodborne zoonosis with high importance for public health (48). In this study, the presence of *E. coli* was detected to be 6.60% in cow milk with mastitis. Messele et al. (44) have reported that the *E. coli* isolation rate was 7.1% from milk. Makolo et al. (41) stated that they isolated six *E. coli* and seven *Klebsiella pneumonia* from 147 milk samples. Zhang et al. (58) reported that they isolated *E. coli* at the rate of 20.1% from 393 milk samples. Klibi et al. (35) stated that they isolated 79 *E. coli* and 30 *Klebsiella pneumonia* in 300 milk samples. Manasa et al. (42) reported that they isolated *E. coli* at the rate of 41%. Considering the mentioned studies, the presence rate of *E. coli* in this study was thought to be significant. In this study, two *K. pneumonia* and three *K. oxytoca* were isolated.

According to the disc diffusion test results, multiple drug resistance was determined in *E. coli* isolates. According to these results, it was considered that multiple drug resistance may spread among *E. coli* isolates. In *K. oxytoca* isolates, the highest resistance was found against cefotaxime, cephalexin, cefoperazone, neomycin, penicillin G, and trimethoprim-sulfamethoxazole, respectively. Dinç et al. (22) were found the resistance rates in 92 *E. coli* strains isolated from mastitis infections of cattle against erythromycin (69.6%), ampicillin (39.1%), tetracycline (34.8%), nalidixic acid (25.0%), chloramphenicol (22.8%), trimethoprim-sulfamethoxazole (21.7%) and amoxicillin-clavulanic acid (21.7%) and also 25.0% of the 92 *E. coli* strains were susceptible to all tested antibiotics. They also reported that 54.3% of *E. coli* strains were resistant to two or more antibiotics, but they could not detect ESBLs in 92 *E. coli* strains. Makolo et al. (41) stated high multiple resistance to tetracycline, penicillin, and erythromycin. Bhat et al. (8) determined that the isolates were susceptible mostly to enrofloxacain and gentamicin but all the isolates were resistant to penicillin. Penicillin and gentamicin results in this study were found to be similar to the results of Bhat et al. (8) and they were found to be compatible with Dinç et al. (22) in terms of *E. coli* isolates with multiple resistance.

ESBL-producing strains have been widespread throughout the world since they were first described in 1983. This spread occurs as a result of clonal replication, transfer of ESBL genes to plasmids, and rarely, the emergence of new enzymes. The most important group among ESBLs is the CTX-M enzymes that emerged in the early 2000s. This group is followed by SHV and TEM-derived ESBLs (9, 11, 14, 26, 40). *Escherichia coli* species where ESBL production is most common are *E. coli* and *Klebsiella pneumoniae*, but other clinically important *Enterobacteriaceae* species are also ESBL producers (9, 15, 26, 39). According to EARS-Net data, rates of invasive *K. pneumoniae* non-sensitive to 3rd generation cephalosporins are more than 25% in most European countries and even more than 50% in many countries. KPC type, except Italy and Greece, where carbapenemase-producing isolates are high. Most local ESBL test results had been considered ESBL positive (25, 26).

Beta-lactam antibiotics are used frequently for treatment purposes in human and animal health. Extended-spectrum beta-lactamases are resistant to penicillin, 1st and 4th generation cephalosporins, and monobactams. Extended-spectrum beta-lactamases are generally associated with the plasmid. The most frequent beta-lactamases groups in *Enterobacteriaceae* isolates are TEM, SHV, and CTX-M. TEM and SHV groups are originated from TEM-1/TEM-2 and SHV-1 (*bla*TEM-1/*bla*TEM-2 and *bla*SHV-1) beta-lactamase genes; the CTX-M gene may be transferred by conjugation (51). Dinç et al. (22) stated that they were not determined ESBL in 92 *E. coli* strains isolated from cattle mastitis. In this study, in one *K. oxytoca* isolates, CTX-M genes were determined. In four *E. coli* isolates, the *bla*CTX-M gene was found. Among the studies conducted in Türkiye last seven years, ESBL increase has been observed in *E. coli* strains which have been the isolates of cattle mastitis infection.

Many researchers have reported that ESBL production among *Enterobacteriaceae* isolates has an increasing prevalence (51). This increase has been associated with the clonal transfer of the strains producing ESBL and the horizontal transfer of these genes carrying plasmids. Dinç et al. (22) conducted a study on milk samples taken from different cities, including Balıkesir in Türkiye and they reported that they could not detect ESBL in *E. coli* strains isolated from mastitis infection of cow’s. In this study, ESBL were identified in one *K. oxytoca* isolates and four *E. coli* isolate in Balıkesir city, which indicated that ESBL genes spread among the strains and the resistance became widespread in seven years. Aslantaş et. al. (4) detected 12 (46.2%) harbored *bla*CTX-M-15, 11 (42.3%) *bla*CTX-M-1, two (7.7%) *bla*CTX-M-3 and one (3.8%) *bla*CMY-2. In addition to ESBL/AmpC genes, other β-lactamase genes were detected in 22 isolates (84.6%), of which 21 isolates harbored *bla*TEM-1b and
one isolate harbored blaOXA-1 in combination with ESBL/AmpC genes.

Today, carbapenem resistance in Gram-negative bacteria is a worldwide problem. (46). Most carbapenemases are encoded by transposable elements on enzymes derived from plasmids. In Enterobacteriaceae members, changes or loss of porin (or possibly PBP) and ESBL or AmpC enzyme production can also be seen with reduced susceptibility to carbapenems. Carbapenemases are particularly sensitive to one of the carbapenems (imipenem, meropenem, ertapenem, doripenem) (26).

In the 1990s, the problem of carbapenem resistance was reported in many Mediterranean countries in Europe, especially in Pseudomonas aeruginosa (13, 26). In the early 2000s, an outbreak of Klebsiella pneumoniae related to metallo-β-lactamase (VIM) encoded by Verona integron and K. pneumoniae carbapenemase (KPC) was reported in Greece (26, 56). Today, OXA-48 carbapenemases are the most common carbapenemase group in Europe (2, 26). Other particularly problematic carbapenemases are New Delhi metallo-β-lactamases (NDMs) (2, 26), which are quite common in the Indian subcontinent and the Middle East and have been reported on several occasions in Europe, as well as instances of regional spread in some countries (5, 27). IMP-carbapenemases are also common in some parts of the world (26, 47).

Nordmann et al. (46) reported that carbapenem nonsusceptibility or resistance rates by region, especially for E. coli, up to 3% in Asia-Pacific, up 34% in India, Nepal, Pakistan, Vietnam, up to 7% in Europe, between 0.2%–0.4% in North America, between 0.4%–9.0% in Latin America (46).

Countries in Africa, including Morocco, Kenya, and South Africa have reported NDM-1 as the most dominant carbapenemase gene. Latin America and China have reported KPC-2 as the most dominant carbapenemase gene (12). In Türkiye, authors reported only AmpC-producing E. coli strains (4).

In Türkiye, there is no study about findings of carbapenem resistance and IMP, OXA-48 like, NDM and KPC genes in animal bacterium isolates. Recently Al et al. (1) reported that no carbapenem resistance in Enterococci strains isolated from raw milk. With this study, carbapenem resistance genes (IMP, OXA-48 like, NDM and KPC) were declared for the first time in Enterobacteriaceae isolates isolated from dairy cows’ mastitis infection in Türkiye.

Köck et al. (36) reported that they isolated carbapenem-resistant Enterobacteriaceae from poultry meat, chicken, pig, cows or raw milk, cattle, and various types of seafood. Pampuntu et al. (50) were reported that they isolated a total of 182 E. coli isolates in 64 water samples obtained from drinking water containers of dairy cattle in 32 dairy farms. Also, they found two isolates resistant to imipenem but showed positive results for only blaNDM gene detection by the PCR in these E. coli strains.

Carbapenemases are a source of concern because they may confer resistance to virtually all β-lactams, and are readily transferable. Because of this, resistance mechanisms to a wide range of antimicrobial agents and their infections with high mortality rates in epidemiologically (10, 26, 43, 55).

Colistin (also known as polymyxin E) and other polymyxins are used for the treatment of severe human infections with Pseudomonas aeruginosa and carbapenem-insensitive ESBL-producing Enterobacteriaceae and Acinetobacter spp. Such strains are also has been found in foods of animal origin and there are signs of mcr-1 gene flow from animals to humans (28). In this study, the mcr-1 gene was found in two E. coli isolates. However, colistin resistance is not very important for cow mastitis cases and colistin has not commonly used for the treatment of cow mastitis infection (28). But this result is important for public health.

Until recently, in Türkiye has not been reported any IMP, OXA-48 like, NDM and KPC carbapenem resistance genes in E. coli and Klebsiella spp. from dairy cows’ mastitis infection isolates. This study results showed that carbapenem resistance was detected for the first time in Türkiye and that occurred in Enterobacteriaceae strains in cow’s mastitis agents in Türkiye. So, this is important for the epidemiology of carbapenem resistance in Türkiye; but more epidemiological studies are needed in animal isolates in Türkiye.

Consequently, determining ESBL and carbapenem resistance in the species especially included in the Enterobacteriaceae family revealed the presence of ESBL and carbapenem resistance, becoming prevalent and increasing, especially among E. coli isolates in Türkiye. Also, the fact that multiple antibiotic active substance resistance was determined in the isolates revealed that antibiotic resistance spread and increased rapidly. For these reasons, it has been considered that researching and monitoring the presence of ESBL and carbapenem resistance genes in the bacteria isolated in animal diseases are essential in terms of obtaining epidemiological data. Finally, this study is evaluated in terms of public health, it demonstrates the importance of control and prevention of antimicrobial resistance with the scope of the One Health concept.

Acknowledgements
The author would like to thank the Republic of Turkey Ministry of Health General Directorate of Public Health Microbiology Reference Laboratory for supplied the control strains used in this study and supported to identification of a few colonies.

DOI: 10.33988/auvfd.828306
Financial Support
This research received no grant from any funding agency/sector.

Conflict of Interest
The author declared that there is no conflict of interest.

Data Availability Statement
The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement
This study does not present any ethical concerns.

Animal Welfare
Not applicable.

References


Publisher’s Note
All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.