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

ARTICLE INFO

Article History
Received : 20.11.2020
Accepted : 18.11.2021
DOI: 10.33988/auvfd.828306

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.

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 *bla*CTX-M, *bla*TEM, and *bla*SHV genes, the *bla*CTX-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.

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-ESBLhas 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 beta-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 monobactams. 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, blaOXA48,* 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 Balıkesir 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 reccommended 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. oxytoca, E. coli, S. aureus, Staphylococcus spp., S. uberis, Streptococcus spp., Micrococcus spp., Pseudomonas spp., Mucor, 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)

DOI: 10.33988/auvfd.828306

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 ruminants (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, cephalexin, 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,), merpenem+dipicolinic acid (Liofilchem, Italy), meropenem+EDTA (Liofilchem, Italy), meropenem+ phenylboronic acid (Liofilchem, Italy), meropenem+ cloxacillin (Liofilchem, Italy), temocillin (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 Bektaş 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 Bektaş 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 ampicon 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.

Primers	Sequence	Target ESBL genes	Base pairs	References
CTX-M-F CTX-M-R	TCTTCCAGAATAAGGAATCCC CCGTTTCCGCTATTACAAAC	*bla*CTX-M	909 bp	Bektaş et al. (7)
TEM-F TEM-R	TCCGCTCATGAGACAATAACC TTGGTCTGACAGTTACCAATGC	*bla*TEM	931 bp	Bektaş et al. (7)
SHV-F SHV-R	TGGTTATGCGTTATATTCGCC GGTTAGCGTTGCCAGTGCT	*bla*SHV	868 bp	Bektaş et al. (7)
IMP-F IMP-R	GGAATAGAGTGGCTTAAYTCTC GGTTTAAYAAAACAACCACC	*IMP*	232 bp	Hatrongjit et al. (32)
OXA-48-like-F OXA-48-like-R	GCGTGGTTAAGGATGAACAC CATCAAGTTCAACCCAACCG	*OXA-48-like*	438 bp	Hatrongjit et al. (32)
NDM-F NDM-R	GGTTTGGCGATCTGGTTTTC CGGAATGGCTCATCACGATC	*NDM*	621 bp	Hatrongjit et al. (32)
KPC-F KPC-R	CGTCTAGTTCTGCTGTCTTG CTTGTCATCCTTGTTAGGCG	*KPC*	798 bp	Hatrongjit et al. (32)
MCR1-F MCR1-R	GGGTGTGCTACCAAGTTTGC CATTGGCGTGATGCCAGTTT	*mcr-1*	1126 bp	Hatrongjit et al. (32)

*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 2. Isolates and milk sample numbers.

Isolates	Number of isolates	Number of milk samples
E. coli	14	14
K. oxytoca	3	3
K. pneumonia	2	2
Staphylococcus aureus	12	12
Staphylococcus spp.	7	7
Enterococcus spp.	3	3
Micrococcus spp.	1	1
Streptococcus spp.	2	2
Streptococcus uberis	7	7
Pseudomonas spp.	1	1
Corynebacterium spp.	7	7
Kocuria spp.	1	1
Mucor spp.	4	4
No bacterial growth	-	148
Total	64	212

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 cefotaxime 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 cefotaxime+clavulanic acid compared with cefotaxime disc.

According to the PCR results of *bla*CTX-M, *bla*TEM, and *bla*SHV genes, the *bla*CTX-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.

	E. coli (n:14)			*K. oxytoca* (n:3)			*K. pneumonia* (n:2)		
Antibiotic discs	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
Amoxicillin (Oxoid, 10µg)	4	-	10	1	-	2	1	-	1
Amoxicillin-clavulanic acid (Oxoid, 30µg)	4	1	9	2	1	-	2	-	-
Ampicillin (Oxoid, 10µg)	6	1	7	2	1	-	1	-	1
Cephalexin (Oxoid, 30µg)	8	1	5	1	1	1	2	-	-
Cephoperazone (Oxoid, 75µg)	3	-	11	1	1	1	1	-	1
Cefotaxime (Liofilchem, 5µg)	7	1	6	1	-	2	1	-	1
Ceftazidime (Liofilchem, 30 µg)	8	1	5	1	1	1	1	-	1
Chloramphenicol (Oxoid, 30µg)	4	-	10	3	-	-	2	-	-
Ciprofloxacin (Oxoid, 5µg)	6	-	8	3	-	-	2	-	-
Doxycyline (Oxoid,	4	3	7	3	-	-	2	-	-
Enrofloxacin (Oxoid, 5µg)	2	1	11	3	-	-	1	-	1
Erithromycin (Oxoid, 15µg)	3	-	11	2	1	-	1	-	1
Gentamicin (Oxoid, 10µg)	9	-	5	3	-	-	2	-	-
Marbofloxacin (5µg)	2	1	11	3	-	-	1	-	1
Neomicin (Oxoid, 10µg)	3	-	11	2	-	1	1	-	1
Ofloxacine (Oxoid, 5µg)	12	-	2	3	-	-	2	-	-
Penicillin G (Oxoid, 10U)	4	-	10	2	-	1	2	-	-
Spiramycin (Oxoid, 100µg)	12	-	2	3	-	-	2	-	-
Streptomycin (Oxoid, 10µg)	12	-	2	3	-	-	2	-	-
Tetracyclin (Oxoid, 30µg)	10	-	4	3	-	-	2	-	-
Trimethoprim-Sulfahamethoxazole (Oxoid, 25µg)	-	-	14	2	-	1	-	-	2

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

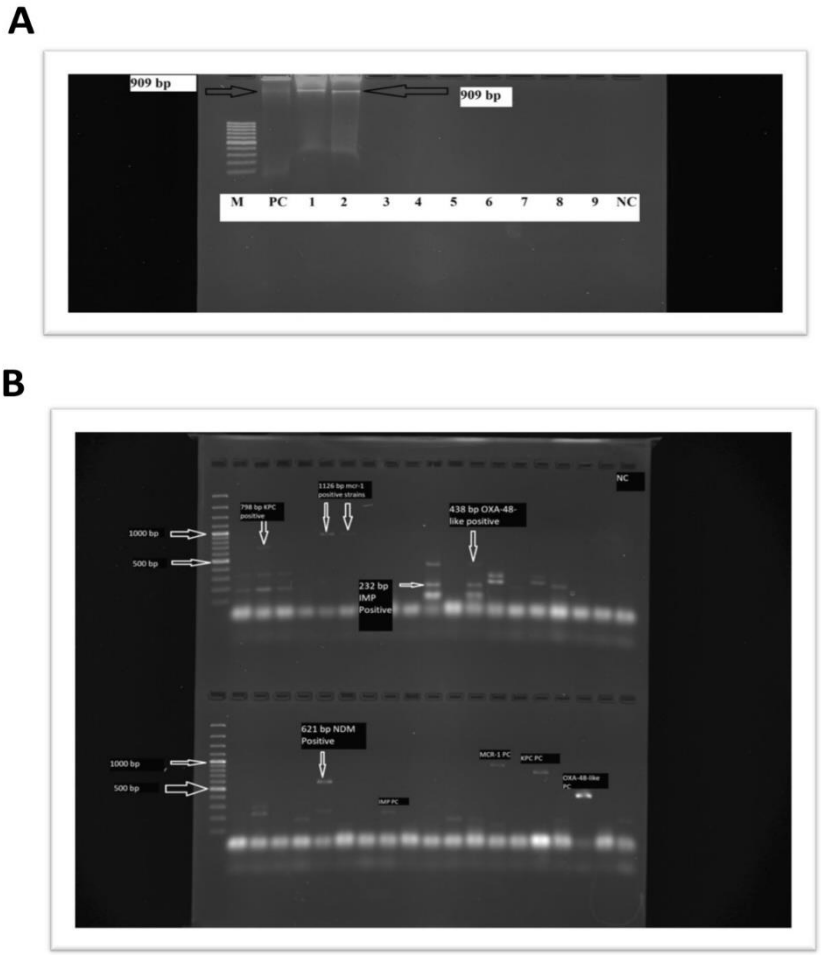

Figure 1. A) PCR results of CTX-M gene for Enterobacteriaceae isolates (M: Marker, PC: Positive control, NC: Negative control: Line 2-3: positive isolates Line 3-9: Negative isolates) B) Multiplex PCR results of carbapenemase and colistin resistance genes for *Enterocteriaceae* isolates with 100 bp plus 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, *mcr-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 the 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 enrofloxacin 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* 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

DOI: 10.33988/auvfd.828306

one isolate harbored *bla*OXA-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 *Enterocateriacae* 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. Pamipuntu 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 supportted to identification of a few colonies.

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

1. **Al S, Hızlısoy H, Ertaş Onmaz E, et al** (2020): *A molecular investigation of carbapenem resistant Enterobacteriaceae and blaKPC, blaNDM and blaOXA-48 genes in raw milk.* Kafkas Univ Vet Fak Derg, **26,** 391-396.
2. **Albiger B, Glasner C, Struelens MJ, et al** (2015): *Carbapenemase-producing Enterobacteriaceae in Europe: assessment by national experts from 38 countries, May 2015.* Euro Surveill, **20.**
3. **Alonso CA, Zarazaga M, Ben Sallem R, et al** (2017): *Antibiotic resistance in Escherichia coli in husbandry animals: the African perspective.* Lett App Microbiol, **64,** 318–334.
4. **Aslantaş Ö, Elmacıoğlu S, Yılmaz EŞ** (2017): *Prevalence and characterization of ESBL- and AmpC-producing Escherichia coli from cattle.* Kafkas Univ Vet Fak Derg, **23,** 63-67.
5. **Baraniak A, Izdebski R, Fiett J, et al** (2016): *NDM-producing Enterobacteriaceae in Poland, 2012-2014: interregional outbreak of Klebsiella pneumoniae ST11 and sporadic cases.* J Antimicrob Chemother, **71**, 85-91.
6. **Bauer AW, Kirby WM, Sherris JC, et al** (1966): *Antibiotic susceptibility testing by a standardized single disk method.* Am J Clin Pathol, **45,** 493- 496.
7. **Bektaş A, Güdücüoğlu H, Gürsoy NC, et al** (2018): *Investigation of extended spectrum beta-lactamase (esbl) genes in esbl-producing Escherichia coli and Klebsiella pneumoniae strains.* Flora, **23,** 116-123.
8. **Bhat AM, Soodan JS, Singh R, et al** (2017): *Incidence of bovine clinical mastitis in Jammu region and antibiogram of isolated pathogens.* Vet World, **10,** 984–989.
9. **Bradford PA** (2001): *Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat.* Clin Microbiol Rev, **14,** 933-951.
10. **Bratu S, Landman D, Haag R, et al** (2005): *Rapid spread of carbapenem-resistant Klebsiella pneumoniae in New York City: a new threat to our antibiotic armamentarium.* Arch Intern Med, **165,** 1430-1435.
11. **Bush K, Jacoby GA, Medeiros AA** (1995): *A functional classification scheme for β -lactamases and its correlation with molecular structure.* Antimicrob Agents Chemother, **139,** 211-1233.
12. **Codjoe FS, Donkor ES** (2018): *Carbapenem resistance: A review.* Med Sci, **6**, 1-28.
13. **Cantón R, Akóva M, Carmeli Y, et al** (2012): *Rapid evolution and spread of carbapenemases among Enterobacteriaceae in Europe.* Clin Microbiol Infect, **18,** 413-431.
14. **Cantón R, Novais A, Valverde A, et al** (2008): *Prevalence and spread of extendedspectrum β-lactamase-producing Enterobacteriaceae in Europe.* Clin Microbiol Infect, **14,** 144-153.
15. **Carattoli A** (2008): *Animal reservoirs for extended-spectrum β-lactamase producers.* Clin Microbiol Infect, **14,** 117-123.
16. **Clinical and Laboratory Standards Institute (CLSI)** (2014): Performance standards for antimicrobial susceptibility testing; twenty forth informational supplement M100-S24. (Vol. 34, No. 1). Wayne, PA, USA.
17. **Clinical and Laboratory Standards Institute (CLSI)** (2017): Performance standards for antimicrobial susceptibility testing; 24th Informational Supplement M100-S26. Wayne, PA, USA.
18. **Clinical and Laboratory Standards Institute (CLSI)** (2018): Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. 5th ed. CLSI standard VET01. Wayne, PA, USA.
19. **Clinical and Laboratory Standards Institute (CLSI)** (2018): Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. 5th ed. CLSI standard VET08 ED4. Wayne, PA, USA.
20. **De Jong A, El Garch F, Simjee S, et al** (2018): Monitoring of antimicrobial susceptibility of udder pathogens recovered from cases of clinical mastitis in dairy cows across Europe: VetPath results. *Vet Microbiol*, **213,** 73–81, 2018.
21. **De Schmitt-van Leemput E, Zadoks RN** (2007): *Genotypic and phenotypic detection of macrolide and lincosamide resistance in Streptococcus uberis.* Dairy Sci, **90,** 5089–5096.
22. **Dinç G, Ata Z, Temelli S** (2012): *Investigation of extended-spectrum beta-laktamase activity and antibiotic resistance profile of Escherichia coli strains isolated from bovine mastitis.* Ank Univ Vet Fak Derg, **59,** 85-88.
23. **Effendi MH, Harijani N** (2019): *Prevalence of pathogenic Escherichia coli isolated from subclinical mastitis in east java province, Indonesia.* Indian Vet J, **96,** 22-25.
24. **Eisenberger D, Carl A, Balsliemke J, et al** (2018): *Molecular characterization of extended-spectrum b-lactamase-producing Escherichia coli isolates from milk samples of dairy cows with mastitis in Bavaria, Germany.* Microb Drug Resist, **24,** 505-510.
25. **European Centres for Disease Prevention and Control (ECDC)** (2014): Antimicrobial resistance surveillance in Europe 2014. Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net).
26. **European Committee on Antimicrobial Susceptability Testing (EUCAST)** (2017): Breakpoint tables for interpretation of MICs and zone diameters. Version 9.0.

Available at http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/ Breakpoint_tables/v_9.0_Breakpoint_Tables.pdf. (Accessed September 29, 2020).

27. **European Committee on Antimicrobial Susceptibility Testing (EUCAST)** (2019): EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance. Version 2.0. Available at https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Resistance_mechanisms/EUCAST_detection_of_resistance_mechanisms_170711.pdf. (Accessed September 29, 2020).
28. **Flioussis G, Kachrimanidou M, Christodoulopoulos G, et al** (2020): *Bovine mastitis caused by a multidrug-resistant, mcr-1-positive (colistin-resistant), extended-spectrum β-lactamase– producing Escherichia coli clone on a Greek dairy farm.* J Dairy Sci, **103,** 852-857.
29. **Fouad Z** (2011): Antimicrobial Disk Diffusion Zone Interpretation Guide. Available at https://doi.org/10.13140/RG.2.2.13801.70240. (Accessed September 29, 2020).
30. **Gao X, Fan C, Zhang Z, et al** (2019): *Enterococcal isolates from bovine subclinical and clinical mastitis: Antimicrobial resistance and integron-gene cassette distribution.* Microb Pathog, **129,** 82–87.
31. **Gemechu T, Yunus HA, Soma M, et al** (2019): *Bovine mastitis: prevalence, isolation and identification of major bacterial pathogens in selected areas of Bench Maji Zone Southwest Ethiopia.* J Vet Med Anim Health, **11,** 30-36.
32. **Hatrongjit R, Kerdsin A, Akeda Y, et al** (2018): *Detection of plasmid-mediated colistin-resistant and carbapenem-resistant genes by multiplex PCR.* MethodsX, **5,** 532- 536.
33. **Horpiencharoen W, Thongratsakul S, Poolkhet C** (2019): *Risk factors of clinical mastitis and antimicrobial susceptibility test results of mastitis milk from dairy cattle in western Thailand: Bayesian network analysis.* Prevent Vet Med, **164,** 49–55.
34. **Jayarao B, Almeida R, Oliver SP** (2019): *Antimicrobial resistance on dairy farms.* Foodborne Pathog Dis, **16,** 1–4.
35. **Klibi A, Jouini A, Boubaker El Andolsi R, et al** (2019): *Epidemiology of β-lactamase-producing staphylococci and gram negative bacteria as cause of clinical bovine mastitis in Tunisia.* Biomed Res Int, **2019,** 1–9.
36. **Köck R, Daniels-Haardt I, Becker K, et al** (2018): *Carbapenem-resistant Enterobacteriaceae in wildlife, food-producing, and companion animals: a systematic review.* Clin Microbiol Infect, **24,** 1241-1250.
37. **Krömker V, Leimbach S** (2017): *Mastitis treatment-reduction in antibiotic usage in dairy cows.* Reprod Domest Anim, **52,** 21–29.
38. **Laven R** (2013): *Treatment of E. coli mastitis: are antibiotics needed?* Livestock, **18,** 59-61.
39. **Livermore DM, Cantón R, Gniadkowski M, et al** (2007): *CTX-M: changing the face of ESBLs in Europe.* J Antimicrob Chemother, **59,** 165-174.
40. **Livermore DM** (1995): *β-Lactamases in laboratory and clinical resistance.* Clin Microbiol Rev, **8,** 557-584.
41. **Makolo D, Suleiman AB, Olonitola OS, et al** (2019): *Antimicrobial Susceptibility Profile of Coliforms from Bovine Mastitis Cases among Pastoral Herds in Parts of Kaduna State , Nigeria : Curbing the Environmental Health Risk.* Asian J Adv Res Rep, **3,** 1–12.
42. **Manasa V, Venkata Sai Kumar T, Prasada Rao T, et al** (2019): *Incidence of bovine clinical mastitis caused by Escherichia coli.* Int J Curr Microbiol Appl Sci, **8,** 1249–1256.
43. **Marchaim D, Navon-Venezia S, Schwaber MJ, et al** (2008): *Isolation of imipenem-resistant Enterobacter species: emergence of KPC-2 carbapenemase, molecular characterization, epidemiology, and outcomes.* Antimicrob Agents Chemother, **52,** 1413-1418.
44. **Messele YE, Abdi RD, Tegegne DT, et al** (2019): *Analysis of milk-derived isolates of E. coli indicating drug resistance in central Ethiopia.* Trop Anim Health Pro, **51,** 661–667.
45. **Naas T, Poirel L, Nordmann P** (2008): *Minor extended-spectrum β-lactamases.* Clin Microbiol Infect, **14,** 42-52.
46. **Nordmann P, Poirel L** (2019): *Epidemiology and diagnostics of carbapenem resistance in Gram- negative bacteria*. Clin Infect Dis, **69,** 521-528.
47. **Nordmann P, Naas T, Poirel L** (2011): *Global spread of Carbapenemase-producing Enterobacteriaceae.* Emerg Infect Dis, **17,** 1791-1798.
48. **Nüesch-Inderbinen M, Kappeli N, Morach M, et al** (2019): *Molecular types, virulence profiles and antimicrobial resistance of Escherichia coli causing bovine mastitis.* Vet Rec Open, **6,** 1-9.
49. **Palmeria JD, Ferreira NDM** (2020): *Extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae in cattle production – a threat around the world.* Heliyon, **6,** e03206.
50. **Pamipuntu N, Pamipuntu S** (2020): *Detection of antimicrobial resistance genes of carbapenem-resistant Enterobacteriaceae in Escherichia coli isolated from the water supply of smallholder dairy farms in Saraburi and Maha Sarakham, Thailand.* Int J of One Health, **6,** 1-5.
51. **Pehlivanoğlu F, Türütoğlu H, Öztürk D, et al** (2017): *Characterization of extended- spectrum beta-lactamase-producing fecal Escherichia coli isolates in laying hens.* Ank Univ Vet Fak Derg, **64,** 301–306.
52. **Pillar CM, Stoneburner A, Shinabarger DL, et al** (2014): *In vitro susceptibility of bovine mastitis pathogens to a combination of penicillin and framycetin: development of interpretive criteria for testing by broth microdilution and disk diffusion.* Dairy Sci, **97,** 6594–6607.
53. **Quinn PJ, Markey BK, Leonard FC, et al** (2004): Veterinary Microbiology and Microbial Disease. Second Edition, Blackwell Science Ltd, Oxford.
54. **Šlosárková S, Nedbalcová K, Bzdil J, et al** (2019): *Antimicrobial susceptibility of streptococci most frequently isolated from Czech dairy cows with mastitis.* Ann Anim Sci, **19,** 679–694.
55. **Souli M, Galani I, Antoniadou A, et al** (2010): *An outbreak of infection due to βlactamase Klebsiella pneumoniae carbapenemase 2-producing K. pneumoniae in a Greek University Hospital: molecular characterization, epidemiology, and outcomes.* Clin Infect Dis, **50,** 364–373.
56. **Vatopoulos A** (2008): *High rates of metallo-β-lactamase-producing Klebsiella pneumoniae in Greece – a review of the current evidence.* Euro Surveill, **13,** 8023.
57. **World Health Organisation** (2017): Available at http://www.who.int/drugresistance/documents/surveillance report/en./. (Accessed October 29, 2019).

58. **Zhang D, Zhang Z, Huang C, et al** (2018): *The phylogenetic group, antimicrobial susceptibility, and virulence genes of Escherichia coli from clinical bovine mastitis.* Dairy Sci, **101,** 572–580.