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Original Article

CTX-M-15-Type Extended-Spectrum Beta-Lactamase-Producing *Escherichia coli* as Causative Agent of Bovine Mastitis

Faruk Pehlivanoglu, Hulya Turutoglu, and Dilek Ozturk

Abstract

In the present study, 3 quarter milk samples from 3 cows showing clinical signs of mastitis were investigated. Three *Escherichia coli* were isolated and were found resistant to ceftiofur and cefquinome. The isolates were detected as extended-spectrum beta-lactamase (ESBL) producers by the Clinical and Laboratory Standards Institute (CLSI) ESBL screening test and combined disc method. CTX-M- and TEM-type beta-lactamase genes ($bla_{\text{CTX-M}}$ bla_{TEM}) were detected by polymerase chain reaction in the isolates. Sequencing of $bla_{\text{CTX-M}}$ and bla_{TEM} genes showed that the isolates were both TEM-1- and CTX-M-15-type beta-lactamase producers. The isolates were tested by agar disc diffusion test for susceptibility to various classes of antibiotics (aminoglycosides, quinolones, phenicols, folate pathway inhibitors, and tetracyclines), and they were determined as multidrug resistant. Therefore, this is the first report indicating the involvement of multidrug-resistant CTX-M-15-type ESBL-producing *E. coli* as a cause of bovine mastitis in Turkey.

Introduction

Emergence and dissemination of extended-spectrum beta-lactamase (ESBL)-producing Gram-negative bacteria of animal origin are on the rise and cause considerable concern for veterinary practitioners across the world. ESBLs are a group of hydrolytic enzymes produced by Gram-negative bacteria and confer resistance to penicillins, first- to fourth-generation cephalosporins, and monobactams. ESBLs do not have activity against carbapenems (e.g., imipenem, meropenem, and ertapenem) or cephamycins (e.g., cefoxitin) and are usually inhibited readily by beta-lactamase inhibitors (e.g., clavulanic acid and tazobactam) (Paterson and Bonomo, 2005; Perez *et al.*, 2007; Pitout and Laupland, 2008).

The most common ESBLs produced by *Enterobacteriaceae* belong to the TEM (temoneira), SHV (sulfhydryl variable), and CTX-M (cefotaximase-Munich) families. The TEM and SHV variants derive from genes for TEM-1, TEM-2, and SHV-1 beta-lactamases, respectively, by point mutations that alter the amino acid configuration around the active site of these beta-lactamases, and this provides an attack on oxyimino-cephalosporins (Paterson and Bonomo, 2005; Livermore, 2012). TEM-1, TEM-2, and SHV-1 beta-lactamases are not ESBLs, but mutations provide them ESBL activities.

CTX-M-type beta-lactamase genes ($bla_{\text{CTX-M}}$) have been captured from the chromosome of *Kluyvera* spp. onto the conjugative plasmids that mediate their dissemination among *Enterobacteriaceae* (Rossolini *et al.*, 2008). CTX-M-type ESBLs are divided into clusters, defined as CTX-M-1 group, CTX-M-2 group, CTX-M-8 group, CTX-M-9 group, and CTX-M-25 group, based on amino acid sequence identity (Bradford, 2001; Bonnet, 2004). The members of each group share >94% identity, whereas ≤90% identity is observed among the members belonging to distinct groups (Bonnet, 2004). ESBLs of the CTX-M-type have been reported increasingly in Gram-negative rods (Bradford, 2001; Bonnet, 2004; Canton and Coque, 2006). CTX-M enzymes confer higher levels of resistance to cefotaxime than to ceftazidime (Bonnet, 2004). The activity of subtypes of CTX-M beta-lactamases against antibiotics differs, such as CTX-M-15 beta-lactamase (belongs to CTX-M-1 group) confers an increased catalytic activity to ceftazidime (Poirel *et al.*, 2002).

Bovine mastitis is one of the most prevalent and costly diseases in the dairy industry (Seegers *et al.*, 2003). *Escherichia coli* is an important cause of mastitis in cattle, and clinical signs vary from severe to mild form in which cows have only local inflammation in the udder (Suojala *et al.*, 2013). In the context of mastitis in cattle, some reports are available for the existence of ESBL-producing *E. coli* as a causative agent in a few countries (Geser *et al.*, 2012; Dahmen *et al.*, 2013; Ohnishi *et al.*, 2013; Timofte *et al.*, 2014); however, it was unknown if the same organism was

Department of Microbiology, Faculty of Veterinary Medicine, Mehmet Akif Ersoy University, Burdur, Turkey.

associated with this condition in Turkey. Therefore, this study was conducted to determine the involvement of ESBL-producing *E. coli* as a cause of bovine mastitis in Turkey.

Materials and Methods

Milk samples and bacterial culture

Three-quarter milk samples from three lactating cows were sampled in the present study. Each milk sample was obtained from individual cows showing repetitive episodes of clinical mastitis in the udder. The cows were from a single farm with 750 lactating cows in Antalya city located off the Mediterranean coast of Turkey. Information about the use of third- and fourth-generation cephalosporins for the treatment of various infections of cattle in the farm was collected. One of the quarter milk samples was collected at the beginning of May 2015 and the other two were collected at the end of May 2015. Affected quarters were hot and swollen. Before taking the milk samples, teats were washed thoroughly and dried. Then, the teats were sprayed with 70% ethanol, the first few squirts of milk were discarded, and ~5 mL milk samples were collected in sterile tubes. The milk samples were transferred to the laboratory in a cooler, and 50 μL of each milk sample was spread on 7% sheep blood agar and MacConkey agar plates in the laboratory within 2 h of collection. After the plates were incubated at 37°C in aerobic atmosphere for 24 h, one type of colony form was seen on each plate. Isolation of the *E. coli* was performed using standard microbiological techniques (Winn *et al.*, 2006), and genetic confirmation was performed by polymerase chain reaction (PCR) amplification of 401 bp fragment of *E. coli* 16S rRNA gene (Wang *et al.*, 2002) (Table 1).

Antibiotic susceptibility testing

The *E. coli* isolates were tested for susceptibilities to beta-lactams, aminoglycosides, quinolones, phenicols, folate pathway inhibitors, and tetracyclines by agar disc diffusion test according to the Clinical and Laboratory Standards Institute (CLSI) protocols (CLSI, 2014). The antibiotics tested were ampicillin (AMP, 10 μg), cefoxitin (FOX, 30 μg), cefquinome (CEQ, 30 μg), ceftiofur (EFT, 30 μg), cefuroxime (CXM, 30 μg), cephalothin (CEF, 30 μg), imipenem (IPM, 10 μg), gentamicin (GEN, 10 μg), kanamycin (KAN, 30 μg), streptomycin (STR, 10 μg), ciprofloxacin (CIP, 5 μg), enrofloxacin (ENR, 5 μg), nalidixic acid (NAL, 30 μg), chloramphenicol (CHL, 30 μg), florfenicol (FFC, 30 μg), sulfamethoxazole–trimethoprim (SXT, 25 μg), and tetracycline (TET, 30 μg) (Oxoid, UK).

In the evaluation of the results, CLSI zone diameters for *Enterobacteriaceae* (CLSI, 2014) were used as criteria for AMP, FOX, CXM, CEF, CHL, CIP, IPM, NAL, and STR; CLSI document VET01-S2 (CLSI, 2013) for EFT, FFC, GEN, and SXT; and CLSI document M31-A3 (CLSI, 2010) for ENR, KAN, and TET. Multidrug resistance was defined as resistance to at least three different classes of antibiotics excluding beta-lactams.

Screening and confirmatory tests for ESBL

Following determination of high resistance to several antibiotics, including CEQ and EFT, the CLSI screening test for ESBLs was performed by using aztreonam (ATM, 30 μg),

TABLE 1. PRIMER PAIRS AND PCR CONDITIONS USED IN THE CURRENT STUDY

Target	*Primer*		*PCR condition*			*Product size (bp)*	*References*
			Dntr./time	*Annl./time*	*Extn./time*		
$bla_{CTX\text{-}M}$	[a]CTX-$M_{universal}$	5′-SCSATGTGCAGYACCAGTAA-3′	94°C/30 s	54°C/30 s	72°C/1 min	543	Saladin *et al.* (2002)
	CTX-M-1 group	5′-CCGCRATATGRTTGGTGGTG-3′	94°C/1 min	55°C/1 min	72°C/1 min	891	Heffernan *et al.* (2009)
bla_{TEM}		5′-CCCATGGTTAAAAAATCACTG-3′					Jeong *et al.* (2005)
		5′-CCGTTTCCGCTATTACAAAC-3′	94°C/1 min	48°C/1 min	72°C/1 min	966	Heffernan *et al.* (2009)
bla_{SHV}		5′-GTATCCGCTCATGAGACAATA-3′					Arpin *et al.* (2003)
		5′-TCTAAAGTATATATGAGTAAAC-3′	94°C/30 s	58°C/30 s	72°C/1 min	1007	Heffernan *et al.* (2009)
ChuA		5′-GCCGGGTTATTCTTATTTGTCGC-3′					Tenover and Rajeed (2004)
		5′-TCTTTCCGATGCCGCCGCCAGTCA-3′	94°C/15 s	60°C/30 s	72°C/45 s	279	Heffernan *et al.* (2009)
YjaA		5′-GACGAACCAACGGTCAGGAT-3′					Clermont *et al.* (2000)
		5′-TGCCGCCAGTACCAAAGACA-3′	94°C/15 s	60°C/30 s	72°C/45 s	211	Higgins *et al.* (2007)
TspE4.C2		5′-TGAAGTGTCAGGAGACGCTG-3′					Clermont *et al.* (2000)
		5′-ATGGAGAATGCGTTCCTCAAC-3′	94°C/15 s	60°C/30 s	72°C/45 s	152	Higgins *et al.* (2007)
16S rRNA		5′-GAGTAATGTCGGGGCATTCA-3′					Clermont *et al.* (2000)
		5′-CGCGCCAACAAAGTATTACG-3′	95°C/30 s	60°C/30 s	72°C/30 s	401	Higgins *et al.* (2007)
		5′-CCCCCTGGACGAAGACTGAC-3′					Wang *et al.* (2002)
		5′-ACCGCTGGCAACAAAGGATA-3′					

[a]S: C and G; Y: C and T; R: A and G.
Annl, annealing; bp, base pair; Dntr, denaturation; Extn, extention; PCR, polymerase chain reaction.

cefotaxime (CTX, 30 μg), cefpodoxime (CPD, 10 μg), ceftazidime (CAZ, 30 μg), and ceftriaxone (CRO, 30 μg) discs (BD, USA) (CLSI, 2014). Then, ESBL production by *E. coli* isolates was confirmed by the combined disc method as recommended by the CLSI (2014) by using CTX (30 μg), CAZ (30 μg), CTX-clavulanic acid (30 + 10 μg), and CAZ-clavulanic acid (30 + 10 μg) discs (BD, USA). *Klebsiella pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were included as positive and negative control strains for ESBL production, respectively, as recommended by the CLSI (2014).

DNA extraction, PCR, and sequencing

The *E. coli* colonies were picked from tryptic soy agar plates and mixed into 1 mL sterile ultrapure water to obtain a turbid suspension (McFarland standard 5.0). Then, the cell suspensions were heated to 100°C in a dry block for 10 min, chilled on ice, and centrifuged for 5 min at 14,000 rpm to pellet cellular debris. The supernatants were used as templates for PCR analysis.

The isolates were subjected to PCR analysis for bla_{TEM}, bla_{SHV}, and $bla_{\text{CTX-M}}$ genes as described elsewhere with slight modifications in the cycling conditions (see Table 1 for the details). *E. coli* ATCC 35218 ($bla_{\text{TEM-1}}$), *K. pneumoniae* ATCC 700603 ($bla_{\text{SHV-18}}$), and *E. coli* NCTC 13461 were used as positive control strains for bla_{TEM}, bla_{SHV}, and $bla_{\text{CTX-M}}$ encoding genes, respectively. As negative control for all PCR protocols, *E. coli* ATCC 25922 was used.

DNA sequencing of PCR products was carried out on both strands using the same primer pairs that were used in the PCR. Sequencing was performed by Refgen Genetical Research and Biotechnology Laboratory (Golbasi-Ankara, Turkey). The obtained sequences were compared with NCBI GenBank sequences using BLASTn to confirm the subtypes of beta-lactamase genes. All gene sequences were submitted to the NCBI GenBank (accession no. for CTX-M-15: KT265729, KT265730, and KT265731).

Phylogenetic typing of E. coli *isolates*

Finally, phylogenetic typing (groups A, B1, B2, and D) of the isolates was performed according to a triplex PCR protocol as described elsewhere (Clermont *et al.*, 2000), with the modified PCR conditions (Higgins *et al.*, 2007). 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). *E. coli* ATCC 25922 was used as positive control strain in the triplex PCR.

PCR for serotype O_{157}:H_7 and EHEC virulence genes

The ESBL-producing isolates were investigated for serotype O*157*:H*7* (*rfbO*$_{157}$ and *fliC*$_{H7}$) (Osek, 2003; Bai *et al.*, 2012) and virulence genes, specific for enterohemorrhagic *E. coli* (EHEC) through PCR as described previously. The EHEC genes investigated were *stx1* and *stx2* (Shiga toxin 1 and 2), *eae* (intimin), *ehxA* (enterohemolysin), *espP* (extracellular serine protease), *katP* (catalase-peroxidase), and *saa* (autoagglutinating adhesin) (Paton and Paton, 2002; Posse *et al.*, 2007; Bai *et al.*, 2012).

Results

E. coli was isolated from all three milk samples analyzed in this study; all three isolates were confirmed as *E. coli* by PCR. All three isolates were found positive for ESBL production as determined by the phenotypic confirmatory test and showed the same antibiotic susceptibility profiles: resistant to aminoglycosides (GEN, KAN, and STR), quinolones (CIP, ENR, and NAL), phenicoles (CHL), folate pathway inhibitors (SXT), tetracyclines (TET), and several beta-lactams (AMP, ATM, CTX, CPD, CEQ, CAZ, EFT, CRO, CXM, and CEF). The isolates were susceptible to FOX, FFC, and IPM. PCR showed that all the isolates possessed both bla_{TEM} and CTX-M group 1 beta-lactamase genes. Sequencing of TEM and CTX-M-1 group PCR products identified all isolates as both TEM-1-type beta-lactamase and CTX-M-15-type ESBL producers. According to phylogenetic grouping, all isolates belonged to group B1. PCR screening for EHEC virulence genes showed that none of the isolates was EHEC.

According to information gathered about the antibiotics used in the farm, the animals have been prescribed commonly with ceftiofur and cefquinome for the treatment of various infections of animals.

Discussion

Cephalosporins and other classes of antibiotics (aminoglycosides, quinolones, phenicols, folate pathway inhibitors, and tetracyclines) are widely used in cattle production for treatment of a variety of infections (e.g., enteritis, mastitis, pneumonia, and septicemia) in Turkey. It is known that the ESBLs that are produced by *E. coli* strains are frequently plasmid encoded, and these plasmids commonly carry genes that also encode resistance to other classes of antibiotics such as aminoglycosides, chloramphenicol, quinolones, sulfamethoxazole–trimethoprim, and tetracyclines (Paterson and Bonomo, 2005; Pitout and Laupland, 2008; Livermore, 2012). Thus, the emergence and dissemination of ESBL-producing *E. coli* strains with multidrug resistance profiles would not be surprising in Turkey. In fact, in the present study, all three ESBL-producing *E. coli* isolates exhibited multidrug resistance phenotypes. To date, only one study examined the presence of ESBL production in *E. coli* isolates (n:92) from bovine mastitis in Turkey (three provinces), but did not find any evidence for that (Dinc *et al.*, 2012). Therefore, this study represents the first report documenting the emergence of CTX-M-type ESBL-producing *E. coli* from bovine mastitis in Turkey.

There have been reports on the association of the use of ceftiofur or cefquinome in farm animals with the emergence and selection of ESBL producers (Jorgensen *et al.*, 2007; Cavaco *et al.*, 2008; Snow *et al.*, 2012). In Turkey, ceftiofur and cefquinome are used by veterinarians for treatment of various infections in cattle. Since ceftiofur and cefquinome have been also used frequently for treatment of infections (enteritis, mastitis, pneumoniae, and septicemia) in cattle in the farm in the present study, the emergence of ESBL-producing *E. coli* can be related to the use of ceftiofur and cefquinome.

Detection of CTX-M-type ESBLs in both commensal and pathogenic *E. coli* strains has increased dramatically during the last 20 years globally (Paterson and Bonomo, 2005; Canton and Coque, 2006; Ewers *et al.*, 2012). In agreement with this trend, in the present study, the $bla_{\text{CTX-M}}$ gene was

detected in the phenotypically confirmed ESBL-producing *E. coli* isolates. In Switzerland, Geser *et al.* (2012) identified one ESBL-producing *E. coli* from bovine mastitis as both CTX-M-14-type ESBL and TEM-1-type beta-lactamase producers. Similarly, in France, CTX-M-1- and CTX-M-14-type ESBL-producing *E. coli* strains were isolated from cattle mastitis (Dahmen *et al.*, 2013). On the contrary, Timofte *et al.* (2014) isolated both CTX-M-15-type ESBL and TEM-1-type beta-lactamase-producing *E. coli* from cattle mastitis in the United Kingdom. In Japan, Ohnishi *et al.* (2013) reported the isolation of CTX-M-2-, CTX-M-14-, and CTX-M-15-type ESBL-producing *E. coli* strains from mastitic milk samples. Similar to these last two reports, we isolated both CTX-M-15-type ESBL and TEM-1-type beta-lactamase-producing *E. coli* from bovine mastitis cases on a single farm.

CTX-M enzymes are the most widespread ESBL in *E. coli*. CTX-M (especially, CTX-M-15) beta-lactamase-producing *E. coli* are responsible for both nosocomial and community-onset infections (mainly urinary tract infection, bacteremia, and gastroenteritis) in humans (Pitout and Laupland, 2008). CTX-M-15-type ESBL-producing *E. coli* ST131 clone has a high virulence potential and has been reported worldwide in humans. This clone belongs to the B2 phylogenetic group and to the O25b serogroup (Rogers *et al.*, 2011). ST405, another *E. coli* clone carrying $bla_{\text{CTX-M-15}}$ gene, belongs to the D phylogenetic group and causes community-onset infections in humans too (Coque *et al.*, 2008). *E. coli* O25b-ST131 and ST405 clones have been responsible for the international spread of $bla_{\text{CTX-M-15}}$ gene that is carried on a conjugative plasmid (Coque *et al.*, 2008). Our isolates belong to B1 phylogenetic group, and thus, they were not pandemic ST131 and ST405 clones. However, our isolates were multidrug resistant similar to *E. coli* ST131 and ST405 clones.

One of the ways for humans to be infected with ESBL-producing *E. coli* is through consumption of food of animal origin. It has been proved that a proportion of human infections due to ESBL-producing *E. coli* originate from food-producing animals (Lazarus *et al.*, 2015). The same ESBL genes, ESBL gene carrying plasmids or ESBL-producing *E. coli* strains were detected from animal meat and humans consuming such meat (Leverstein-van Hall *et al.*, 2011; Overdevest *et al.*, 2011). To our knowledge, there has been no report indicating the transmission of ESBL-producing *E. coli* to humans through bovine mastitic milk and milk products. However, Dahmen *et al.* (2013) reported the isolation of CTX-M-producing *E. coli* strains belonging to ST10, ST23, and ST58 clones from bovine mastitis cases, which were all identified previously in human infections, including as ESBL producers. Therefore, further investigations are needed to prove the risk for humans due to consumption of milk from mastitis cases caused by ESBL-producing *E. coli* isolates.

Phylogenetic typing of *E. coli* strains shows that most commensal strains generally belong to groups A and B1, whereas group B2 and, to a lesser extent, group D are usually associated with virulent extraintestinal strains (Picard *et al.*, 1999; Clermont *et al.*, 2000). Phylogenetic typing showed that all three *E. coli* isolates belonged to the same phylogenetic group B1, suggesting their low virulence potential. This is in line with the fact that none of the isolates carried any of the potential virulence genes tested through PCR, including O157 (*rfbO157*), H7 (*fliCH7*), *eae* (intimin), *ehxA* (enterohemolysin), *stx1* (Shiga toxin 1), *stx2* (Shiga toxin 2), *espP* (extracellular serine protease), *katP* (catalase-peroxidase), and *saa* (autoagglutinating adhesin). It is known that cattle and sheep play an important role as a reservoir for EHEC/Shiga-toxigenic *E. coli* (STEC) isolates, and similarity among EHEC/STEC strains isolated from animals, humans, and food of animal origin has been shown (Söderlund *et al.*, 2012; Ateba and Mbewe, 2014). Although isolation of ESBL-producing EHEC/STEC isolates from animals is still rare (Valat *et al.*, 2012; Mandakini *et al.*, 2015), it is important to investigate the EHEC/STEC isolates for ESBL production for timely treatment of the infections in animals and humans, and to reduce the fecal shedding in animals that prevents fecal contamination of food of animal origin. We can state that our isolates cannot cause an EHEC infection (diarrheagenic infection) in humans if they are transmitted through the food chain to humans. However, to exclude the isolates of the present study as nonvirulent for humans, additional investigations should be performed, such as for extraintestinal *E. coli*-related virulence genes.

FFC is a broad-spectrum antibiotic and derived from thiamphenicol. FFC contains a fluorine atom instead of hydroxyl group located at C-3 of CHL. FFC can be successfully used against many Gram-negative and Gram-positive bacteria, including CHL-resistant (by acetyltransferase production or CmIA efflux pump) strains (Cloeckaert *et al.*, 2001; Schwarz *et al.*, 2004). In the mastitis cases in our study, FFC was the only option for the treatment, because the other antibiotics (FOX and IPM) to which the isolates were found susceptible are not available for veterinary use in Turkey. Thus, the very limited option for the treatment of clinical mastitis caused by CTX-M-15-type ESBL-producing *E. coli* isolates shows the magnitude of antimicrobial resistance problem.

Conclusion

In conclusion, according to the antibiotic susceptibility and phylogenetic typing results of the three CTX-M-15 ESBL-producing *E. coli* isolates with multidrug resistance phenotype, it can be stated that most probably a single *E. coli* strain caused the mastitis cases in all the three cows on this farm. Thus, to prevent further dissemination of ESBL-producing strain among the cows and to humans through the food chain, mastitis control measures should be applied strictly on the farm, and the cows should be isolated, treated, or culled. Also, prudent use of cephalosporins, particularly ceftiofur and cefquinome, should be encouraged to preserve their therapeutic efficacy in animals in Turkey. In the aspect of public health, these animals and milk products may create a public health risk because *E. coli* strains producing CTX-M-15 have been known as an important causative agent of community-onset infections.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Faruk Pehlivanoglu, DVM, PhD
Department of Microbiology
Faculty of Veterinary Medicine
Mehmet Akif Ersoy University
Istiklal Campus
Burdur 15030
Turkey

E-mail: pehlivanoglu@mehmetakif.edu.tr