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# A molecular investigation of Extended Spectrum Beta-Lactamase genes in *Escherichia coli* and *Klebsiella spp.* in raw cow milk

**Mehmet Demirci**[1] **Akın Yigin**[2] **Serap Kiliç Altun**[3] **Seda Ekici**[4]

[1] *Department of Medical Microbiology, Faculty of Medicine, Kırklareli University, Kırklareli, Türkiye*
[2] *Department of Genetics, Faculty of Veterinary Medicine, Harran University, Şanlıurfa, Türkiye*
[3] *Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Harran University, Şanlıurfa, Türkiye*
[4] *Veterinary Control Central Research Institute, Ankara, Türkiye*

***Correspondence:*** *Seda Ekici (seda.ergen@hotmail.com)*

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## ABSTRACT

**Objective:** Raw milk is an important source of nutrients. Therefore, today, there is a great demand for raw milk consumption. The positive side of milk consumption on growth and development cannot be ignored, but unfortunately, pathogens in raw milk are always potential public health risks for transmission pathogens. Bacteria such as Enterobacteriaceae in normal flora can cause serious problems due to their extended-spectrum beta-lactamase (ESBL) production. These bacteria and their resistance genes have been reported in raw milk. In this matter, the aim of the study is to determine the status of blaCTX-M-1, blaCTX-M-2, blaTEM and blaSHV genes responsible for the production of ESBL enzyme in Escherichia coli and Klebsiella spp. strains to identify risk factors in raw milk consumption and to gain an understanding of the epidemiology of these resistant strains.

**Materials and methods:** A total of different 50 raw milk samples were collected and subjected to phenotypic microbiological analysis and Real-time PCR targeting blaCTX-M-1, blaCTX-M-2, blaTEM and blaSHV genes. In the phenotypic analyses, suspicious isolates were identified by classical microbiological methods and antibiotic resistance profiles were revealed.

**Results:** These results indicated that raw milk is a potential reservoir for ESBL producing E. Coli, Klebsiella spp. strains are obviously significant. And It was determined that CTX-M-basedESBL genes are predominant in ESBL production.

**Conclusion:** The present study revealed that raw milk is epidemiologically involved in the transmission of ESBL genes. Raw milk could be distributed to ESBL genes widely and is consumed in Şanlıurfa.

***Keywords:*** *E. coli, ESBL genes, Klebsiella spp., Raw Cow milk, qPCR*

## INTRODUCTION

Raw milk is a particularly nutritious food having proteins, fats, carbohydrates, vitamins, minerals, and essential amino acids. Due to its near-neutral pH level and high-water activity, raw milk also provides ideal conditions for the growth of many microorganisms (Altun et al., 2002; Kim et al., 2017). More and more people are choosing to consume unpasteurized raw milk. The thought that some nutrients in raw milk will be lost after pasteurization is advocated as the reason for the increased interest in raw milk consumption. However, many epidemiological studies expressly show that raw milk can be contaminated with pathogens, some of which are incorporated with human diseases, as it also provides optimal

conditions due to the growth of many microorganisms (Oliver et al., 2009; Yun et al., 2020). While most studies about foods such as raw milk focus on zoonotic pathogens, there is a lack of data focused on bacteria associated with antimicrobial resistance in the normal flora (Gaffer et al., 2019; Shi et al., 2020; Plassard et al., 2021). In contrast, Gram-negative (GN) bacteria, such as Extended-spectrum beta-lactamase (ESBL), cephalosporins (AmpC), and carbapenemase (CP)-producing *Enterobacteriaceae*, have been identified in numerous environments worldwide, including bovine (Tóth et al., 2020). ESBL/AmpC/CP producing GN bacteria have also been reported in raw milk (Ansharieta et al., 2021). The increasing prevalence of antimicrobial resistance (AMR) continues to be a significant threat to global health. The extensive use of antibiotics in both human health and control of animal diseases is gradually reducing the time it takes for resistant strains to develop and multidrug-resistant strains of bacteria may cause life-threatening infections (Jena et al., 2017; Baran, et al., 2020; Tóth et al., 2020; Ansharieta et al., 2021). *Escherichia coli* (*E. coli*) and *Klebsiella spp.* are major pollutants in the environment that are often associated with ESBL-encoding genes (Jena et al., 2017). Milk is a food source of animal origin that can deed as a reservoir for infectious bacterial diseases. The presence of *E. coli* and *Klebsiella* in raw milk is generally reported as sources of foodborne illness (Badri et al., 2017; Athanasakopoulou et al., 2021). The prevalence of ESBL-producing *E. coli* and *Klebsiella* are very high in animal-derived food products (Odenthal et al., 2016). In the light of this information, we aim to determine the status of blaCTX-M-1, blaCTX-M-2, blaTEM and blaSHV genes, which are responsible for extended-spectrum beta-lactamase enzyme production in *E. coli* and *Klebsiella* strains detected in raw cow milk, by real-time PCR method and to present molecular epidemiological data.

## MATERIALS and METHODS

### *Sample collection*

In our study, 50 raw cow milk offered for sale in markets in Şanlıurfa province and surrounding districts were included. All samples were taken in sterile glass bottles and quickly transferred to the laboratory in the cold chain.

### *Isolation and identification of E. coli and Klebsiella spp*

In the laboratory, 50 milk samples were examined for *Enterobacteriaceae* using the ISO 21528-1 method. 10 ml milk samples were pre-enriched using a liquid medium containing 90 ml of Tryptic Soy Broth, the samples diluted at a 1/10 ratio were inoculated on MacConkey agar medium after enrichment. After 24 hours of incubation at 37°C, the proliferated colonies were evaluated by Gram staining (Diassa et al., 2017). From suspected colonies, for the identification of the *E. coli* and *Klebsiella* strains, inoculation was done on a TSI medium, lactose and sugar fermentation and biochemical properties were utilized. Antimicrobial susceptibility tests were performed by Kirby Bauer disc diffusion method by using ampicillin, trimethoprim-sulfamethoxazole, ceftazidime, cefotaxime, meropenem, ceftriaxone, chloramphenicol, gentamicin and tetracycline antibiotic discs in Mueller-Hinton medium (Plassard et al., 2021).

### *Phenotypic ESBL detection*

The double disc synergy method was used to detect the phenotypic ESBL in strains. For this, ceftazidime, cefotaxime, ceftriaxone and aztreonam antibiotic discs were placed in Mueller hinton medium around the amoxicillin-clavulanic acid antibiotic disc and incubated at 37°C overnight. Enlargement of the inhibition zone mediated by amoxicillin-clavulanate around the antibiotic discs of ceftazidime, cefotaxime, ceftriaxone and aztreonam was accepted as phenotypic confirmation of the presence of ESBL (EUCAST, 2016).

### *DNA Extraction*

For the detection of the ESBL Genes which are *blaCTX-M-1, blaCTX-M-2, blaSHV* and *blaTEM*, the manufacturer's instructions for High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany) were followed in order to isolate DNA. DNAs were stored at -20°C.

### *Detection of blaCTX-M1, blaCTX-M2, blaSHV and blaTEM Genes with real-time PCR*

Primers used for the detection of *blaCTX-M-1*, *blaCTX-M-2*, *blaSHV* and *blaTEM*, are shown in Table 1 (Casti et al., 2016; Demirci et al., 2020). qPCR protocols to detect *blaCTX-M-1, blaCTX-M-2, blaSHV* and *blaTEM* genes were described by Demirci et al. (2020) on LightCycler 480 real-time PCR system according to manufacturer's instructions.

**Table 1.** Primers used in study to amplify the ESBL genes

| Gene | Oligo |
|---|---|
| *CTX-M1* | F: GCGTGATACCACTTCACCTC |
| | R: TGAAGTAAGTGACCAGAATC |
| *CTX-M2* | F: TGATACCACCACGCCGCTC |
| | R: TATTGCATCAGAAACCGTGGG |
| *blaTEM* | F: AGTATTCAACATTTYCGTGT |
| | R: TAATCAGTGAGGCACCTATCTC |
| *SHV* | F: ATGCGTTATATTCGCCTGTG |
| | R: TTAGCGTTGCCAGTGCTC |

## RESULTS

*Enterobacteriaceae* species were detected in 22 (44%) of 50 raw cow milk samples included in our study. Out of these strains, 18 (36%) were identified as *E. coli*, while 4 (8%) were found to be *Klebsiella* spp. were detected. When the antibiotic susceptibility profiles of the reproducing strains were examined, it was found that there was no resistance to the meropenem which belongs to the carbapenem group in the isolates. After meropenem, it was determined that the highest sensitivity was to quinolone group antibiotics such as ciprofloxacin. Total sensitivity to ampicillin was 5%. While all *Klebsiella* spp. strains showed ampicillin resistance, only one *E. coli* strain was found susceptible. Table 2 shows the susceptibility profiles of all strains against all antibiotics. In our study, we detected phenotypical ESBL production in 12 (54.5%) of 22 *Enterobacteriaceae* strains detected in raw cow milk. 10 of them were E. coli and 2 of them were Klebsiella spp. all of these strains were found to contain at least one ESBL gene. Table 3 shows the ESBL gene distribution in ESBL positive strains.

**Table 2.** *E. coli* and *Klebsiella* spp. detected in raw milk. distribution of antibiotic susceptibility status of the strains.

| Antibiotic | *E. coli* (n=18) | | *Klebsiella* spp. (n=4) | | Total (n=22) | |
|---|---|---|---|---|---|---|
| | n | % | n | % | n | % |
| Ampicillin | 1 | 6 | 0 | 0 | 1 | 5 |
| Trimethoprim-sulfamethoxazole | 4 | 22 | 1 | 25 | 5 | 23 |
| Ceftazidime | 8 | 44 | 1 | 25 | 10 | 45 |
| Cefotaxime | 10 | 56 | 2 | 50 | 13 | 59 |
| Meropenem | 18 | 100 | 4 | 100 | 22 | 100 |
| Ceftriaxone | 11 | 61 | 2 | 50 | 14 | 64 |
| Chloramphenicol | 16 | 89 | 3 | 75 | 19 | 86 |
| Gentamicin | 16 | 89 | 3 | 75 | 19 | 86 |
| Tetracycline | 14 | 78 | 2 | 50 | 16 | 73 |
| Ciprofloxacin | 17 | 94 | 3 | 75 | 20 | 91 |

**Table 3.** Distribution of ESBL genes in ESBL positive strains.

| ESBL production genes | *E. coli* | *Klebsiella* spp. | Total |
|---|---|---|---|
| *blaCTX-M-1* | 1 | 0 | 1 |
| *blaTEM* | 1 | 0 | 1 |
| *blaCTX-M-1* & *blaTEM* | 3 | 0 | 3 |
| *blaCTX-M-2* & *blaTEM* | 2 | 1 | 3 |
| *blaCTX-M-1* & *blaSHV* | 1 | 0 | 1 |
| *blaCTX-M-1* & *blaTEM* & *blaSHV* | 2 | 1 | 3 |
| Total | 10 | 2 | 12 |

## DISCUSSION

Contaminated food consumption is the most effective reason for the emergence and spread of antimicrobial resistance genes and resistant bacteria. In addition to animal husbandry, the occurrence of multidrug-resistant bacteria in the community and hospitals has increased rapidly in the last decade (Waade et al., 2021). The increase of *Enterobacteriaceae* strains, especially multidrug-resistant Gram-negative bacteria, such as *Klebsiella pneumoniae* and *Escherichia coli*, is a growing concern all over the world (Yao et al., 2007; Odenthal et al., 2016; Paghdar et al., 2020; Widodo et al., 2020; Waade et al., 2021; Salinas et al., 2021). Resistance in ESBL-producing *Enterobacteriaceae* strains is predominantly formed by the plasmid-mediated *blaSHV*, *blaTEM* and *blaCTX-M* beta-lactamase genes. While TEM and SHV genes were dominant in the 1990s and early 2000s, it is known that the balance has shifted towards the newly discovered family of CTX-M enzymes in recent years (Yao et al., 2007). ESBL-producing *Enterobacteriaceae* strains have been reported in raw milk (Uraz and Aslan, 1998; Vendramin et al., 2014; Odenthal et al., 2016; Tekiner and Özpınar, 2016; Tóth et al., 2020; Athanasakopoulou et al., 2021; Ramos et al., 2021; Waade et al., 2021). When the prevalence studies in raw cow milk were examined, Diassa et al. (2017) reported *E. coli* at a rate of 39% in Ethiopia in 2017. Vendramin et al. (2014) reported *E. coli* at a rate of 53.5% in Brazil. Altun et al. (2002) detected *E. coli* at a rate of 72.6% and *Klebsiella* spp. at a rate of 41.3% in the milk they examined in Ankara. We detected *Enterobacteriaceae* species in 22 (44%) of 50 raw cow milk samples included in our study. Eighteen (36%) of these strains were found to be *E. coli*, while 4 (8%) were found to be *Klebsiella* spp. It has been concluded that there are differences in the results of the study and that the rates may be affected by the hygiene conditions, and therefore there may be similarities or differences with our study results.

When the studies examining the phenotypic ESBL productions were controlled, Uraz and Arslan (1998) found a rate of 13.99% in *E. coli* and 12.59% in *Klebsiella* spp. Tekiner and Özpınar (2016) detected 80% in *E. coli* and 3.6% in *Klebsiella* spp. In our study, we detected ESBL production in 12 (54.5%) of 22 *Enterobacteriaceae* strains detected in raw cow milk that we included in our study. It is thought that the results may contain regional differences.

When the studies examining the genes causing ESBL production were controlled, Tekiner and Özpınar (2016) found *blaTEM*, *blaCTX-M* and *blaSHV* to be 96.4%, 53.7% and 34.5% respectively. In this study, the highest rate of togetherness of *blaTEM* and *blaCTX-M* was reported at 52% (14). Jouini et al. (2007) detected 10 (26%) of 38 *E. coli* strains in Tunisia. They detected *blaCTX-M-1* in 5 of these strains. When we analyzed the genes of 12 *E. coli* strains that we detected in our study, we found *blaCTX-M-1* in 7 isolates (58.3%), *blaCTX-M-2* in 2 strains (16.7%), *blaTEM* in 8 strains (66.7%), and *blaSHV* in 3 strains (25%). Table 3 shows the genes of 12 *E. coli* strains that we detected in our study. Similar to the studies we examined in our country, it is seen that the CTX-M-based ESBL production genes are predominant.

## CONCLUSION

In conclusion, our data show that there are flora originated strains such as *E. coli* and *Klebsiella* spp. and it was determined that these strains can produce ESBL. It was also determined that CTX-M-based ESBL genes predominated in ESBL productions and there is a coexistence of different ESBL production genes in the isolates. We believe that in our world where antibiotic resistance is a problem, molecular surveillance of ESBL genes in frequently used foods such as raw milk should be done routinely to determine epidemiological data.

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