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First detection of carbapenem resistance in *Enterobacteriaceae* isolates isolated from dairy cows' mastitis infection in Turkey

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Abstract: With this study, carbapenem resistance genes were declared for the first time in *Enterobacteriaceae* isolates isolated from dairy cows' mastitis infection in Turkey. 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.

Keywords: Antimicrobial resistance, carbapenem resistance, colistin, *E. coli*, esbl, mastitis.

Türkiye'de karbapenem direncinin süt ineklerinin mastitis infeksiyonlarından izole edilen *Enterobacteriaceae* izolatlarında ilk teşhisi

Özet: Bu çalışma ile Türkiye'de karbapenem direnç genleri süt ineklerinin mastitis infeksiyonlarından izole edilen *Enterobacteriaceae* izolatlarında ilk kez bildirildi. Süt örneklerinin bakteriyolojik incelenmesi sonucunda 212 örneğin 14 (%6,60)'ünde *E. coli* ve üç (1,41%)'ünde *Klebsiella oxytoca* ve ikisinde *Klebsiella pneumonia* (%0,94) izole edildi. En az iki *E. coli* izolatı antibiyogram testinde kullanılan tüm antibiyotiklere dirençli bulundu. *K. oxytoca* izolatlarında ise en yüksek direnç sefotaksim ve amoksisilin'e karşı bulundu. *bla*CTX-M, *bla*TEM ve *bla*SHV

genlerinin PCR sonuçlarına göre, GSBL pozitif bulanan bir *K. oxytoca* ve dört *E.coli* izolatında *bla*CTX-M geni saptandı. Karbapenem ve kolistin direnç genlerinin PZR sonuçlarına göre, IMP geni dört *E.coli*, bir *K. oxytoca* ve bir *K. pneumonia* izolatında bulundu. OXA-48 benzeri gen iki *E. coli* izolatında bulundu. Bu iki *E. coli* izolatında ayrıca IMP geni de bulundu. NDM geni iki *E. coli* izolatında bulunurken, bir *E. coli* izolatında KPC geni bulundu. PCR sonucunda, kolistin direnç genlerinden olan *mcr-1* geni iki *E. coli* izolatında bulundu. Sonuç olarak *Enterobacteriaceae* familyasına bağlı türlerde GSBL ve karbapenem direnci saptanması özellikle *E. coli* izolatları arasında yaygınlaşan ve artan GSBL ve karbapenem direnç varlığını göstermektedir. Ayrıca izolatlarda çoklu antibiyotik etken madde direnci saptanması antibiyotik direncinin de hızla yayıldığını ve arttığını göstermektedir.

Anahtar sözcükler: Anmikrobiyal direnç, *E. coli*, gsbl, karbapenem direnci, kolistin, mastitis.

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 (27).

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. (27). In Turkey, 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 has 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 about 5 ml into sterile sample containers after teats were cleaned with antiseptic and first milk discarded by veterinarians. 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, paini 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, 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., *E. coli*, *Staphylococcus* spp., *Streptococcus* spp. *Enterococcus* spp. and all 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, 26). 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 and Vitek II, then antibiogram tests were performed (26).

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 (26). 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 (27), 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 (27).

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 7.5 µL DreamTaq PCR Master Mix (2X) Kit (Thermo Scientific, US), 1.5 µL DEPC water, 0.4 µL each Primer F (100 pmol/µL), 0.4 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.

E.coli (ATCC ® 25922™), *K. pneumoniae* (ATCC ® 700603™), and *E.coli* (CCUG 62975; CTX-M positive), *E.coli* (NTCC 13846; colistin resistant mcr-1 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 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 (27). 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.

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, 27, 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, 27, 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

304 and Greece, where carbapenemase-producing isolates are high. Most local ESBL test
305 results had been considered ESBL positive (25, 27).

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

318 Many researchers have reported that ESBL production among *Enterobacteriaceae*
319 isolates has an increasing prevalence (51). This increase has been associated with the
320 clonal transfer of the strains producing ESBL and the horizontal transfer of these genes
321 carrying plasmids. Dinç et al. (22) conducted a study on milk samples taken from
322 different cities, including Balıkesir in Turkey and they reported that they could not
323 detect ESBL in *E. coli* strains isolated from mastitis infection of cow's. In this study,
324 ESBL were identified in one *K. oxytoca* isolates and four *E. coli* isolate in Balıkesir
325 city, which indicated that ESBL genes spread among the strains and the resistance
326 became widespread in seven years. Aslantaş et. al. (4) detected 12 (46.2%) harbored
327 *bla*CTX-M-15, 11 (42.3%) *bla*CTX-M-1, two (7.7%) *bla*CTX-M-3 and one (3.8%)

328 *bla*CMY-2. In addition to ESBL/AmpC genes, other β-lactamase genes were detected in
329 22 isolates (84.6%), of which 21 isolates harbored *bla*TEM-1b and one isolate harbored
330 *bla*OXA-1 in combination with ESBL/AmpC genes.

331 Today, carbapenem resistance in Gram-negative bacteria is a worldwide problem.
332 (46). Most carbapenemases are encoded by transposable elements on enzymes derived
333 from plasmids. In *Enterobacteriaceae* members, changes or loss of porin (or possibly
334 PBP) and ESBL or AmpC enzyme production can also be seen with reduced
335 susceptibility to carbapenems. Carbapenemases are particularly sensitive to one of the
336 carbapenems (imipenem, meropenem, ertapenem, doripenem) (27).

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

347 Nordmann et al. (46) reported that carbapenem nonsusceptibility or resistance
348 rates by region, especially for *E. coli*, up to 3% in Asia-Pacific, up 34% in India, Nepal,
349 Pakistan, Vietnam, up to 7% in Europe, between 0.2%–0.4% in North America,
350 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 Turkey, authors reported only AmpC-producing *E. coli* strains (4).

In Turkey, 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 Turkey.

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

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 Turkey. 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.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The author declared that there is no conflict of interest.

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

Primers	Sequence	Target ESBL genes	Base pairs	References
CTX-M-F	TCTTCCAGAATAAGGAATCCC	*bla*CTX-M	909 bp	Bektaş et al. 2018
CTX-M-R	CCGTTTCCGCTATTACAAAC			
TEM-F	TCCGCTCATGAGACAATAACC	*bla*TEM	931 bp	Bektaş et al. 2018
TEM-R	TTGGTCTGACAGTTACCAATGC			
SHV-F	TGGTTATGCGTTATATTCGCC	*bla*SHV	868 bp	Bektaş et al. 2018
SHV-R	GGTTAGCGTTGCCAGTGCT			
IMP-F	GGAATAGAGTGGCTTAAYTCTC	*IMP*	232 bp	Hatrongjit et al. 2018
IMP-R	GGTTTAAYAAAACAACCACC			
OXA-48-like-F	GCGTGGTTAAGGATGAACAC	*OXA-48-like*	438 bp	Hatrongjit et al. 2018
OXA-48-like-R	CATCAAGTTCAACCCAACCG			
NDM-F	GGTTTGGCGATCTGGTTTTC	*NDM*	621 bp	Hatrongjit et al. 2018
NDM-R	CGGAATGGCTCATCACGATC			
KPC-F	CGTCTAGTTCTGCTGTCTTG	*KPC*	798 bp	Hatrongjit et al. 2018
KPC-R	CTTGTCATCCTTGTTAGGCG			
MCR1-F	GGGTGTGCTACCAAGTTTGC	*mcr-1*	1126 bp	Hatrongjit et al. 2018
MCR1-R	CATTGGCGTGATGCCAGTTT			

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590 **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	-	68
Total	64	212

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599 **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	1	-
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

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

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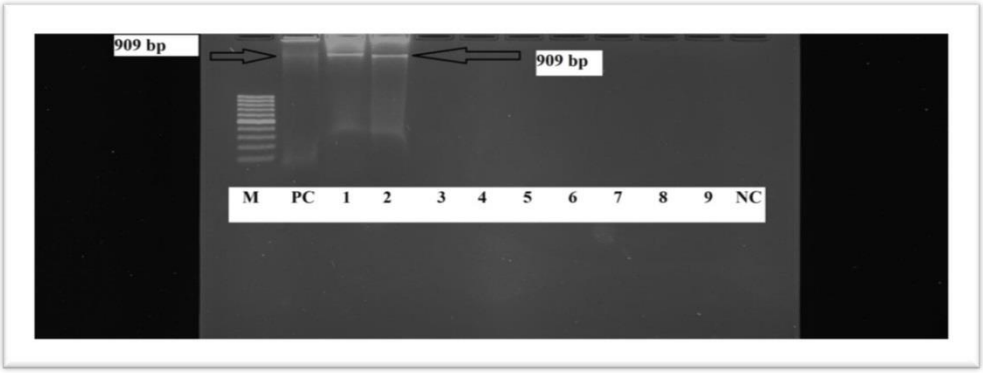
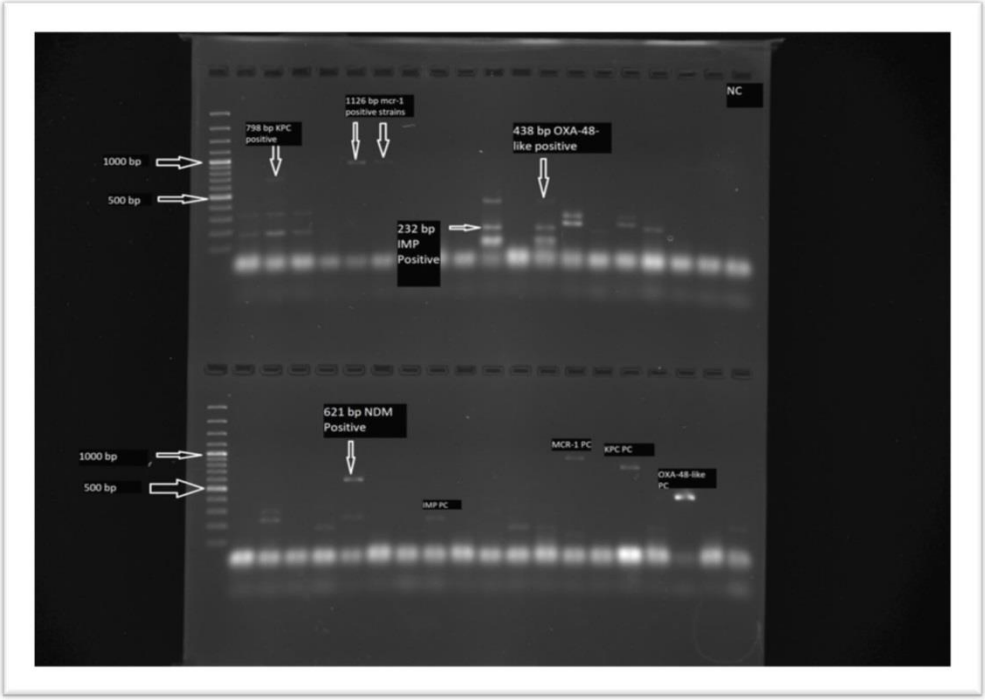
A



B



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603 **Figure 1.** A) PCR results of CTX-M gene for Enterobacteriaceae isolates (M: Marker,
604 PC: Positive control, NC: Negative control: Line 2-3: positive isolates Line 3-9:
605 Negative isolates) B) Multiplex PCR results of carbapenemase and colistin resistance
606 genes for *Enterocteriaceae* isolates with 100 bp plus ladder (PC: Positive control, NC:
607 Negative control, Marked Lines: positive *Enterobacteriaceae* strains with marked
608 gene).