FOODBORNE PATHOGENS AND DISEASE
Volume 7, Number 12, 2010

DOI: 10.1089/fpd.2010.0615

CTX-M-Type Extended-Spectrum β-Lactamases Present in *Escherichia coli* from the Feces of Cattle in Ohio, United States

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Abstract

CTX-M extended-spectrum β-lactamases are enzymes produced by bacteria that are capable of inhibiting the antimicrobial effects of cephalosporin drugs. Recently, the first domestically acquired *Salmonella* in the United States expressing $bla_{\text{CTX-M}}$ was reported. This is a concern because expanded-spectrum cephalosporins are the treatment of choice for invasive Gram-negative infections, including salmonellosis in children. Because *Salmonella* transmission is primarily foodborne, there is also concern that resistant enteric bacteria from livestock can be transferred through the food supply chain to consumers. $bla_{\text{CTX-M}}$ has not been previously identified in bacterial isolates from food animal populations in the United States. We report the recovery of CTX-M-type extended-spectrum β-lactamases from fecal *Escherichia coli* of sick and healthy dairy cattle in Ohio. Four individual fecal samples yielded *E. coli* isolates representing three clonal strains that carried $bla_{\text{CTX-M}}$ on transferable plasmids. Two distinguishable plasmids were identified, each encoding $bla_{\text{CTX-M-1}}$ or $bla_{\text{CTX-M-79}}$. Transferrable $bla_{\text{CTX-M}}$ genes in bovine *E. coli* have the potential to serve as a reservoir of resistance for pathogens and may represent a public health concern.

Introduction

CTX-M extended-spectrum β-lactamases confer resistance to penicillins, and to the first-, third-, and fourth-generation cephalosporins, but to neither cephamycins nor β-lactamase inhibitors (Bonnet, 2004). Since first emerging in the late 1980s, $bla_{\text{CTX-M}}$ alleles have become the predominant genes encoding the ESBL phenotype isolated from human clinical isolates of *Escherichia coli* and *Klebsiella* spp. in healthcare settings in many parts of the world (Bonnet, 2004; Livermore *et al.*, 2007). Recently, the first domestically acquired *Salmonella* in the United States expressing $bla_{\text{CTX-M-5}}$ was recovered from a child in Georgia (Sjolund *et al.*, 2008). This is a concern because expanded-spectrum cephalosporins are the treatment of choice for invasive Gram-negative infections, including salmonellosis in children. Because *Salmonella* transmission is primarily foodborne, there is also concern that resistant enteric bacteria from livestock can be transferred through the food supply to consumers. $bla_{\text{CTX-M}}$ has not been previously identified in bacterial isolates from food animal populations in the United States. However, *E. coli* carrying $bla_{\text{CTX-M}}$ have been isolated from livestock in other regions of the world, including Europe (Brinas *et al.*, 2005; Meunier *et al.*, 2006) and Asia (Duan *et al.*, 2006; Tian *et al.*, 2009). We report the recovery of *E. coli* containing $bla_{\text{CTX-M}}$ on transferable plasmids from the feces of both sick and healthy cattle in Ohio.

Methods

In the spring of 2009, fresh bovine fecal samples ($n = 50$) were collected from the floor of 15 holding pens at a livestock market in Ohio 1 day after the weekly auction when 439 cattle had occupied the facility. Sampling was conducted in all pens that had contained mature dairy cows being sold as beef animals. Fifty fresh fecal samples were also collected from the floor of freestall barns that housed mature lactating cows at 3 dairy farms in Ohio with a mean herd size of 172 head in the spring of 2009. Aliquots (4 g) of individual fecal samples were incubated overnight in nutrient broth (EMD Chemicals) containing cefotaxime 2 μg/mL and then streaked onto MacConkey agar (EMD Chemicals) containing cefepime 4 μg/mL. In addition, historical *Salmonella* spp. ($n = 16$) and *E. coli* ($n = 2$) isolates recovered from bovine or porcine diagnostic submissions to the Ohio Animal Disease Diagnostic Laboratory

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Table 1. Minimum Inhibitory Concentrations of Antimicrobial Agents for Four *Escherichia coli* Isolates Containing bla_{CTX-M} and Their Transconjugants

	Wild-type strains[a]				*Transconjugants*				
Antimicrobial	*DL-1*	*LA-1*[b]	*LA-2*	*LA-3*[b]	*tDL-1*	*tLA-1*	*tLA-2*	*tLA-3*	*Recipient MG1655*
Amikacin	≤0.5	1	1	1	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Amoxicillin/clavulanic acid	2/1	8/4	4/2	4/2	4/2	4/2	8/4	4/2	2/1
Ampicillin	>32	>32	>32	>32	>32	>32	>32	>32	≤8
Cefazolin	>16	>16	>16	>16	>16	>16	>16	>16	≤8
Cefepime	>16	>16	>16	>16	>16	>16	>16	>16	≤1
Cefotaxime	>64	>64	64	>64	>64	64	64	64	≤0.25
Cefotaxime/clavulanic acid[c]	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12
Cefoxitin	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4
Cefpodoxime	>32	>32	>32	>32	>32	>32	>32	>32	≤0.25
Ceftazidime	16	2	8	2	16	8	8	4	≤0.25
Ceftazidime/clavulanic acid[c]	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12
Ceftiofur	>8	>8	>8	>8	>8	>8	>8	>8	≤0.12
Ceftriaxone	>128	>128	>128	>128	>128	64	>128	>128	≤1
Cephalothin	>16	>16	>16	>16	>16	>16	>16	>16	≤8
Chloramphenicol	>32	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2
Ciprofloxicin	2	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1
Gentamicin	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4
Imipenem	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Kanamycin	>64	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤8
Meropenem	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1
Naladixic acid	>32	≤0.5	≤0.5	≤0.5	2	2	4	2	2
Piperacillin/tazobactam[d]	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4
Streptomycin	≤32	≤32	≤32	≤32	≤32	≤32	≤32	≤32	≤32
Sulfasoxazole	>256	≤16	≤16	≤16	≤16	≤16	≤16	≤16	≤16
Tetracycline	32	≤4	32	≤4	≤4	≤4	≤4	≤4	≤4
Trimethoprim/sulfamethoxazole	>4/76	≤0.12/2.38	≤0.12/2.38	≤0.12/2.38	≤0.12/2.38	≤0.12/2.38	≤0.12/2.38	≤0.12/2.38	≤0.12/2.38

[a]Isolate DL-1 originated from a bovine clinical diagnostic submission to the Ohio Animal Disease Diagnostic Laboratory. Isolates LA-1, LA-2, and LA-3 originated from bovine fecal samples collected at a livestock auction in Ohio.

[b]Isolates LA-1 and LA-3 were determined to be clonal *Escherichia coli* strains based on results of pulsed-field gel electrophoresis with a single enzyme, *Spe*I.

[c]Clavulonic acid at a fixed concentration of 4 μg/mL.

[d]Tazobactam at a fixed concentration of 4 μg/mL.

Table 2. Primers Used for Sequencing Four *Escherichia coli* Isolates and Their Transconjugants

Primer name	Oligonucleotide sequence		Product size (bp)	Reference
	Forward (5′ to 3′)	Reverse (5′ to 3′)		
CTXM–Global	TTTGCGATGTGCAGTACC AGTAA	CGATATCGTTGGTGGTGC CATA	544	Edelstein *et al.* (2003)
CTXM-1-Amp	GACGATGTCACTGGCTGAGC	AGCCGCCGACGCTAATACA	499	Pitout *et al.* (2004)
CTXM-1 upstream	ATGTTGTTGTTAATTCGTCTC	CGTTATCGCTGTACTGTAG	446	This study
CTXM-2 downstream	TTAACTATAATCCGATTGCG	TTTCTGCCTTAGGTTGAG	507	This study
TEM	ATAAAATTCTTGAAGACGAAA	GACAGTTACCAATGCTTAATCA	1074	Ma *et al.* (2005)

(ADDL) in 2008 and 2009 that were classified as resistant (minimum inhibitory concentration [MIC] $\geq 8\,\mu g/mL$) to ceftiofur were identified from the laboratory database. These isolates were recovered from storage and streaked onto MacConkey agar containing cefepime 4 μg/mL and incubated overnight.

MICs were determined to standard panels of antimicrobials detailed in Table 1 using broth microdilution (CMV1AGNF and ESB1F panels, Sensititre; Trek Diagnostic Systems) following manufacturer's instructions and Clinical and Laboratory Standards Institute guidelines (CLSI, 2008). The presence of $bla_{\text{CTX-M}}$ was established by polymerase chain reaction (PCR) using available protocols (Pitout *et al.*, 2004; Lewis *et al.*, 2007) and amplicons from positive PCR reactions were sequenced. Isolates were also screened for the presence of TEM-family, SHV-family, and OXA-1-family β-lactamase genes by PCR (Siu *et al.*, 2000; Ma *et al.*, 2005) and confirmed by sequencing. Conjugation experiments were accomplished by mating putative donor strains with a rifampicin-resistant derivative of *E. coli* strain MG1655 (Gebreyes and Thakur, 2005). Transconjugants were detected by plating mating mixtures on Luria-Bertani agar supplemented with cefepime 16 μg/mL and rifampicin 100 μg/mL and incubated overnight at 37°C. Additional conjugation experiments were performed using rifampicin-resistant mutants of *Salmonella infantis* originally recovered from a porcine field sample as recipients. Transconjugants were re-streaked on Luria-Bertani agar with cefepime 16 μg/mL and rifampicin 100 μg/mL to obtain isolated colonies used to derive template DNA for PCR using the CTX-M primer set (Lewis *et al.*, 2007). Pulsed-field gel electrophoresis was performed on total genomic DNA using *Spe*I (New England Biolabs) following Centers for Disease Control and Prevention–recommended procedures (Ribot *et al.*, 2006). Clonality was assessed visually by examination of the macrorestriction patterns, and confirmed using Bionumerics software (version 4.6; Applied Math Inc.). Plasmid detection was accomplished using the method described by Kado and Liu (1981). Plasmid restriction fragment analysis was performed using *Acc*I (New England Biolabs). Sequencing primers (Table 2) were designed (Premier Biosoft International) and amplicons were sequenced bi-directionally using the CEQ 8000 capillary electrophoresis system (Beckman Coulter) and analyzed using the basic local alignment search tool (BLAST).

Results and Discussion

E. coli carrying $bla_{\text{CTX-M-1}}$ were recovered from 3 of 50 (6%) fecal samples collected at the livestock market. Two isolates were clones by pulsed-field gel electrophoresis analysis. While these clonal isolates originated from different samples collected from different pens, it is unknown if they came from the same animal. These isolates were resistant to ampicillin, cefazolin, cephalothin, cefotaxime, cefpodoxime, ceftriaxone, ceftiofur, and cefepime, and had ceftazidime MICs that ranged from 2 to 8 μg/mL (Table 1). These isolates were susceptible to cefoxitin and to beta-lactam drug combinations that included clauvulanic acid or tazobactam.

No cefepime-resistant *E. coli* were recovered from the fecal samples collected from freestall barns at the three Ohio dairy farms, all of which were known by the investigators to use ceftiofur for treatment of various health conditions in cows.

None of the 16 ceftiofur-resistant *Salmonella* historical isolates from the Ohio ADDL were resistant to cefepime. However, one of the two ceftiofur-resistant diagnostic *E. coli* isolates had a cefepime MIC > 16 and was positive for both $bla_{\text{CTX-M-79}}$ and $bla_{\text{TEM-1}}$ by PCR. This isolate was originally recovered from a dairy calf fecal sample submitted as part of an outbreak investigation of diarrhea at an Ohio calf rearing facility. A single *Salmonella* Muenster and a second *E. coli* recovered from other diarrheic calves from this outbreak were not resistant to cefepime. All sick calves during the outbreak were treated with ceftiofur before submission of the diagnostic samples to the Ohio ADDL.

All 4 field isolates transferred cefepime resistance to the *E. coli* recipient by conjugation, but only the 3 isolates from the livestock market transferred cefepime resistance to the *Salmonella* recipient. This is consistent with the hypothesis that commensal *E. coli* in the intestinal flora of food animals can serve as a reservoir of resistance genes for pathogens. PCR for $bla_{\text{CTX-M}}$ performed on DNA isolated from MG1655 and *Salmonella* transconjugants yielded amplicons of the expected

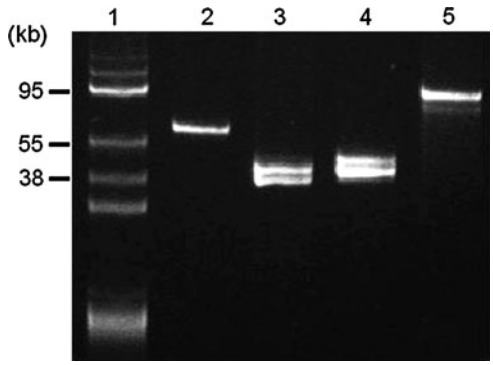

FIG. 1. Plasmid content of four *Escherichia coli* transconjugants containing $bla_{\text{CTX-M}}$. Lanes: 1, BacTracker supercoiled DNA ladder; 2, tDL-1 ($bla_{\text{CTX-M-79}}$); 3, tLA-1 ($bla_{\text{CTX-M-1}}$); 4, tLA-2 ($bla_{\text{CTX-M-1}}$); 5, tLA-3 ($bla_{\text{CTX-M-1}}$).

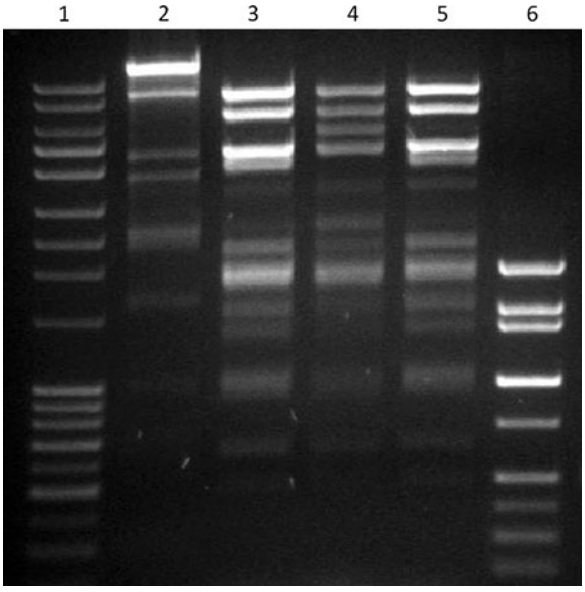

FIG. 2. *Acc*I restriction analysis of plasmid DNA isolated from four *E. coli* transconjugants containing bla_{CTX-M}. Lanes: 1, Lonza DNA marker 1-10 kb; 2, tDL-1 ($bla_{CTX-M-79}$); 3, tLA-1 ($bla_{CTX-M-1}$); 4, tLA-2 ($bla_{CTX-M-1}$); 5, tLA-3 ($bla_{CTX-M-1}$); 6, exACTGene 1 kb Plus DNA ladder.

size of 544 bp (Lewis *et al.*, 2007). Subsequent sequencing of these amplicons corresponded with the bla_{CTX-M} alleles that were identified in their respective parental donor strains. Plasmids ranging in size from ~40 to 100 kb were observed on plasmid profiles of the *E. coli* transconjugants (Fig. 1). The plasmid recovered from the diagnostic isolate carrying $bla_{CTX-M-79}$ was distinguishable from the three plasmids carrying $bla_{CTX-M-1}$ using restriction analysis with *Acc*I (Fig. 2). MICs for the field strains and their transconjugants are reported in Table 1. Resistance to ampicillin, as well as first-, third-, and fourth-generation cephalosporins was transferred to each of the transconjugants.

Before this report, bla_{CTX-M} has not been recognized in enteric bacteria of animal origin in the United States. *Salmonella* carrying $bla_{CTX-M-5}$ have been recovered in the United States from an infant recently adopted from Russia (Zirnstein *et al.*, 2000) and from an infant in Georgia without a history of travel (Sjolund *et al.*, 2008). Beyond these reports, other studies of Salmonellae from humans (Whichard *et al.*, 2007) and livestock (Frye *et al.*, 2008) in the United States have not implicated bla_{CTX-M} as a basis for cephalosporin resistance.

E. coli carrying bla_{CTX-M} have been previously recovered from calves with diarrhea in the United Kingdom (Liebana *et al.*, 2006). In addition, *E. coli* with bla_{CTX-M} have been recovered from cattle and other food animals in Europe (Brinas *et al.*, 2005; Meunier *et al.*, 2006; Jørgensen *et al.*, 2007) and Asia (Duan *et al.*, 2006; Tian *et al.*, 2009). The $bla_{CTX-M-1}$ allele has been previously reported in *E. coli* isolates recovered from bovine diagnostic submissions in France (Meunier *et al.*, 2006), but $bla_{CTX-M-79}$ has not been previously reported in livestock species. *E. coli* carrying $bla_{CTX-M-1}$ have been reported from seven human diagnostic submissions from five U.S. states (Moland *et al.*, 2003).

It has been hypothesized that ceftiofur use in livestock populations may provide selection pressure leading to the dissemination of bla_{CTX-M} (Jørgensen *et al.*, 2007; Tian *et al.*, 2009). Our results are consistent with this hypothesis given that we recovered *E. coli* with bla_{CTX-M} from a calf that had recently received ceftiofur therapy. Likewise, cefquinome is available for use in Europe and Asia, where the recovery of bla_{CTX-M} from livestock has been reported (Brinas *et al.*, 2005; Duan *et al.*, 2006; Meunier *et al.*, 2006; Jørgensen *et al.*, 2007; Tian *et al.*, 2009). However, the veterinary use of ceftiofur or cefquinome has not been associated with the emergence or dissemination of bla_{CTX-M} in livestock populations.

Disclosure Statement

No competing financial interests exist.

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