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Investigation of Extended-Spectrum Beta-Lactamase Producing *Escherichia Coli* in Milk on Dairy Cattle Farm

Çiftliklerden Alınan İnek Sütlerinde Genişlemiş Spektrumlu Beta-Laktamaz Üreten *Escherichia Coli*'nin Araştırılması

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ABSTRACT

The present study was conducted to investigate the presence of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* (*E. coli*) in cattle farm milk mixtures. Thirty two farm milks from 32 farms in Gölhisar district of Burdur city (Türkiye) were concurrently cultured on MacConkey agar plates supplemented with either 2 µg/mL cefotaxime or 2 µg/mL ceftazidime. After incubation at 37°C for 24 h under aerobic conditions, biochemical/physiological tests were applied to the lactose positive colonies for identification of *E. coli* in the cultures. Subsequently, determination of ESBL production in *E. coli* isolate was done by the combined disk method. In order to determine the ESBL class, the isolates were screened for CTX-M, SHV and TEM genes by polymerase chain reaction (PCR). After the selective culture of the milk samples, one (1/32, 3.12%) E. coli was isolated. The isolate confirmed as an ESBL procedure by the combined disc method harbored the CTX-M group 1 ESBL gene, but tested negative for TEM and SHV genes. This study showed that ESBL-producing *E. coli* could be found at low level in farm milks of the cattle farms in the region. Although the source of the ESBL-producing *E. coli* isolated from the farm milk mixture was not identified in this study, it is important that this bacterium was detected in the milk to be consumed

Keywords: Beta-lactamase, cattle milk, CTX-M gene, extended-spectrum beta-lactamase, *Escherichia coli*

ÖZ

Bu çalışma, sığır çiftliği süt karışımlarında genişlemiş spektrumlu beta-laktamaz (ESBL) üreten Escherichia coli'nin varlığını araştırmak amacıyla yapıldı. Burdur ili Gölhisar ilçesindeki 32 çiftlikten alınan 32 çiftlik sütü, 2 µg/ml sefotaksim veya 2 µg/ml seftazidim ilave edilmiş MacConkey agar besi yerlerine eş zamanlı olarak ekildi. Aerobik şartlarda 37°C'de 24 saat inkübasyonun ardından, kültürlerde *E. coli*'nin tanımlanması amacıyla laktoz pozitif kolonilere biyokimyasal/fizyolojik testler uygulandı. Daha sonra *E. coli* izolatında ESBL üretiminin tespiti kombine disk yöntemiyle yapıldı. ESBL sınıfını belirlemek amacıyla, izolatlarda CTX-M, SHV ve TEM genleri polimeraz zincir reaksiyonu (PZR) yöntemiyle tarandı. Süt örneklerinden selektif kültür yapıldıktan sonra 1 adet (1/32, %3,12) *E. coli* izole edildi. Kombine disk yöntemiyle ESBL ürettiği doğrulanan izolatın CTX-M grup 1 sınıf ESBL genini taşıdığı, TEM ve SHV sınıflarına sahip olmadığı belirlendi. Bu çalışma, bölgedeki sığır işletmelerinin çiftlik sütlerinde ESBL üreten *E. coli*'nin düşük düzeyde bulunabileceğini göstermiştir. Bu çalışmada bir çiftlikten elde edilen çiftlik sütü karışımında tespit edilen ESBL üreten *E. coli* izolatının kaynağı araştırılmamış olsa da, tüketilecek sütte bu bakterinin tespit edilmiş olması önemlidir.

Anahtar Kelimeler: Beta-laktamaz, CTX-M geni, *Escherichia coli*, genişlemiş spektrumlu beta-laktamaz, sığır sütü

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INTRODUCTION

The emergence of multidrug resistance in Gram-negative bacteria of animal origin, driven by antibiotic use in livestock, poses a significant threat not only to animal health but also to human health and ecological balance. The resistance developed by gram-negative bacteria by producing beta-lactamase enzymes, which destroy beta-lactam class antibiotics in particular, is very critical because such resistance is genetically mediated and the beta-lactamase genes can be horizontally transferred among bacteria. In addition, the high diversity of beta-lactamase enzymes makes the situation even worse because the substrate (antibiotic) specificity of each beta-lactamase variety varies.[1] Extended-spectrum beta-lactamases (ESBL), which constitute an important group in this diversity, are produced especially by bacteria belonging to the *Enterobacterales* family and have increased in both microflora and pathogenic strains in recent years.[2,3]

ESBL production by bacteria makes them resistant to 1st-4th generation cephalosporins, penicillins and monobactams.[1] In recent years, the increased use of 3rd and 4th generation cephalosporins in farm animals is thought to play an additional role in the increase of ESBL-producing Gram negatives.[4]

There are multiple classes of ESBL genes identified in gram-negative bacteria. The most common of these in *E. coli* isolates are TEM (temoneira), SHV (sulfhydryl variable genes) and CTX-M (cefotaximase) and the isolation proportion of *E. coli* isolates, especially those carrying variants in the CTX-M class, has increased in the last 20 years.[5] This is the case for *E. coli* strains isolated from both farm animals and humans.[6]

The presence of ESBL-producing *E. coli* has been demonstrated in cattle populations in different parts of Türkiye in the last 15 years.[7-10] Although there are some studies in Türkiye on the presence of ESBL-producing *E. coli* in milk obtained from farms, there are not sufficient numbers.[11-14] In terms of cattle farm milks of Burdur city of Türkiye, there is no research conducted or data available on the presence of ESBL-producing *E. coli* and/or other Gram negatives. Given that approximately two-thirds of the population in Burdur is engaged in agriculture and animal husbandary, understanding the microbiological quality of dairy products is particularly relevant for regional public health. Burdur, with 40% of its economy based on milk production, has an important share in this field with over a thousand tons of raw milk production per day. Burdur livestock sector has reached 195 thousand cattle and 365 thousand sheep by 2023.[15] Gölhisar district has an important place in Burdur livestock and the cattle population has reached 15,500 according to 2022 data. The area of Gölhisar district of Burdur city is 575.820 km².[16]

The presence of ESBL-producing *E. coli* in farm milks may be due to various reasons. One of these may be subclinical mastitis caused by ESBL-producing *E. coli*. Others may be fecal-environmental contamination due to poor milking hygiene, cross contamination due to poor cleaning and disinfection of milk tanks and post-milking contamination of bacteria from humans into milk. For whatever reason, the presence of ESBL-producing *E. coli* in milk to be consumed is not a desirable situation and should be evaluated from One-health perspective.

By this study, we aimed to investigate the presence of ESBL-producing *E. coli* in farm milk mixtures brought to a milk collection center which is in Gölhisar district of Burdur city (Türkiye) and to determine the class of ESBL genes in the isolate(s) to be determined.

MATERIALS AND METHODS

This study was approved by the Mehmet Akif Ersoy University Animal Experiments Local Ethics Committee (Date: 02.10.2024; Decision Number:1373).

Samples

In our study sampling unit consisted of farm milk which is mixture of milk from the cows on a farm. A visit was conducted to a milk collection unit which is in Golhisar district of Burdur province where 32 farm milk samples were collected from 32 dairy cattle farms located in Gölhisar district. At least 5 milliliters of milk were taken into sterile tubes from the containers of farmers and the milk samples were transferred to the laboratory under cold conditions within 2 hours at most.

Selective Isolation and Identification

Milk samples were simultaneously plated on MacConkey agar (MCA) medium (Merck) supplemented with 2 µg/ml ceftazidime (Sigma-Aldrich) or 2 µg/ml cefotaxime (Sigma-Aldrich). After the cultures were incubated at 37°C for 24 h under aerobic conditions, the lactose-positive colonies were passaged onto Tryptic Soy agar (TSA) medium (Merck) to obtain pure culture. Then, A Gram negative rod with positive lactose and glucose utilization and no H_2S production in TSIA test tube, positive mannitol utilization, positive indole test, negative urease test, positive catalase test, negative citrate test, negative oxidase test and positive nitrate reductation test results was accepted as *E. coli*.[17]

ESBL Confirmation Test

Subsequently, ESBL production of the isolates was determined by the combined disc method, following the Clinical and Laboratory Standards Institute Standards (CLSI).[18] First, a few colonies were taken from the 24-hour culture of the isolate on TSA medium, its suspension was prepared in Tryptic Soy broth (TSB)(Merck) and turbidity was adjusted to 0.5 McFarland standard. After this suspension is spread on Mueller Hinton Agar (MHA) medium (Merck), cefotaxime (30 µg), cefotaxime-clavulanic acid (30/10 µg), ceftazidime (30 µg) and ceftazidime-clavulanic acid (30/10 µg) discs (Bioanalyse, Türkiye) with a distance of at least 3 cm between them were placed. Then, the petri dish was incubated at 37°C for 24 h and the inhibition zone diameters around the discs were measured. The inhibition zone around at least one of the discs containing clavulanic acid was at least 5 mm wider than the inhibition zone around the disc without clavulanic acid was accepted as evidence for ESBL production. In the combined disc method, *K. pneumoniae* ATCC 700603 was used as the positive control strain producing ESBL, and *E. coli* ATCC 25922 strain was used as ESBL nonproducer for negative control.[18] In the evaluation of zone diameters, values recommended by CLSI as ≤27 mm for cefotaxime and ≤22 mm for ceftazidime were accepted as evidence of ESBL production.[18]

DNA Extraction

A suspension at McFarland 5.0 turbidity was prepared by taking colonies from 24 h culture of the isolate on TSA medium. After the suspension was frozen at -20°C for 24 h, it was kept in the heating block set at 100°C for 10 min. Then, the suspension was cooled on ice, it was centrifuged at 14.000 rpm for 5 min and finally 20 µl of the supernatant from upper phase was collected, taking care not to shake the tube, and stored at -20°C to use as template DNA in PCR tests.

PCR for Identification of Esbl Genes

Information about the primers used in PCR tests performed for the detection of CTX-M, SHV and TEM class ESBL genes is presented in Table 1.[19-23] As the control strains in PCR; DNA of *E. coli* NCTC 13461 strain was used in both CTX-M gene and CTX-M group 1 PCR, DNA of *K. pneumoniae* ATCC 700603 strain was used for SHV gene PCR, and DNA of *E. coli* ATCC 35218 strain was used for TEM gene PCR. DNA of *E. coli* ATCC 25922 strain was used as negative control for all PCRs.

Table 1. Details of the primers used in PCR tests performed for ESBL genes

Target gene	Nucleotide sequence of primer	Amplicon	Reference
CTX-M universal	F: 5'-SCS ATG TGC AGY ACC AGT AA-3' R: 5'-CCG CRA TAT CRT TGG TGG TG-3'	543 bp	Heffernan et al.[19] ,Saladin et al.[22]
CTX-M group 1	F: 5'-CCC ATG GTT AAA AAA TCA CTG-3' R: 5'-CCG TTT CCG CTA TTA CAA AC-3'	891 bp	Heffernan et al.[19], Jeong et al.[23]
SHV	F: 5'-GCC GGG TTA TTC TTA TTT GTC GC-3' R: 5'-TCT TTC CGA TGC CGC CGC CAG TCA-3'	1007 bp	Heffernan et al.[19], Tenover and Rasheed[20]
TEM	F: 5'-GTA TCC GTC CAT GAG ACA ATA-3' R: 5'-TCC AAA GTA TAT ATG AGT AAA C-3'	966 bp	Heffernan et al.[19], Arpin et al.[21]

F: Forward primer; R: Reverse primer; S:G/C; Y:C/T; R:A/G; bp: Base pair

In PCR test to determine the CTX-M gene performed with CTX-M universal primers (Table 1); PCR mix consisted of 2.5 µl 10xKCl buffer, 1.5 µl $MgCl_2$ (25 mM), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 2.5 µl dNTP mix (2 mM each), 1.5 Units (0.3 µl) Taq DNA polymerase, 13.5 µl ddH_2O and 3 µl template DNA. The thermocycler program consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, primer binding at 54°C for 30 sec, and extension at 72°C for 1 min, and lastly a final extension step of 7 min was performed at 72°C.[8]

In PCR test for CTX-M group 1; the PCR reaction was performed with 2.5 µl 10xKCl buffer, 1.5 µl $MgCl_2$ (25 mM), 0.5 µl forward primer (10 µM) and 0.5 µl reverse primer (10 µM)(*Table 1*), 2.5 µl dNTP mixture (2 mM each), 1.5 Units (0.3 µl) Taq DNA polymerase, 15 µl ddH_2O and 2.5 µl template DNA. The thermocycler program consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, extension at 72°C for 1 min, and finally final extension at 72°C for 7 min[8].

In PCR test for the SHV gene; the PCR reaction was performed with 2.5 µl 10xKCl buffer, 1.5 µl $MgCl_2$ (25 mM), 1 µl forward primer (10 µM) and 1 µl reverse primer (10 µM)(Table 1), 2.5 µl dNTP mixture (2 mM each), 1.5 U (0.3 µl) Taq DNA polymerase, 13.5 µl ddH_2O and 3 µl template DNA. The thermocycler program consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of

denaturation at 94°C for 30 sec, primer annealing at 58°C for 30 sec, and extension at 72°C for 1 min, and finally a final elongation at 72°C for 7 min.[8]

In TEM gene specific PCR test; PCR mix contained 2.5 µl 10xKCl buffer, 1.5 µl $MgCl_2$ (25 mM), 1 µl forward primer (10 µM) and 1 µl reverse primer (10 µM)(Table 1), 2.5 µl dNTP mixture (2 mM each), 1.5 U (0.3 µl) Taq DNA polymerase, 14 µl ddH_2O and 2.5 µl template DNA. The thermocycler program was as follow: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer binding at 48°C for 1 min, and extension at 72°C for 1 min, and finally a final elongation at 72°C for 10 min.[8]

PCR products were run in a prepared 1% agarose gel and a constant voltage of 85 V for 45 minutes. DNA bands were visualized and photographed by a gel imaging system (EDAS 290-Kodak) under ultraviolet light.

Susceptibility to Other Classes of Antibiotics

The susceptibility of ESBL-producing *E. coli* in our study to beta-lactam antibiotics and antibiotics belonging to other classes was investigated by agar disk diffusion test (ADDT) on MHA according to CLSI protocols.[18] The antibiotic disks, containing beta lactams, used in the test were: ampicillin (AMP, 10 µg), aztreonam (ATM, 30 µg), cefepime (FEP, 30 µg), cefpodoxime (CPD, 10 µg), ceftriaxone (CRO, 30 µg), cefuroxime (CXM, 30 µg), cephalothin (CEF, 30 µg), ceftiofur (FUR, 30 µg) and imipenem (IPM, 10 µg). Antibiotics from other classes tested were; chloramphenicol (CHL, 30), ciprofloxacin (CIP, 5 µg), enrofloxacin (ENR, 5 µg), florfenicol (FFC, 30 µg), gentamicin (GEN, 10 µg), kanamycin (KAN, 30 µg), nalidixic acid (NAL, 30 µg), streptomycin (STR, 10 µg), sulfamethoxazole-trimethoprim (SXT, 25 µg) and tetracycline (TET, 30 µg)(Bioanalyse, Türkiye). An isolate resistant to at least one agent in three or more antibiotic classes was accepted as multidrug resistant isolate.

The evaluation of the infection zone diameters was performed according to the following criteria: Growth inhibition zone of AMP, CAZ, CHL, IPM, STR, GEN, SXT, KAN, FFC, TET, CPD, ENR and FUR discs were evaluated according to CLSI document VET01S-ED7[24]; zone of ATM, CIP, CRO, CTX and CXM and NAL discs were evaluated according to CLSI document M 100 ED34.[25]

RESULTS

As a result of the simultaneous culture of 32 farm milk samples in the study on MCA medium supplemented with 2 µg/ml cefotaxime or 2 µg/ml ceftazidime, *E. coli* suspicious colonies in red colour (lactose positive) were seen in both selective cultures of only one sample. After obtaining the pure culture of the isolate and performing identification tests, it was determined that the isolate was *E. coli*.

After the combined disk method, the *E. coli* isolate was confirmed to be ESBL producer. In the PCR performed for CTX-M, SHV and TEM, it was found that the isolate carried the CTX-M gene but did not posses the TEM and SHV genes. Then, in the PCR specific for CTX-M group 1, it was determined that the isolate carried the group 1 CTX-M gene.

In the ADDT performed to determine the susceptibility of the isolate to various antibiotics belonging to the beta lactam group and other classes of antibiotics, the isolate was found resistant to tested beta lactams (AMP, ATM, CAZ, CEF, CPD, CRO, CTX, FEP and FUR) except IPM, aminoglycosides (GEN, KAN and STR), phenicols (CHL and FFC), tetracyclines (TET) and folate pathway inhibitors (SXT) but sensitive to fluoroquinolones (CIP, ENR and NAL). The isolate possesed a multiple antibiotic resistance phenotype, due to its resistance to more than two classes of antibiotics.

DISCUSSION

In studies conducted in different parts of Türkiye, the presence of ESBL-producing *E. coli* in cattle milk tank samples was investigated. Kurekci et al.[13] identified ESBL-producing *E. coli* in 14 (22.6%) of 62 milk tank milk samples in Hatay province, and the highest proportion of isolates (12 out of 14 ESBL-producing *E. coli*) contained CTX-M group 1 genes (CTX-M-15 and CTX- M-1). Özpinar et al.[11] isolated a total of 23 (17.04%) ESBL-producing *E. coli* in 135 milk tank milk samples collected from farms in Istanbul, Kocaeli and Sakarya cities. Tepeli et al.[12] isolated ESBL-producing *E. coli* in 5 (29.41%) of 17 milk tank milk samples collected from Çanakkale city. In our study, only 1 (3.12%, 1/32) ESBL-producing *E. coli* was isolated from 32 farm milk mixture samples, which seems considerably low compared to the research results in the mentioned cities in Türkiye.[11-13] However, it is not entirely possible to compare our results with the results of the mentioned studies since in our study, a milk sample is a mixture of milks from cows in one farm, while in others it is most likely a mixture of milk from many farms.[11-13] Therefore, we can think that the reason for the low prevalence in our study is that the milk samples in the study were collected by different approach. In the studies performed with similar sampling method for milk to our study, ESBL-producing *E. coli* was not detected in 100 milk tank samples from 100 farms in Switzerland[26] and in 85 farm milk tanks in Çanakkale city in Türkiye.[14] Similarly, in a study in Colombia, ESBL-producing *E. coli* was detected in 3.3% of 120 milk samples taken from 120 farm milk tanks[27], similar to low level like our study. As a result, it will be possible to state that the probability of isolating ESBL-producing *E. coli*

in the tank milk sample may vary depending on how many farm milks that a tank milk contains.

In the present study, ESBL-producing *E. coli* isolated from milk was CTX-M type ESBL producer, not TEM or SHV type ESBL producer. Similarly, Kurekci et al.[13] identified 14 ESBL-producing *E. coli* in tank milk samples in Hatay province in Türkiye, and 12 out of them contained CTX-M group 1 genes (CTX-M-15 and CTX- M-1) and it was highest proportion among the ESBL genes they detected. Vazquez et al.[27] found that all four *E. coli* isolates from farm milk tanks in their study in Colombia carried the CTX-M group 1 gene. Moreover, studies worldwide show also that CTX-M genes have been detected more frequently in normal flora and pathogenic *E. coli* isolates in both humans and animals and *E. coli* isolates causing foodborne infections in human than TEM and SHV type ESBL genes.[28-31] In a study covering 3 years (July 2015 and June 2018) in India, Devi et al.[28] showed that the proportions of *E. coli* isolated from community-acquired infections in humans carrying only CTX-M gene were 78.9% in Year 1, 80.8% in Year 2 and 91.1% in Year 3. Heldall et al.[29] compared *E. coli* strains isolated from urinary tract infections in elderly humans in Sweden between 2003-2005 and 2008-2009 time periods and showed that the proportion of CTX-M beta-lactamase gene detection increased significantly. Park et al.[30] reported that 80 ESBL-producing *E. coli* strains isolated from humans with foodborne diarrhea carried CTX-M gene the most (79/80) among ESBL genes. Mandujano-Hernández et al.[31] reported in their review article that the CTX-M beta-lactamase gene is found to be more prevalent in domestic and farm animals compared to other ESBL genes.

In the present study, ESBL producing *E. coli* was found resistant to aminoglycosides (GEN, KAN and STR), phenicols (CHL and FFC), tetracyclines (TET) and folate pathway inhibitors (SXT) in addition to beta-lactams, therefore it was a multidrug resistant ESBL-producing *E. coli* isolate. Similarly, multidrug characteristics of ESBL producing *E. coli* isolates have been shown in many studies.[32-34] The tendency of ESBL producing *E. coli* to show multiple antibiotic resistance is attributed to the fact that they are encoded on common plasmids with resistance genes that confer resistance to other antibiotics. Therefore, widespread use of antibiotics increases the selection of these plasmids and causes their spread in Gram-negative bacteria, including *E. coli*.[32-34]

The increase in ESBL-producing bacteria in farm animals has been found to be associated with the increase in ESBL-producing bacteria in humans. The role of consumption of animal foods (milk, meat and eggs) in this relationship is also obvious.[35] Transmission of ESBL-producing *E. coli* into cow milk may be caused by mastitis or through environmental contamination during milking/transportation or human originated contamination during milking/processing. In this study, the milk samples were taken from farm milk containers at the milk collection center, so ESBL-producing *E. coli* we detected may have been caused by one of the mastitis, environmental or human originated contamination sources. Thus, determining the source of ESBL-producing *E. coli* detected in farm milk through more detailed studies will pave the way for the necessary measures to be taken to obtain better quality cow milk for human consumption.

In conclusion, the detection of low-level ESBL-producing *E. coli* in farm milk in Burdur province is a positive finding. However, continuous monitoring of ESBL-producing *E. coli* levels in farm milk in Burdur province is important for both animal and public health.

Ethics Committee Approval: This study was approved by the Mehmet Akif Ersoy University Animal Experiments Local Ethics Committee (Date: 02.10.2024; Decision Number:1373).

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