MOLECULAR CHARACTERIZATION OF ESBL-PRODUCING ESCHERICHIA COLI ISOLATED FROM HEALTHY CATTLE AND SHEEP

PEHLIVANOGLU Faruk1*, TURUTOGLU Hulya1, OZTURK Dilek1, YARDIMCI Hakan2

1Department of Microbiology, Faculty of Veterinary Medicine, Mehmet Akif Ersoy University, Burdur, Turkey; 2Department of Microbiology, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey

(Received 28 March; Accepted 26 September 2016)

The present study aims to characterize ESBL-producing Escherichia coli isolated from healthy cattle and sheep in the Burdur province of Turkey. Fecal samples from a total of 200 cattle and 200 sheep were tested and ESBL-producing E. coli was isolated from 31 (15.5%) cattle and three (1.5%) sheep samples using the Clinical and Laboratory Standards Institute’s combined disk method. Among the ESBL gene classes detected by PCR, \( \text{bla}_{\text{CTX-M}} \) was the most frequent type, followed by the \( \text{bla}_{\text{TEM}} \) and \( \text{bla}_{\text{SHV}} \) families. ESBL-producing E. coli isolates showed co-resistance to multiple classes of antibiotics including aminoglycosides, phenicols, quinolones, folate pathway inhibitors and tetracyclines. The resistance rates were higher in the cattle isolates than in the sheep isolates. Phylogenetic grouping of the E. coli isolates indicated group A (particularly A1) was the predominant phylogenetic group (19/34, 55.9%), followed by groups B1 (9/34, 26.5%) and D (6/34, 17.6%); none of the isolates belonged to group B2. The study shows that ESBL-producing E. coli isolates exist in the intestinal flora of healthy cattle and sheep in the Burdur province of Turkey. This is the first report showing the emergence of CTX-M type ESBL-producing E. coli in sheep farms in Turkey.

Key words: cattle, ESBL, Escherichia coli, multidrug-resistance, sheep

INTRODUCTION

Extended-spectrum beta-lactamases (ESBLs) are hydrolytic enzymes produced by Gram-negative bacteria, and they confer resistance to many important antibiotics including penicillins, 1st - 4th generation cephalosporins and monobactams; ESBLs are not active against carbapenems (e.g., imipenem, meropenem and ertapenem) or cephemycins (e.g., cefoxitin). ESBLs are usually inhibited by beta-lactamase inhibitors (e.g., clavulanic acid and tazobactam), which are commonly utilized for laboratory

*Corresponding author: e-mail: pehlivanoglu@mehmetakif.edu.tr

520
detection and confirmation of ESBLs [1-3]. In the recent years, there has been a steady increase in the emergence of ESBL-producing members of *Enterobacteriaceae* around the globe, which presents a major challenge for healthcare and is in part a consequence of selective pressure generated by the extensive use of oximino-cephalosporins in the treatment of bacterial infections [4]. The most frequently encountered ESBLs in *Enterobacteriaceae* belong to the TEM, SHV and CTX-M families [1,3]. TEM and SHV variants with ESBL activity have been largely derived from TEM-1/TEM-2 and SHV-1 beta-lactamases respectively [5]. On the other hand, *bla* 

CTX-M genes have been captured from the chromosome of *Kluyvera* spp. onto the conjugative plasmids that mediate their dissemination among *Enterobacteriaceae* [6]. CTX-M enzymes can be subclassified into clusters 1, 2, 8, 9 and 25, based on similarities in amino acid sequences [7].

The presence of ESBL-producing *Escherichia coli* has been described in cattle and sheep populations around the world [8-12]. However, very limited information is available on the presence and extent of ESBL-producing bacteria in cattle and sheep populations in Turkey. To date, only a few local studies [13-15] have been conducted, and the majority focused only on the phenotypic detection of ESBL-producing *E. coli*, without detailed characterization of the ESBL types involved. However, in a small-scale study conducted by Kucukbasmaci et al. [15], ESBLs detected in fecal *Enterobacteriaceae* isolates from cattle and sheep in northwest of Turkey were identified, and none of them were of the CTX-M type. This finding was somewhat surprising considering that CTX-M has been increasingly identified in many different sources including humans, animals and the environment and that it has virtually displaced the other ESBLs within *Enterobacteriaceae* during the last decade [16]. Therefore, the present study was conducted to characterize the ESBL genes found in fecal *E. coli* isolated from healthy cattle and sheep.

**MATERIAL AND METHODS**

*Study population and sampling*

The present study was conducted on dairy cattle and sheep populations in Burdur province located in the southwest of Turkey. The study included 16 herds of dairy cattle (Holstein) and 12 flocks of sheep (Awassi) selected using the random sampling method. For sample collection, 200 healthy cattle (≥ 12 months of age) and 200 healthy sheep (≥ 6 months of age) were selected by random sampling. Fecal samples from each cow and sheep were taken directly from the rectum.

*Selective isolation and confirmation of ESBL-producing isolates*

An enrichment procedure was performed to increase the total bacterial population before culturing the fecal samples for ESBL-producing *E. coli*. A 10% suspension of fecal sample in buffered peptone water (Lab M, UK) was prepared and mixed using a vortex mixer. After incubation of the suspension at 37°C for 24 hours under
aerobic conditions, 50 μl was evenly spread onto Brilliance *E. coli*/coli form selective agar (Oxoid, UK) supplemented with cefotaxime (CTX, 2 μg/ml) (Sigma-Aldrich, Germany) or ceftazidime (CAZ, 2 μg/ml) (Sigma-Aldrich, Germany) at the same time and incubated for another 24 hours at 37°C under aerobic conditions.

One colony from each plate (one colony from the selective agar supplemented with CTX and one from the selective agar supplemented with CAZ) per positive sample was selected randomly and subcultured on Tryptic Soy agar (Oxoid, UK) for identification. After *E. coli* identification using conventional methods (Gram staining, acid and gas from glucose, catalase test, citrate utilization, decarboxylation of lysine, hydrogen sulphide production, indole production, methyl red-voges proskauer test, orthonitrophenyl-beta-D-galactopyranoside activity, oxidase test and urease production) [17], the isolates were subjected to genetic confirmation by PCR amplification of a 401 bp fragment of the *E.coli* 16S rRNA gene [18].

ESBL production by *E. coli* isolates was confirmed using the combined disc method recommended by the Clinical and Laboratory Standards Institute (CLSI)[19].

**Antibiotic susceptibility testing**

One isolate from each medium supplemented with CTX or CAZ per positive sample was subjected to susceptibility testing against nine beta-lactam antibiotics using the agar disc diffusion test following CLSI protocols [19]. The tested antibiotic discs (Oxoid, UK) that were: ampicillin (AMP 10 μg), aztreonam (ATM 30 μg), cefepime (FEP 30 μg), cefoxitin (FOX 30 μg), cefpodoxime (CPD 10 μg), ceftiraxone (CRO 30 μg), cefuroxime (CXM, 30 μg), cephalothin (CEF 30 μg), and imipenem (IPM 10 μg). Results were evaluated in accordance with CLSI criteria [19, 21].

In addition to susceptibility to beta-lactam antibiotics, the isolates were also tested for susceptibility to aminoglycosides (gentamicin: GEN, kanamycin: KAN, streptomycin: STR), quinolones (ciprofloxacin: CIP, enrofloxacin: ENR and nalidixic acid: NAL), folate pathway inhibitors (sulfamethoxazole-trimethoprim: SXT), phenicols (florfenicol: FFC) and tetracyclines (tetracycline: TET) using the agar disc diffusion test recommended by CLSI [19]. The antibiotic discs (Oxoid) that were tested were: CIP (5 μg), ENR (5 μg), FFC (30 μg), GEN (10 μg), KAN (30 μg), NAL (30 μg), STR (10 μg), SXT (23.75 + 1.25 μg) and TET (30 μg). Results were evaluated using CLSI criteria [19-21].

The isolates were classified as resistant, intermediate or susceptible [19-21]. *E. coli* isolates of a single fecal sample cultured on the two selective media containing CTX or CAZ and with the same antibiotic susceptibility profile were considered to be the same isolate in this study. Multidrug-resistance was defined as resistance to at least 3 different classes of antibiotics excluding beta-lactams.
Polymerase chain reaction and sequencing

DNA from *E. coli* isolates with confirmed ESBL production was extracted using a genomic DNA purification kit (Thermo Fisher Scientific Inc., Massachusetts, USA) and tested by PCR with specific primers for the *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> and *bla*<sub>CTX-M</sub> genes as described elsewhere [22-27] with slight modifications in cycling conditions. Tag DNA polymerase enzyme, deoxyribonucleotide triphosphates and buffers used in the PCR mixture were obtained from Thermo Fisher Scientific Inc. (Massachusetts, USA). The cycling conditions for detection of the *bla*<sub>TEM</sub> gene were as follows: initial denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C for 1 min, with a final elongation at 72 °C for 10 min. The cycling conditions for *bla*<sub>SHV</sub> gene detection were initial denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 30 sec, 58 °C for 30 sec and 72 °C for 1 min, with a final elongation at 72 °C for 7 min. The cycling conditions for *bla*<sub>CTX-M</sub> gene (universal) detection were initial denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 30 sec, 54 °C for 30 sec and 72 °C for 1 min, with a final elongation at 72 °C for 7 min. The cycling conditions for detection of *bla*<sub>CTX-M</sub> group 1, 2, 8/25 and 9 genes were as follows: initial denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, with a final elongation at 72 °C for 7 min.

*E. coli* ATCC 35218 (*bla*<sub>TEM</sub>) and *K. pneumoniae* ATCC 700603 (*bla*<sub>SHV</sub>) were used as positive control strains for *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> encoding genes. *E. coli* NCTC 13461, *E. coli* NCTC 13462, *E. coli* NCTC 13463, *Enterobacter cloacae* NCTC 13464 and *K. pneumoniae* NCTC 13465 were used as positive controls for the detection of *bla*<sub>CTX-M</sub> group 1, group 2, group 8, group 9 and group 25 genes, respectively. *E. coli* ATCC 25922 was used as a negative control for all PCRs.

To demonstrate *bla*<sub>CTX-M</sub> and *bla*<sub>TEM</sub> gene diversity in the cattle population, sequence analysis of the respective genes was performed. *E. coli* isolates were selected according to their antibiotic susceptibility profiles and phylogenetic groups. Nine *E. coli* isolates belonging to three phylogenetic groups (A, B1 and D) with nine different antibiotic susceptibility profiles were selected from all of the cattle farms positive for ESBL (*n* = 8) for further study. For sequence analysis of the *bla*<sub>TEM</sub> gene, 10 *E. coli* isolates (from five farms) belonging to three phylogenetic groups (A, B1 and D) with nine different antibiotic susceptibility profiles were also included in the study. To determine *bla*<sub>SHV</sub> gene diversity, we sequenced all of the PCR products (*n* = 3) that were obtained even if the *E. coli* isolates were from a single farm and belonged to the same phylogenetic group. All PCR products (3 *bla*<sub>CTX-M</sub> and 2 *bla*<sub>TEM</sub>) from *E. coli* isolates from sheep were sequenced. DNA sequencing of PCR products was performed by Refgen Genetical Research and Biotechnology (Golbasi-Ankara, Turkey). Sequencing was carried out on both strands using the same primer pairs that were used in the PCR. These sequences were then compared to the NCBI GenBank sequences using BLAST to confirm the subtypes of beta-lactamase genes. Finally, these sequences were submitted to the NCBI GenBank.
Phylogenetic analysis

To reveal whether ESBL-producing *E. coli* isolates belonging to a particular phylogenetic group were more likely to carry ESBL genes, phylogenetic typing (A, B1, B2 and D) of the isolates was performed according to a triplex PCR protocol as described [28] with modified PCR conditions [29]. To enhance strain discrimination, subgroups (A: A₀ and A₁; B₂: B₂₁ and B₂₂; D: D₁ and D₂) were also identified as previously described [30].

RESULTS

Detection of ESBL-producing *E. coli* from cattle and sheep feces

*E. coli* grew on both types of selective media (supplemented with CTX or CAZ) in 47 of the fecal samples (45 cattle and 2 sheep). The number of isolates grown on only medium containing CTX was five cattle and one sheep isolate but with only medium containing CAZ, there was only one cattle isolate. Overall, presumptive ESBL-producing *E. coli* were isolated from the fecal samples of 51 cattle and 3 sheep. Further characterization using the combined disk method confirmed that 31 of the 51 *E. coli* cattle isolates and all of the *E. coli* sheep isolates produced ESBL. Therefore, 15.5% (31/200) and 1.5% (3/200) of cattle and sheep fecal samples, respectively, were positive for ESBL-producing *E. coli*. Of the farms tested in this study, ESBL-producing *E. coli* was obtained from 50% (8/16) of the cattle herds and 25% (3/12) of the flocks of sheep.

Antimicrobial susceptibility of ESBL-producing *E. coli* strains

In antibiotic susceptibility testing for the nine beta-lactams, high resistance rates were detected in the ESBL-producing *E. coli* isolates from both cattle and sheep (Table 1). The resistance rates in the cattle isolates against ATM, CPD, CTX, CAZ and CRO, which are used in CLSI initial screening test for ESBL-producing *E. coli*, were 100%, 96.8%, 100%, 80.6% and 96.8%, respectively (Table 1).

Among the *E. coli* cattle isolates confirmed as ESBL-producing, the highest resistance rate against aminoglycosides was found for STR (71.0 %, 22/31). NAL resistance was found in 38.7% of the isolates (12/31), followed by ENR (35.5%, 11/31) and CIP (29.0%, 9/31). In addition, 48.4% (15/31) and 93.5% (29/31) of the cattle isolates were resistant to SXT and TET, respectively (Table 1). Among the ESBL-producing *E. coli* isolates from sheep, resistance was found against GEN only (66.7%, 2/3). None of the sheep isolates showed resistance against CIP, ENR, NAL, TET, SXT and FFC (Table 1). While 45.2% (14/31) of cattle isolates showed multidrug-resistance phenotypes, none of the sheep isolates were multidrug-resistant.
Table 1. Susceptibility of ESBL-producing fecal *E. coli* isolates (n = 34) from cattle and sheep against beta-lactams and other classes of antibiotics

| Beta-lactams | Cattle (n = 31) | Sheep (n = 3) | Other antibiotics | Cattle (n = 31) | Sheep (n = 3) |
|--------------|----------------|--------------|-------------------|----------------|--------------|
|              | R (n) | I (n) | R (n) | I (n) | R (n) | I (n) | R (n) | I (n) | R (n) | I (n) | R (n) | I (n) |
| AMP          | 31    | 0     | 3     | 0     | 11   | 3     | 2     | 0     |
| ATM          | 31    | n/a   | 3     | n/a   | 14   | 10    | 0     | 2     |
| FEP          | 7     | 10    | 3     | 0     | 22   | 9     | 0     | 2     |
| CTX          | 31    | n/a   | 3     | n/a   | 9    | 1     | 0     | 0     |
| FOX          | 0     | 0     | 0     | 0     | 11   | 5     | 0     | 0     |
| CPD          | 30    | n/a   | 3     | n/a   | 12   | 3     | 0     | 0     |
| CAZ          | 25    | n/a   | 3     | n/a   | 2    | 8     | 0     | 0     |
| CRO          | 30    | n/a   | 3     | n/a   | 15   | 1     | 0     | 0     |
| CXM          | 31    | 0     | 3     | 0     | 29   | 1     | 0     | 0     |
| CEF          | 31    | 0     | 3     | 0     |      |       |       |       |
| IPM          | 0     | 0     | 0     | 0     |      |       |       |       |

R, resistant; I, intermediate; n/a, not applicable.

**Molecular characterization of ESBL types**

PCR screening for the *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> and *bla*<sub>CTX-M</sub> genes in phenotypically-confirmed ESBL-producing *E. coli* isolates of cattle origin indicated that CTX-M was the most common ESBL type, detected in 87.1% (27/31) of the isolates, followed by TEM (77.4%, 24/31) and SHV (9.7%, 3/31). In the ESBL-producing *E. coli* isolates from sheep, *bla*<sub>CTX-M</sub> (100%, 3/3) and *bla*<sub>TEM</sub> genes (66.7%, 2/3) were detected, but none of the isolates carried *bla*<sub>SHV</sub> genes. Group-specific PCR indicated that all of the *bla*<sub>CTX-M</sub> genes detected in *E. coli* isolates of both cattle and sheep belonged to CTX-M group 1.

Multiple beta-lactamase genes were detected in the majority of the *E. coli* isolates tested in the study. It was determined that 67.7% (21/31) of the isolates from cattle and 66.7% (2/3) of the isolates from sheep were carriers of both *bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub> genes. Each of the *bla*<sub>CTX-M</sub> + *bla*<sub>SHV</sub> and *bla*<sub>TEM</sub> + *bla*<sub>SHV</sub> gene combinations were found in a single cattle isolate while the sheep isolates did not carry these gene combinations. None of the isolates tested in the study included the *bla*<sub>CTX-M</sub>, *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> genes together.

Of the *bla*<sub>CTX-M</sub> genes detected in the 27 *E. coli* isolates from cattle, nine were selected for further DNA sequencing. One isolate was CTX-M-3, two isolates were CTX-M-1 and six isolates were CTX-M-15 type ESBL-producers (Table 2). All PCR products of *bla*<sub>CTX-M</sub> genes (n = 3) from the sheep isolates were also sequenced; one isolate was CTX-M-3 and two isolates were CTX-M-15 producers (Table 2). Since all three of
these CTX-M types belong to the CTX-M-1 cluster, this finding indicates agreement between sequencing and group-specific PCR. Among the 24 \( \text{bla}_{\text{TEM}} \) genes detected in cattle isolates, 10 were also selected for sequence analysis, and all were found to encode TEM-1 type beta-lactamase (Table 2). Furthermore, sequence analysis of two \( \text{bla}_{\text{TEM}} \) genes detected from sheep isolates confirmed to have the TEM-1 genotype (Table 2). Sequencing of the three \( \text{bla}_{\text{SHV}} \) genes from the cattle isolates indicated the presence of SHV-12 type ESBL (Table 2).

Table 2. Distribution of ESBL types of fecal \textit{E. coli} isolates from cattle and sheep according to animal farms and phylogenetic groups

| Farm of isolates | Number of isolates | Phylogenetic group | ESBL Type |
|------------------|--------------------|--------------------|-----------|
| **Cattle**       |                    |                    |           |
| A                | 3                  | D (subgroup D\(_2\)) | CTX-M-1 (n = 1); CTX-M group 1\(^*\)(n = 2) |
| B                | 1                  | D (subgroup D\(_1\)) | CTX-M-1    |
| C                | 1                  | D (subgroup D\(_3\)) | CTX-M-15   |
| D                | 12                 | A (subgroup A\(_1\)) (n = 8) | TEM-1 (n = 1); CTX-M group 1\(^*\)+ TEM\(^*\) (n = 5); CTX-M group 1\(^*\)+ TEM-1 (n = 1); CTX-M-3 + TEM\(^*\) (n = 1) |
|                  |                    | B1 (n = 3)          | SHV-12 (n = 1); SHV-12 + TEM\(^*\) (n = 1); SHV-12 + CTX-M group 1\(^*\) (n = 1) |
|                  |                    | D (subgroup D\(_3\)) (n = 1) | TEM-1 |
| E                | 3                  | A (subgroup A\(_1\)) | CTX-M-15 + TEM-1 (n = 1); CTX-M group 1\(^*\)+ TEM\(^*\) (n = 2) |
| F                | 2                  | B1                 | CTX-M-15 + TEM-1 (n = 1); CTX-M group 1\(^*\)+ TEM\(^*\) (n = 1) |
| G                | 2                  | B1                 | CTX-M group 1\(^*\)+ TEM-1 (n = 1); CTX-M-15 + TEM\(^*\) (n = 1) |
| H                | 7                  | A (subgroup A\(_1\)) (n = 2) | CTX-M group 1\(^*\)+ TEM-1 (n = 2) |
|                  |                    | A (subgroup A\(_1\)) (n = 4) | CTX-M group 1\(^*\)+ TEM\(^*\) (n = 1); CTX-M-15 + TEM\(^*\) (n = 2); CTX-M group 1\(^*\)+ TEM-1 (n = 1) |
|                  |                    | B1 (n = 1)          | CTX-M group 1\(^*\)+ TEM-1 |
| **Sheep**        |                    |                    |           |
| A                | 1                  | A (subgroup A\(_1\)) | CTX-M-15 + TEM-1 |
| B                | 1                  | B1                 | CTX-M-15 + TEM-1 |
| C                | 1                  | A (subgroup A\(_1\)) | CTX-M-3 |

\(^*\)not sequenced

Assigned accession numbers for \( \text{bla}_{\text{CTX-M-1}} \), \( \text{bla}_{\text{CTX-M-3}} \), \( \text{bla}_{\text{CTX-M-15}} \) and \( \text{bla}_{\text{SHV-12}} \) gene nucleotide sequence data submitted in GenBank are as follows. \( \text{bla}_{\text{CTX-M-1}} \): F11 (KP162338) and F23 (KP162339). \( \text{bla}_{\text{CTX-M-3}} \): F62 (KP303590) and F187 (KP303592). \( \text{bla}_{\text{CTX-M-15}} \): F50 (KP325140), F54 (KP325141), F85 (KP325142), F97 (KP325143), F11.
F128 (KP325144), F130 (KP325145), F147 (KP325146) and F170 (KP325147). *bla*<sub>SHV-12</sub>: F57 (KP100155), F58 (KP100154) and F68 (KP162337).

**Phylogenetic types of ESBL-producing *E. coli* strains**

Of the 31 *E. coli* isolates of cattle origin that were analyzed, 17 (54.8%) belonged to phylogenetic group A, eight (25.8%) to group B1, and six (19.4%) to group D. Most of the group A isolates of cattle origin (15/17, 88.2%) belonged to subgroup A<sub>1</sub>. Of the three *E. coli* isolates of sheep origin, two (66.7%) were in group A (subgroup A<sub>1</sub>) and the third strain (33.3%) was in group B1. None of the cattle and sheep isolates belonged to group B2, the phylogenetic group most likely to be highly virulent. Distribution of the isolates according to phylogenetic groups along with the included ESBL types is given in Table 2.

Of the eight cattle herds which were positive for ESBL-producing *E. coli*, six farms had more than one isolate. Three isolates which belonged to phylogenetic group D (subgroup D<sub>2</sub>) and three isolates which belonged to group A (subgroup A<sub>1</sub>) were identified on farm A and E, respectively. On farms F and G, two isolates from phylogenetic group B1 were identified. Nevertheless on farm D, 12 isolates from three different phylogenetic groups were detected and they belonged to group A (subgroup A<sub>1</sub>, n = 8), group B1 (n = 3) and group D (subgroup D<sub>1</sub>, n = 1). On farm H, seven isolates were distributed in two different phylogenetic groups, A (subgroup A<sub>0</sub>, n = 2; and subgroup A<sub>1</sub>, n = 4) and B1 (n = 1). The SHV-12 type ESBL-producing *E. coli* isolates were from Farm D, and all isolates belonged to phylogenetic group B1. However, the additional beta-lactamase genes they carried were different; one strain carried only the *bla*<sub>SHV-12</sub> gene, the second carried both the *bla*<sub>SHV-12</sub> and *bla*<sub>TEM</sub> gene and the third had the *bla*<sub>SHV-12</sub> and *bla<sub>CTX-M</sub>* gene (Table 2).

**DISCUSSION**

Emergence and dissemination of ESBL-producing *Enterobactericeae* of animal and human origin is increasing, which is a cause for considerable concern to both medical and veterinary practitioners around the world. A number of investigations have been conducted in various parts of the world to investigate the presence and types of ESBL in cattle [8-12, 31-33], but research on ESBL in sheep is limited [8,11,33]. In Turkey, only one study has been conducted so far in which both the presence and types of ESBLs in cattle and sheep were investigated, and this was in the northwest of Turkey [15]. That study reportedly identified only three ESBL-producing *E. coli* isolates in cattle and none in sheep. Therefore, our study represents the first report of the presence of ESBL-producing *E. coli* isolates from sheep in Turkey.

The increase in the prevalence of ESBL-producing *E. coli* may be due to the clonal spread of certain ESBL-producing strains and/or horizontal transfer of ESBL-plasmids between strains of different genomic background [5]. Although the types of
ESBLs produced by *E. coli* differ depending on the animal population and geographical areas, detection rates of CTX-M type ESBLs have increased dramatically around the world during the last several years [1, 16, 34]. In line with this trend, the present study found that the *bla*<sub>CTX-M</sub> gene was the most common ESBL type detected in the phenotypically confirmed ESBL-producing *E. coli* isolates.

Among CTX-M type ESBLs, CTX-M-1, CTX-M-14 and CTX-M-15 are the most widespread and predominant ones detected in many studies reported from various countries [33,35-37]. In bovine *E. coli* strains, CTX-M-1, -14, and -15 types in France [36,37], CTX-M-14 and -15 types in the UK [35], and CTX-M-14 and -15 types in Wales [33] have been reported. In sheep, *E. coli* strains producing CTX-M-1, -14 and -15 types were detected in Switzerland [11]. Similar to the findings of these studies, CTX-M-15 was also found to be the most common ESBL-CTX-M type detected in fecal *E. coli* isolates from cattle and sheep in our study.

DNA sequencing of the *bla*<sub>TEM</sub> genes identified in *E. coli* isolates from cattle and sheep has shown that all of the isolates are TEM-1 type, which is not considered an ESBL [1]. However, of the 10 *E. coli* isolates of cattle origin carrying *bla*<sub>TEM-1</sub>, nine also carried the *bla*<sub>CTX-M</sub> gene and all of the *E. coli* isolates (n= 2) of sheep origin with *bla*<sub>TEM-1</sub> also carried the *bla*<sub>CTX-M</sub> gene. Only two cattle strains had *bla*<sub>TEM-1</sub> alone, yet exhibited the ESBL phenotype. This is likely due to the production of other ESBL types that were not investigated in the present study.

Intensive use of beta-lactams and other classes of antibiotics in the livestock industry may have contributed to the emergence of multidrug-resistant bacterial phenotypes. In Turkey, beta-lactams, aminoglycosides, phenicols, quinolones, folate pathway inhibitors and tetracyclines are widely used in cattle and sheep production for the treatment of a variety of infections (for example, enteritis, mastitis, pneumonia and septicemia). Studies performed in Turkey show that *E. coli* isolates of cattle origin are generally more resistant to various antibiotics than isolates of sheep origin [13, 38]. Likewise, we found that the overall antibiotic resistance rates of other classes in the ESBL-producing *E. coli* isolates of cattle origin were higher than those of the sheep isolates. While resistance was observed against CIP, ENR, NAL, FFC, SXT and TET in the cattle isolates, the sheep isolates were not resistant to these antibiotics. Additionally, multidrug-resistant phenotypes were observed in only *E. coli* isolates of cattle origin in the present study. The higher resistance in the cattle isolates can be attributed to use of these antibiotics more widely in the treatment of a wide variety of infections in the cattle population and co-selection of resistant isolates.

Phylogenetic grouping of *E. coli* strains shows that most commensal strains generally belong to groups A and B1, whereas group B2, and to lesser extent group D, are generally associated with virulent extraintestinal strains [28,39]. In our study, the predominant phylogenetic group was group A (particularly subgroup A1), followed by group B1 and group D. Even though none of the isolates in our study belonged to the B2 phylogenetic group, which represents the highly virulent extraintestinal *E.
coli strains, we found six E. coli isolates in the group D cluster, meaning that some of
the isolates may be also pathogenic. On the other hand, Milanov et al. [40] reported
E. coli strains from phylogenetic groups A and B1 isolated from bovine mastitis cases,
which shows that commensal E. coli strains from group A and B1 can cause various
infections in cattle.

The presence of ESBL-producing E. coli isolates from more than one phylogenetic
group indicates that there is significant diversity among E. coli isolates carrying ESBL
genes in the cattle herds and sheep flocks in this region. This is especially supported
by the presence of E. coli isolates from three different phylogenetic groups on cattle
farm D and two different phylogenetic groups on cattle farm H.

In conclusion, our study shows that bla\textsubscript{CTX-M} group 1 ESBL genes (especially bla\textsubscript{CTX-M-15})
are predominant in commensal E. coli isolates in cattle and sheep in Burdur province.
This is the first report of the presence of this gene in E. coli isolated from sheep in
Turkey. However, additional studies using a broader population should be conducted
in order to better understand the epidemiology of ESBL genes in animals in Turkey.
Furthermore, the veterinary practitioners and farmers should be informed of this
important problem and encouraged to be prudent in the use of antimicrobials for
animals.

Acknowledgements

The present study was funded by The Scientific and Technological Research Council
of Turkey (TUBITAK project number, 112O 820) and partially by Mehmet Akif
Ersoy University, Scientific Research Projects Unit (Turkey) (Project number, 0158
KAYDEP-13). We thank biologist Mert Sudagidan (Scientific and Technological
Application and Research Center, Mehmet Akif Ersoy University, Burdur, Turkey) for
technical assistance.

Authors’ contributions

FP designed the study, and carried out the sample collection, isolation and identification
of the bacterial isolates and molecular experiments (PCR and DNA sequencing), and
drafted the manuscript. HT participated in the design of the study and the laboratory
experiments and helped to draft the manuscript. DO participated in the sample
collection and the laboratory experiments, and helped to draft the manuscript. HY
participated in the design of the study and helped to draft the manuscript. All authors
read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research,
authorship, and/or publication of this article.
REFERENCES

1. Paterson DL, Bonomo RA: Extended-spectrum beta-lactamases: a clinical update. Clin Microbiol Rev 2005, 18:657–686.

2. Perez F, Endimiani A, Hujer KM, Bonomo RA: The continuing challenge of ESBLs. Curr Opin Pharmacol 2007, 7:459–469.

3. Pitout JD, Laupland KB: Extended-spectrum beta-lactamase-producing Enterobacteriaceae: an emerging public-health concern. Lancet Infect Dis 2008, 8:159–166.

4. Rossolini GM, Mantengoli E: Antimicrobial resistance in Europe and its potential impact on empirical therapy. Clin. Microbiol Infect 2008, 14 Suppl 6:2–8.

5. Livermore DM: Current epidemiology and growing resistance of gram-negative pathogens. Korean J Intern Med 2012, 27:128–142.

6. Rossolini GM, D’Andrea MM, Mugnaioli C: The spread of CTX-M-type extended-spectrum beta-lactamases. Clin Microbiol Infect 2008, 14 Suppl 1:33-41.

7. Bonnet R: Growing group of extended-spectrum beta-lactamases: The CTX-M enzymes. Antimicrob Agents Chemother 2004, 48:1–14.

8. Ben Sallem R, Ben Slama K, Sáenz Y, Rojo-Bezares B, Estepa V, Jouini A, Gharsa H, Klibi N, Boudabous A, Torres C: Prevalence and characterization of extended-spectrum beta-lactamase (ESBL-) and CMY-2-producing Escherichia coli isolates from healthy food-producing animals in Tunisia. Foodborne Pathog Dis 2012, 9:1137–1142.

9. Duan RS, SitTH, WongSS, WongRC, ChowKH, MakGC, YamWC, NgLT, YuenKY, HoPL: Escherichia coli producing CTX-M beta-lactamases in food animals in Hong Kong. Microb Drug Resist 2006, 12:145–148.

10. Friese A, Schulz J, Laube H, von Salvati C, Hartung J, Roesler U: Faecal occurrence and emissions of livestock-associated methicillin-resistant Staphylococcus aureus (laMRSA) and ESBL/AmpC-producing E. coli from animal farms in Germany. Berl Munch Tierarztl Wochenschr 2013, 126:175–180.

11. Geser N, Stephan R, Hächler H: Occurrence and characteristics of extended-spectrum beta-lactamase (ESBL) producing Enterobacteriaceae in food producing animals, minced meat and raw milk. BMC Vet Res 2012, 8:21.

12. Hiroi M, Yamazaki F, Harada T, Takahashi N, Iida N, Noda Y, Yagi M, Nishio T, Kanda T, Kawamori F, Sugiyama K, Masuda T, Hara-Kudo Y, Ohashi N: Prevalence of extended-spectrum beta-lactamase-producing Escherichia coli and Klebsiella pneumoniae in food-producing animals. J Vet Med Sci 2012, 74:189–195.

13. Aksoy A, Yildirim M, Kacmaz B, Apan TZ, Gocmen JS: Verotoxin production in strains of Escherichia coli isolated from cattle and sheep and their resistance to antibiotics. Turk J Vet Anim Sci 2007, 31:225–231.

14. Dinc G, Ata Z, Temelli S: Investigation of extended-spectrum beta-lactamase activity and antibiotic resistance profile of Escherichia coli strains isolated from bovine mastitis. Ankara Univ Vet Fak Derg 2012, 59:85–88.

15. Kucukbasmaci O, Ciftcioglu G, Midilli K, Issa G: Detection of extended spectrum beta-lactamase producing Enterobacteriaceae from food animals in Turkey. Revue Méd Vét 2008, 159:586–592.

16. Canton R, Gonzalez-Alba JM, Galan JC: CTX-M-Enzymes: Origin and Diffusion. Front Microbiol 2012, 3:110.
17. Winn, W, Allen S, Janda W, Koneman E, Procop G, Schreckenberger P, and Woods G: The Enterobacteriaceae. In: Koneman’s Color Atlas and Textbook of Diagnostic Microbiology. Sixth Edition, Lippincott Williams and Wilkins, Philadelphia, USA; 2006, 211-302.

18. Wang G, Clark CG, Rodgers FG: Detection in Escherichia coli of the genes encoding the major virulence factors, the genes defining the O157:H7 serotype, and components of the type 2 shiga toxin family by multiplex PCR. J Clin Microbiol 2002, 40:3613–3619.

19. CLSI (Clinical and Laboratory Standards Institute): Performance standards for antimicrobial susceptibility testing, 26th edition, CLSI document M100S, Wayne, Pennsylvania, USA; 2016.

20. CLSI (Clinical and Laboratory Standards Institute): Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals, approved standard, 3rd edition, CLSI document M31-A3, Wayne, Pennsylvania, USA; 2010.

21. CLSI (Clinical and Laboratory Standards Institute): Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals, 2nd informational supplement, CLSI document VET01-S2, Wayne, Pennsylvania, USA; 2013.

22. Arpin C, Dubois V, Coulange L, Andre C, Fischer I, Noury P, Grobost F, Brochet JP, Jullin J, Dutilh B, Larribet G, Lagrange I, Quentin C: Extended-spectrum beta-lactamase-producing Enterobacteriaceae in community and private health care centers. Antimicrob Agents Chemother 2003, 47:3506–3514.

23. Costa D, Poeta P, Saenz Y, Vinue L, Rojo-Bezares B, Jouini A, Zarazaga M, Rodrigues J, Torres C: Detection of Escherichia coli harbouring extended-spectrum beta-lactamases of the CTX-M, TEM and SHV classes in faecal samples of wild animals in Portugal. J Antimicrob Chemother 2006, 58:1311–1312.

24. Heffernan HM, Woodhouse RE, Pope CE, Blackmore TK: Prevalence and types of extended-spectrum beta-lactamases among urinary Escherichia coli and Klebsiella spp. in New Zealand. Int J Antimicrob Agents 2009, 34:544–549.

25. Jeong SH, Bae IK, Kwon SB, Lee JH, Song JS, Jung HI, Sung KH, Jang SJ, Lee SH: Dissemination of transferable CTX-M-type extended-spectrum beta-lactamase-producing E. coli in Korea. J Appl Microbiol 2005, 98:921–927.

26. Saladin M, Cao VT, Lambert T, Donay JL, Herrmann JL, Ould-Hocine Z, Verdet C, Delisle F, Philippon A, Arlet G: Diversity of CTX-M beta-lactamases and their promoter regions from Enterobacteriaceae isolated in three Parisian hospitals. FEMS Microbiol Lett 2002, 209:161–168.

27. Tenover FC, Rasheed JK: Detection of antimicrobial resistance genes and mutations associated with antimicrobial resistance in microorganisms. In: Molecular microbiology: diagnostic principles and practice, Washington DC, USA, ASM Publishing; 2004, 391–406.

28. Clermont O, Bonacorsi S, Bingen E: Rapid and simple determination of the Escherichia coli phylogenetic group. Appl Environ Microbiol 2000, 66:4555–4558.

29. Higgins J, Hohn G, Hornor S, Frana M, Denver M, Joerger R: Genotyping of Escherichia coli from environmental and animal samples. J Microbiol Methods 2007, 70:227–235.

30. Escobar-Páramo P, Le Menac’h A, Le Gall T, Amorin C, Gouriou S, Picard B, Skurnik D, Denamur E: Identification of forces shaping the commensal Escherichia coli genetic structure by comparing animal and human isolates. Environ Microbiol 2006, 8:1975–1984.

31. Ho PL, Chow KH, Lai EL, Lo WU, Yeung MK, Chan J, Chan PY, Yuen KY: Extensive dissemination of CTX-M-producing Escherichia coli with multidrug resistance to ‘critically important’ antibiotics among food animals in Hong Kong, 2008-10. J Antimicrob Chemother 2011, 66:765–768.
32. Ohnishi M, Okatani AT, Esaki H, Harada K, Sawada T, Murakami M, Marumo K, Kato Y, Sato R, Shimura K, Hatanaka N, Takahashi T: Herd prevalence of Enterobacteriaceae producing CTX-M-type and CMY-2 β-lactamases among Japanese dairy farms. J Appl Microbiol 2013, 115:282–289.

33. Snow LC, Wearing H, Stephenson B, Teale CJ, Coldham NG: Investigation of the presence of ESBL-producing Escherichia coli in the North Wales and West Midlands areas of the UK in 2007 to 2008 using scanning surveillance. Vet Rec 2011, 169:656.

34. Ewers C, Bethe A, Semmler T, Guenther S, Wieler LH: Extended-spectrum beta-lactamase-producing and AmpC-producing Escherichia coli from livestock and companion animals, and their putative impact on public health: a global perspective. Clin Microbiol Infect 2012, 18:646–655.

35. Horton RA, Randall LP, Snary EL, Cockrem H, Lotz S, Wearing H, Duncan D, Rabie A, McLaren I, Watson E, La Ragione RM, Coldham NG: Fecal carriage and shedding density of CTX-M extended-spectrum beta-lactamase-producing Escherichia coli in cattle, chickens, and pigs: Implications for environmental contamination and food production. Appl Environ Microbiol 2011, 77:3715–3719.

36. Madec JY, Lazizzera C, Chatre P, Meunier D, Martin S, Lepage G, Menard MF, Lebreton P, Rambaud T: Prevalence of fecal carriage of acquired expanded-spectrum cephalosporin resistance in Enterobacteriaceae strains from cattle in France. J Clin Microbiol 2008, 46:1566–1567.

37. Meunier D, Jouy E, Lazizzera C, Kobisch M, Madec JY: CTX-M-1- and CTX-M-15-type beta-lactamases in clinical Escherichia coli isolates recovered from food-producing animals in France. Int J Antimicrob Agents 2006, 28:402–407.

38. Mustak HK, Ica T, Ciftci A, Diker KS: Plasmid-mediated quinolone resistance in Escherichia coli strains isolated from animals in Turkey. Ankara Univ Vet Fak Derg 2012, 59:255–258.

39. Picard B, Garcia JS, Gouriou S, Duriez P, Brahimi N, Bingen E, Elion J, Denamur E: The link between phylogeny and virulence in Escherichia coli extraintestinal infection. Infect Immun 1999, 67:546–553.

40. Milanov D, Prunić B, Velhner M, Todorović D, Polaček V: Investigation of biofilm formation and phylogenetic typing of Escherichia coli strains isolated from milk of cows with mastitis. Acta Vet-Beograd 2015, 65: 202-216.

MOLEKULARNA KARAKTERIZACIJA ESBL-PRODUKUJUĆIH ESCHERICHIA COLI IZOLATA IZ ZDRAVIH GOVEDA I OVACA

PEHLIVANOGLU Faruk, TURUTOGLU Hulya, OZTÜRK Dilek, YARDIMCII Hakan

Cilj studije je bio karakterizacija Escherichia coli izolata koji proizvode ESBL, a koji su izolovani iz zdравих goveda i ovaca u Burdur provinciji u Turkosji. Uzorci fecesa od ukupno 200 goveda i 200 ovaca su ispitivani pri čemu je 31 uzorak od goveda (15,5%) i 3 uzoraka od ovaca (1,5%), bilo pozitivno na ESBL-produkujuće E. coli. Upotrebljen je kombinovani klinički i laboratorijski standardni metod Instituta. Od svih ESBL klasa
Pehlivanoglu et al.: Molecular characterization of ESBL-producing *Escherichia coli* isolated from healthy cattle and sheep

genetics, which were established by PCR method, bla\textsubscript{CTX-M} type was the most frequent, followed by bla\textsubscript{TEM} and bla\textsubscript{SHV} families. Isolates *E. coli* which produce ESBL showed multi-resistance to a large number of antibiotics, including aminoglycosides, fenicol, quinolones, inhibitors of the folate pathway and tetracyclines. Resistance level was higher in isolates of cattle compared to sheep isolates. Phylogenetic grouping of *E. coli* isolates indicates that group A (especially A1) was dominant (19/34, 55.9%), followed by group B1 (9/34, 26.5%) and D (6/34, 17.6%); no isolates belonged to B2 group. The study shows that ESBL-producing *E. coli* isolates are found in the intestinal flora of healthy cattle and sheep in Burdur province of Turkey. This is the first report that indicates the occurrence of CTX-M type ESBL-producing *E. coli* isolates in sheep farms in Turkey.