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Research Article

Carriage of Plasmidic AmpC Beta-Lactamase Producing *Escherichia coli* in Cattle and Sheep and Characterisation of the Isolates in Terms of Antibiogram Profiles, Phylogeny and Virulence [1]

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Abstract

AmpC type beta-lactamase enzyme production by *Escherichia coli* confers resistance to penicillin and cephalosporins including oxyimino-cephalosporin, cephamycin and aztreonam (variably). Screening of AmpC beta-lactamase determinants in both commensal and pathogenic *E. coli* isolates in livestock is important to reveal the resistance status of the bacteria. Therefore, we aimed to investigate the AmpC beta-lactamase producing *E. coli* isolates in cattle and sheep populations in Burdur, Turkey. The fecal samples were collected from 250 Holstein cows, older than 12 months of age and apparently healthy, and from 225 sheep from different breeds, older than 6 months of age and apparently healthy. After selective isolation and identification of the agent in coliform/*E. coli* selective medium supplemented with cefotaxime (2 µg/mL) or ceftazidim (2 µg/mL), the cefoxitin resistant *E. coli* isolates were determined by agar disc diffusion test (ADDT). Then, *pAmpC* beta-lactamase genes were determined by multiplex polimerase chain reaction (PCR) as gold standart test for *pAmpC* beta-lactamase producing *E. coli*. Finally, the isolates were characterized by PCR for phylogeny and Enterohemorrhagic/Shiga toxin producing *E. coli* (EHEC/STEC) related virulence genes. Totally 17 (6.8%) cattle fecal samples were found positive for *pAmpC* beta-lactamase producing *E. coli.* None of the sheep fecal samples yielded culture positive results for the bacteria of interest. Among the *E. coli* isolates, only *CIT* (origin, *Citrobacter freundii)* family *pAmpC* gene was found. The predominant phylogenetic group was found as group A and only *eae* gene was detected in only one *E. coli* isolate. Multidrug resistance (MDR) was observed in 7 (41.2%) isolates. Consequently, the present study revealed that *pAmpC* beta-lactamase producing *E. coli*, with MDR and low phylogenetic group diversity, exists in cattle population, but not in sheep population.

Keywords: *Beta-lactamase, Escherichia coli, intimin, pAmpC, Phylogeny, Ruminant*

Sığır ve Koyunlarda Plasmidik AmpC Beta Laktamaz Üreten *Escherichia coli* Taşıyıcılığı ve İzolatların Antibiyogram Profilleri, Filogenetik ve Virulans Yönünden Karakterizasyonu

Öz

Escherichia coli tarafından AmpC beta laktamaz enzim üretimi penisilinlere, oksiimino sefalosporinler dahil tüm sefalosporinlere, sefamisinlere ve değişken olmakla birlikte aztreonama direnç sağlar. Çiftlik hayvanlarında komensal ve patojenik *E. coli* izolatlarında AmpC beta laktamazların taranması bakterilerdeki antibiyotik dirençliliğinin durumunu göstermesi açısından önemlidir. Bu nedenle, Burdur ilindeki sığır ve koyun popülasyonunda AmpC beta laktamaz üreten *E. coli* yaygınlığını ortaya çıkarmayı amaçladık. Bu çalışmada, 12 aylık yaştan daha büyük ve sağlıklı görünümdeki 250 Holştayn ırkı sığır ve 6 aylıktan daha büyük ve sağlıklı görünümdeki 225 değişik ırktan koyundan dışkı örneği toplandı. Sefotaksim (2 µg/mL) veya seftazidim (2 µg/mL) ilave edilmiş Koliform/*E. coli* besi yerinde selektif izolasyon ve identifikasyon gerçekleştirildikten sonra, agar disk difüzyon testi (ADDT) ile sefoksitine dirençli *E. coli* izolatları belirlendi. Takiben *pAmpC* beta laktamaz genleri, *pAmpC* beta laktamaz üreten *E. coli* belirlenmesi için altın standart test olan multipleks polimeraz zincir reaksiyonu (PZR) ile belirlendi. Son olarak, izolatlar PZR ile filogenetik ve enterohemorajik/Siga toksin üreten *E. coli* (EHEC/STEC) virulans genleri açısından karakterize edildi. Toplam 17 (%6.8) sığır dışkı örneği *pAmpC* beta laktamaz üreten *E. coli* yönünden pozitif bulunurken koyun örneklerinin tümü negatif bulundu. Izolatlarda sadece *CIT* ailesi pAmpC geni tespit edildi. İzolatlarda en yaygın filogenetik grubun grup A olduğu ve sadece 1 izolatta eae virulans geninin olduğu tespit edildi. Çoklu antibiyotik dirençliliği 7 (%41.2) izolatta tespit edildi. Sonuç olarak, bu çalışma pAmpC beta laktamaz üreten *E. coli*'nin koyunlarda bulunmadığı ve sığırlarda çoklu antibiyotik dirençliliğine sahip ve az sayıda filogenetik çeşitlilikte var olduğu belirlendi.

Anahtar sözcükler: *Beta laktamaz, Escherichia coli, İntimin, pAmpC, Filogenetik, Ruminant*

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INTRODUCTION

Beta-lactamase enzymes that inactivate the beta-lactam antibiotics by hydrolysing the beta-lactam ring of the antibiotic [1,2] posses highly heterogenic character in nature. They were divided to several groups for classification that is updated regularly by the researchers [3,4]. Several classification schemes for bacterial beta-lactamases have been described. One of them was based on the activity of the beta-lactamases against different beta-lactam antimicrobials [5]. The other scheme developed by Ambler divides the beta-lactamase enzymes into four classes as A, B, C, and D, according to their amino acid sequence differences [6]. The group of beta-lactamases classified as Ambler Class C and named AmpC beta-lactamases can confer resistance to penicillins and cephalosporins including oxyimino-cephalosporins (e.g., cefotaxime, ceftazidime and ceftriaxone), cephamycins (e.g., cefoxitin and cefotetan), and aztreonam (variably) [5,7]. It has been reported that use of beta-lactams for treatment of several infections causes development of AmpC beta-lactamase producing *E. coli* isolates in animal and human intestinal microflora. This casual use also triggers an increase in AmpC beta-lactamase production in Gram-negative pathogens in humans and animals due to the horizontal transfer of resistance genes [8,9].

The genes encoding AmpC beta-lactamases can be located on a conjugative plasmid or chromosome of a Gram-negative bacterium [7]. Plasmid mediated AmpC beta-lactamases (pAmpC) are composed of 6 families which were formed based on amino acid sequences. These families are named as ACC (Ambler class C), CIT (origin, *Citrobacter freundii)*, DHA (site of discovery, Dhahran hospital in Saudi Arabia), EBC (origin, *Enterobacter claocae*), FOX (resistance to cefoxitin) and MOX (resistance to moxalactam). Unlike to extended-spectrum beta-lactamases (ESBL), AmpC beta-lactamases can not be inhibited by beta-lactamase inhibitors (clavulanic acid and tazobactam) [7,8].

Escherichia coli strains causing diarrhea in human have been classified into several pathotypes based on virulence characteristics and infection mechanisms. There have been described 5 main intestinal pathogenic *E. coli* strains named as enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC) and enteroinvasive *E. coli* (EIEC). EHEC strains are responsible from bloody diarrhoea, haemorrhagic colitis (HC) and the Haemolytic Uraemic Syndrome (HUS) in human and can be transmitted to human by consumed food [10]. All are life threating infections for human. On the other hands, animals especially cattle are known the reservoir of EHEC/Shiga toxin producing strains (STEC) and especially carriage status of O157:H7 strains have been revealed by several researchers in cattle [11]. Therefore, it is important to investigate the multidrug resistant *E. coli* isolates, such as ampC beta-lactamase producing *E. coli*, for EHEC/STEC virulence determinants to reduce the fecal shedding of such *E. coli* isolates in animals that prevents fecal contamination of food of animal origin.

The studies conducted in several countries in the world report the existence and prevalence of AmpC beta-lactamase producing Gram-negative bacteria isolated from live-stock [2,12-15]. In Turkey, Pehlivanoglu [16] reported the presence of pAmpC producing *E. coli* in laying hens, Aslantaş et al.[17] reported in cattle, Gumus et al.[18] reported in dogs and cats and Aslantas and Yilmaz [19] reported in dogs, but more studies are needed to reveal the true prevelance of AmpC producing *E. coli* in Turkey. Hence, the present study was carried out to investigate the existence of pAmpC beta-lactamase producing *E. coli* isolates in healthy cattle and sheep in Burdur, Turkey and to characterize the isolates for antibiotic susceptibility pattern, phylogeny and virulence.

MATERIAL and METHODS

Sampling

Approximate sample size of the study was determined to be 138 using a 10% expected field prevalence [16,17] at the 95% confidence level and the desired absolute precision of 5% [20], but more animals were included to the present study for higher precise results. Twenty dairy cattle and 12 sheep farms with no close contact to cattle farms were selected randomly from different locations of Burdur. Approximately 50% of animals were selected randomly from each cattle and sheep farms. Total 250 Holstein cows, older than 12 months of age and apparently healthy, and 225 sheep from different breeds, older than 6 months of age and apparently healthy, were included in the study. Fecal samples (at least 5 grams) were collected from rectum of the animals by using separate disposable examination gloves for each animals. The fecal samples were put into sterile screw-top vials, transported to the laboratory on ice in a cooler within 2 h and kept at 4ºC until processing within 24 h. The protocol for fecal collection from animals in the present study was approved by Burdur Mehmet Akif Ersoy University (Turkey) Animal Care and Use Committee (approval number: 07.09.2012/05).

Selective Isolation

Isolation was initiated with preparation of a 10% suspension of each fecal sample in buffered peptone water (Lab M, UK) and incubation at 37ºC for 24 h under aerobic conditions. Fifty microliters from each suspension was plated onto Brilliance *E. coli*/coliform Selective Agar (Oxoid, UK) supplemented with cefotaxime (CTX, 2 µg/mL) (Sigma Aldrich, Germany) or ceftazidime (CAZ, 2 µg/mL) (Sigma Aldrich, Germany) and the plates were incubated at 37ºC for 24 h under aerobic conditions.

Presumptive *E. coli* colonies (purple or blue colour) from each plate (one colony from the selective agar with CTX and one colony from the selective agar with CAZ) per culture

positive fecal sample were selected randomly. Identification of the suspicious colonies were carried out by the biochemical tests [21]. Finally, molecular confirmation of the *E. coli* isolates were performed by PCR [22] after DNA extraction yielded by boiling method [23] *(Table 1)*.

Determination of the Presumptive AmpC Beta-lactamase Producing E. coli

Firstly, the *E. coli* isolates were tested in terms of ESBL production by agar disc diffusion test (ADDT) [24]. In this test, aztreonam (ATM, 30 µg), cefotaxime (CTX, 30 µg), cefpodoxime (CPD, 10 µg), ceftazidime (CAZ, 30 µg) and ceftriaxone (CRO, 30 µg) discs were used [24]. The isolate resistant to at least one of them were further tested with ESBL confirmatory test [24] and 34 isolates found positive for ESBL production were excluded from the study. Then, non-ESBL-producing isolates were tested for cefoxitin resistance by ADDT for phenotypic determination of AmpC beta-lactamase producers [24,25]. ADDT was performed by plating of each *E. coli* isolates with an inoculum (McFarland turbidity 0.5) on Mueller Hinton Agar (MHA) (Oxoid, UK) plates followed by the disc placement of cefoxitin (FOX, 30 µg) (Oxoid, UK). The plates were incubated at 37ºC for 24 h. Inhibition zone diameter lower than 18 mm was accepted for the evidence of FOX resistance [24].

PCR Analysis of Plasmid Mediated AmpC Beta-lactamase Genes (pAmpC)

As the gold standard test for detemination of pAmpC producing *E. coli*, PCR was performed for *pAmpC* genes

Table 1. *Primers used in the present study*

Target Gene	Primer Sequence (5'----------3')	Amplicon (bp)	Reference
MOX	F-GCTGCTCAAGGAGCACAGGAT R-CACATTGACATAGGTGTGGTGC	520	Perez-Perez and Hanson [8]
CIT	F-TGGCCAGAACTGACAGGCAAA R-TTTCTCCTGAACGTGGCTGGC	462	Perez-Perez and Hanson [8]
DHA	F-AACTTTCACAGGTGTGCTGGGT R-CCGTACGCATACTGGCTTTGC	405	Perez-Perez and Hanson [8]
ACC	F-AACAGCCTCAGCAGCCGGTTA R-TTCGCCGCAATCATCCCTAGC	346	Perez-Perez and Hanson [8]
EBC	F-TCGGTAAAGCCGATGTTGCGG R-CTTCCACTGCGGCTGCCAGTT	302	Perez-Perez and Hanson [8]
FOX	F-AACATGGGGTATCAGGGAGATG R-CAAAGCGCGTAACCGGATTGG	190	Perez-Perez and Hanson [8]
chuA	F-GACGAACCAACGGTCAGGAT R-TGCCGCCAGTACCAAAGACA	279	Clermont et al.[26]
YjaA	F-TGAAGTGTCAGGAGACGCTG R-ATGGAGAATGCGTTCCTCAAC	211	Clermont et al.[26]
TspE4.C2	F-GAGTAATGTCGGGGCATTCA R-CGCGCCAACAAAGTATTACG	152	Clermont et al.[26]
16S rRNA	F-CCCCCTGGACGAAGACTGAC R-ACCGCTGGCAACAAAGGATA	401	Wang et al.[22]
rfbO157	F-CAGGTGAAGGTGGAATGGTTGTC R-TTAGAATTGAGACCATCCAATAAG	296	Bai et al.[27]
fliCH7	F-GCTGCAACGGTAAGTGAT R-GGCAGCAAGCGGGTTGGT	948	Wang et al.[22], Osek [28]
stx1	F-TGTCGCATAGTGGAACCTCA R-TGCGCACTGAGAAGAAGAGA	655	Bai et al.[27]
stx2	F-CCATGACAACGGACAGCAGTT R-TGTCGCCAGTTATCTGACATTC	477	Bai et al.[27]
eae	F-CATTATGGAACGGCAGAGGT R-ACGGATATCGAAGCCATTTG	375	Bai et al.[27]
ehxA	F-GCGAGCTAAGCAGCTTGAAT R-CTGGAGGCTGCACTAACTCC	199	Bai et al.[27]
espP	F-GATTACAGCACGCATTCATGGTAT R-TCCAGGCATCCTCAGTGACA	73	Posse et al.[29]
katP	F-GAAGTCATATATCGCCGGTTGAA R-GTCATTTCAGGAACGGTGAGATC	73	Posse et al.[29]
saa	F-CGTGATGAACAGGCTATTGC R-ATGGACATGCCTGTGGCAAC	119	Paton and Paton [30]

F: Forward, R: Reverse, bp: base pair

according to the method developed by Perez-Perez and Hanson [8]. The PCR protocol was modified slightly in our laboratory as follow: Two sets of triplex PCR (set 1: ACC, CIT, FOX and set 2: DHA, EBC, MOX) were established for detection of *pAmpC* genes. The first triplex PCR was adjusted as 25 µL consisted of; 50 mM KCl, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.4-0.6 µM primer sets (specific for FOX, ACC and CIT, respectively), 1.25 U of Taq DNA polymerase (Thermo Scientific) and 2 µL template DNA. The second triplex PCR was adjusted as 25 µL consisted of; 50 mM KCl, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.5-0.6 µM primer sets (specific for EBC, DHA and MOX, respectively), 1.25 U of Taq DNA polymerase (Thermo Scientific) and 2 µL template DNA. Thermal cycling conditions for both of triplex PCRs were 5 min at 94ºC for initial denaturation, followed by 35 cycles of 45 sec at 94ºC, 45 sec at 64ºC and 1 min at 72ºC, and a final elongation step of 7 min at 72ºC. The primer sequences used were presented in *Table 1*.

Antibiotic Susceptibility Profiles, Phylogroups and Virulence Genes of the Isolates

Susceptibility of pAmpC beta-lactamase producing *E. coli* isolates to beta-lactam antibiotics and to other classes of antibiotics were determined by ADDT [24,31,32]. The beta-lactams antibiotic discs (Oxoid, UK) tested were ampicillin (AMP, 10 µg), cefepime (FEP, 30 µg), cefuroxime sodium (CXM, 30 µg), cephalothin (CEF, 30 µg) and imipenem (IPM, 10 µg). The antibiotics (Oxoid, UK) from other classes tested were chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg), enrofloxacin (ENR, 5 µg), florfenicol (FFC, 30 µg), gentamicin (CN, 10 µg), kanamycin (K, 30 µg), nalidixic acid (NA, 30 µg), streptomycin (S, 10 µg), sulfamethoxazole-trimethoprim (SXT, 25 µg) and tetracycline (TE, 30 µg). *E. coli* ATCC 25922 strain was used as the control strain in ADDT. The inhibition zone diameters were evaluated according to CLSI critical zone diameters in CLSI document M10 0-S26 [24], M31-A3 [31] and VET01-S2 [32]. Based on the results, the isolates were classified as resistant, intermediate or susceptible. In the present study, a pAmpC beta-lactamase producing *E. coli* isolate that was resistant to at least 3 different classes of antibiotics excluding beta-lactams was accepted as multidrug-resistant (MDR) strain.

The phylogroups (groups A, B1, B2 and D) of the *E. coli* isolates were determined according to a triplex PCR protocol as described elsewhere [26], with the modified PCR conditions by Higgins et al.[33]. The triplex PCR is based on the amplification of a 279 bp fragment of the *chuA* gene, 211 bp fragment of the *yjaA* gene and 152 bp fragment of TspE4.C2 (a noncoding DNA region of *E. coli* genome). The phylogenetic groups of the isolates were assigned according to following criteria: the phylogenetic group A (*chuA*-, TspE4.C2-), B1 (*chuA*-, TspE4.C2+), B2 (*chuA*+, *yjaA*+), or D (*chuA*+, *yjaA*-). Additionally, phylogenetic subgroups (A: A_0 and A_1; B2: $B2_2$ and $B2_3$; D: D_1 and D_2) were investigated as described by Escobar-Páramo et al.[34]. *E. coli* ATCC 25922 was used as positive control strain (*chuA*+, *yjaA*+ and TspE4.C2+) in the triplex PCR.

The pAmpC beta-lactamase producing *E. coli* isolates were screened by PCR for serotype O157:H7 (*rfbO157* and *fliCH7* genes) [27,28] and *eae* (intimin, attaching and effacing protein), *ehxA* (enterohemolysin), *espP* (extracellular serine protease), *katP* (catalase-peroxidase), *saa* (autoagglutinating adhesin), *stx1* (Shiga toxin 1) and *stx2* (Shiga toxin 2) to determine if the isolates were Enterohemorrhagic *E. coli* (EHEC) [27,29,30].

RESULTS

As the results of culture of cattle fecal samples, presumptive *E. coli* colonies were observed on at least one of both media (supplemented with CTX or CAZ) from 51 fecal samples. All colonies were identified as *E. coli* by phenotypic tests and PCR. After ESBL confirmatory test, *E. coli* isolates from 34 fecal samples were separated as ESBL-producing isolates. The remaining *E. coli* isolates (non-ESBL producers) were from 17 fecal samples and growth of *E. coli* colonies on these fecal samples (n=17) were observed on both medium (supplemented with CTX or CAZ) and therefore total 34 non-ESBL-producing *E. coli* were obtained. All of 34 non-ESBL- producing *E. coli* isolates were found to be resistant to cefoxitin by ADDT and therefore they were accepted as potential AmpC producers. In the present study, both potential AmpC beta-lactamse producing *E. coli* isolates (one from selective agar with CTX and the other one from the selective agar with CAZ) from a single fecal sample showed the same antibiotic susceptibility profile and the same phylogenetic group in all of the fecal samples. Therefore, the prevalence was estimated as 6.8% (17/250) for cattle in the study. *E. coli* was not isolated from sheep fecal samples.

All *E. coli* isolates from cattle harbored only *CIT* family *pAmpC* gene *(Fig. 1)*. In total, 5 (25%, 5/20) cattle herds were found positive in terms of carrying pAmpC beta-lactamase producing *E. coli* isolates *(Table 2)*.

According to phylogenetic analysis by PCR, 11 (11/17, 64.7%) *E. coli* isolates were found belong to group A (subgroup A_1), 3 (3/17, 17.6%) isolates to group D (subgroup D_1), 2 (2/17, 11.8%) isolates to group B2 (subgroup $B2_2$) and 1 (1/17, 5.9%) isolate to group B1 *(Table 2)*.

In total, 7 of 17 (41.2%) pAmpC beta-lactamase producing *E. coli* isolates were found MDR. Among the MDR isolates, 3 isolates belonged to phylogoup A (subgroup A_1), one isolate to phylogoup B1 and one isolate to D (subgroup D_1) *(Table 2)*. The highest resistance was found against TET (9/17, 52.9%) whereas the lowest resistance was against CIP, ENR and NA (1/17, 5.9%). All isolates were susceptible to FEP and IPM *(Table 3)*.

Among the virulence genes screened among the isolates, only *eae* (intimin) gene was detected in only one isolate

Table 2. *Distrubition of the pAmpC beta-lactamase producing E. coli isolates (cattle) according to herds, phylogenetic group and antibiogram profiles*

Farm No	*E. coli* Isolates (n)	*E. coli* Isolates (n): Phylogenetic Group	Resistance Profile	*blapAmpC* Gene Family
3	8	7:A (A_1)	-	CIT
		1:B2 ($B2_2$)	-	CIT
6	1	A (A_1)	CN, S, K, FFC, C, TE*	CIT
7	6	1:A (A_1)	TE	CIT
		1:B1	CN, S, K, SXT, NA, ENR, CIP, FFC, C, TE*	CIT
		1:B2 ($B2_2$)	S, K, TE	CIT
		3:D (D_1)	S, SXT, TE*	CIT
8	1	A (A_1)	CN, S, K, SXT, FFC C, TE*	CIT
9	1	A (A_1)	CN, S, K, FFC, C, TE*	CIT

** Multidrug resistant isolate (MDR, resistant to at least 3 classes of antibiotics except beta-lactam antibiotics). C: chloramphenicol, CIP: ciprofloxacin, ENR: enrofloxacin, FFC: florfenicol, CN: gentamicin, K: kanamycin, NA: nalidixic acid, S: streptomycin, SXT: sulfamethoxazole-trimethoprim, TE: tetracycline*

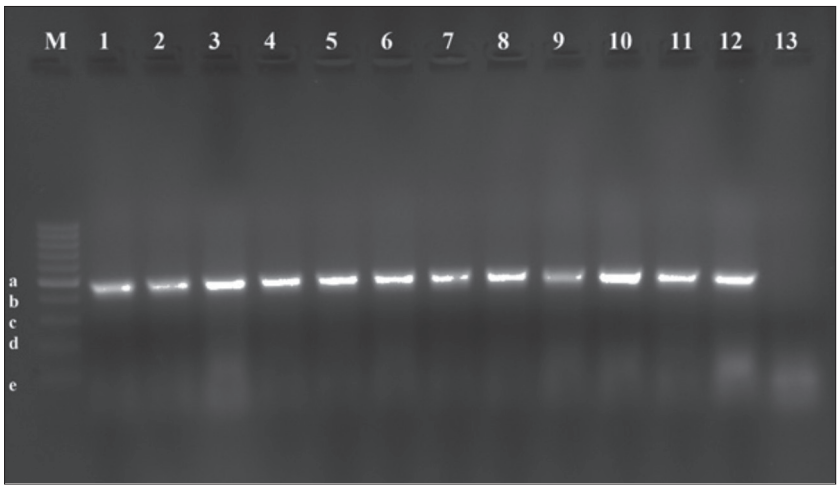

Fig 1. Results of PCR test performed for pAmpC beta-lactamase genes of *E. coli* isolates (cattle). M: Marker (a:500 bp, b:400 bp, c:300 bp, d:200 bp, e:100 bp), Lane 1-12: *E. coli* isolates positive for CIT type pAmpC beta-lactamase gene (462 bp), Lane 13: negative control

Table 3. *Antibiotic susceptibilities of pAmpC beta-lactamase producing E. coli isolates (cattle)*

Beta- Lactams	pAmpC Producing *E. coli* (n=17)		Other Antibiotics	pAmpC Producing *E. coli* (n=17)	
	R (n)	I (n)		R(n)	I (n)
AMP	17	0	CN	4	0
ATM	17	n/a	K	5	0
FEP	0	0	S	8	1
CTX	17	n/a	CIP	1	0
CPD	17	n / a	ENR	1	0
CAZ	17	n/a	NA	1	0
CRO	17	n /a	TE	9	0
CXM	13	0	SXT	5	0
CEF	17	0	FFC	4	0
IPM	0	0	C	4	0

n/a: not applicable, R: resistant, I: intermediate, AMP: ampicillin, ATM: aztreonam, CAZ: ceftazidime, FEP: cefepime, CPD: cefpodoxime, CRO: ceftriaxone, CXM: cefuroxime, CEF: cephalothin, CTX: cefotaxime, FOX: cefoxitin, IPM: imipenem, C: chloramphenicol, CIP: ciprofloxacin, ENR: enrofloxacin, FFC: florfenicol, CN: gentamicin, K: kanamycin, NA: nalidixic acid, S: streptomycin, SXT: sulfamethoxazole-trimethoprim, TE: tetracycline

from Farm 3. This *E. coli* isolate belonged to $B2_2$ phylogenetic group and was not resistant to any of the antibiotics (except beta lactams) tested. None of the pAmpC beta-lactamase producing *E. coli* isolates was O157:H7 serotype of *E. coli*.

DISCUSSION

The real prevalence of pAmpC beta-lactamase producing *E. coli* is still unknown in healthy cattle and sheep in Turkey. There is only one local study conducted on this topic in healthy cattle in Turkey and it was found only one *E. coli* isolate carrying *ampC (cmy)* gene out of 312 cattle rectal swab samples [17]. On the other hand, there is no report for pAmpC beta-lactamase producing *E. coli* in healthy sheep in Turkey. Even though some studies have shown the existence of AmpC beta-lactamase producing *E. coli* and *AmpC* genes on cattle origin food products (cheese, meat, and milk) in different parts of Turkey [35-37], these studies do not reflect exactly the extent of these isolates in live animals in Turkey due to possible contamination of the animal origin food during processing. Therefore, the present study conducted have given additional information about the presence of AmpC beta-lactamase producing *E. coli* in cattle and sheep in Turkey.

Although we detected AmpC beta-lactamase producing *E. coli* in 17 cattle, no sheep was detected positive for AmpC beta-lactamase producing *E. coli*. This can be attributed to use of the beta-lactams and other classes of antibiotics (aminoglycosides, beta-lactams, phenicols, quinolones, sulfamethoxazole-trimethoprim and tetracycline) more widely in the prevention and treatment of wide variety of infections (mastitis, lameness, calf diarrhea, metritis, arthritis, pneumoniae, salmonellosis, urinary tract infections, septisemia, etc.) in cattle population than sheep population in Turkey and some of them may cause co-selection of AmpC beta-lactamase producing *E. coli* isolates in gut microflora. It has been known that *ampC* genes are located on a large plasmid together with other antimicrobial resistance genes such as the genes responsible for aminoglycosides, phenicols, quinolones, sulfamethoxazole-trimethoprim and tetracycline resistances and frequent use of these antibiotics in livestock for several purposes leads the selection pressure for AmpC beta-lactamase producing Gram-negative bacteria in gut microflora [2]. In line with this it was detected co-resistance to at least one of these antibiotics in 9 (9/17, 52.9%) isolates and MDR in 7 (41.2%) isolates in this study.

Escherichia coli isolates obtained in this study were found fall into four main phylogenetic groups (A, B1, B2 and D). It is known that *E. coli* strains belonging to group A and B1 are primarily found in the commensal microflora [26,34]. The pathogen *E. coli* strains associated with extra-intestinal infections and diarrhea mainly belong to B2 and D groups [26,34]. Later, Escobar-Paramo et al.[34] stated phylogenetic subgroups (A: A_0 and A_1; B2: $B2_2$ and $B2_3$; D: D_1 and D_2). Likewise, in the present study, phylogentic analysis of *E. coli* isolates showed that group A (subgroup A_1) (n=11) is the predominant group, followed by group D (subgroup D_1) (n=3), group B2 (subgroup $B2_2$) (n=2) and group B1 (n=1). On the other hand, the reports indicate that *E. coli* strains from B2 and D phylogroups possess more virulence factors but less MDR pattern than A and B1 phylogroups [38,39]. Similarly, in the present study the *E. coli* isolates from A and B1 phylogroups showed resistance to more antibiotics than the B2 and D phylogroup isolates and EHEC/STEC related virulence gene (*eae*, intimin) detected was found in the *E. coli* isolate belonging to $B2_2$ phylogroup with no co-resistance to other classes of antibiotics. The *eae* gene encodes the intimin protein on the surface of EPEC/STEC/EHEC isolates and it is located on the locus of enterocyte effacement (LEE) pathogenicity island. The intimin protein is important for intimate attachment to the intestinal mucosa and the formation of the attaching and effacing lesions in EPEC and STEC/EHEC infections [40]. Therefore, it is possible to state that the *eae* gene positive and pAmpC beta-lactamase producing *E. coli* isolate from $B2_2$ phylogenetic group determined in cattle in this study can be pathogenic for both human and calf even thogh this isolate is not a STEC/EHEC isolate.

Overall, based on the similarity of phylogenetic analysis results and antibiogram profiles of the isolates, three farms (Farm 6, 8 and 9) had only one isolate, one farm (Farm 3) had 2 different isolates and one farm (Farm 7) had 4 different isolates. Additionally, all isolates had the same *blapAmpC* gene family (*CIT*). Hence, it can be stated that few parent *E. coli* strains with the same *pAmpC* gene (*CIT*) were circulating in the cattle farms in Burdur, Turkey.

It is generally accepted that food producing animals serve as reservoir for MDR *E. coli* strains and they can be transmitted to human by direct contact and/or via food chains. This issue has also been considered for AmpC beta-lactamase producing *E. coli* since there are many studies showing the similar *pAmpC* genes and plasmids in both animal and animal owners or farm workers [41-43]. Therefore, we can mention the possible health risk for people close contact to the cattle population in the present study.

In conclusion, the present study showed the absence of pAmpC beta-lactamase producing *E. coli* in sheep population and presence of few multi-drug resistant pAmpC beta-lactamase producing *E. coli* strains with only one type *pAmpC* gene family (*CIT*) in cattle population in Burdur, Turkey. However, more studies are needed to reveal and understand the course of prevalence of pAmpC beta-lactamase producing *E. coli* (pathogen and commensal) and diversity in *pAmpC* genes in livestock populations in Turkey. The other point that should be considered, as known, emergence of *E. coli* isolates possesing *pAmpC* genes may also bring the increase in production of the AmpC beta-lactamases in other Gram-negative bacteria due to horizontal

transfer of plasmids between Gram-negative bacteria species. Hence, the necessary preventive measurements should be taken, for examples, livestock sector workers and veterinarians should be informed regularly about increase in antimicrobial-resistant strains and proper selection of antimicrobials should be provided in the treatment of the infections in animals. Also, monitoring should be performed for antimicrobial resistance levels to limit the escalating trend in antimicrobial resistance and the emergence of resistance traits in genetic material of Gram-negatives.

Declaration of Conflicting Interests

The authors declare no conflict of interest.

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