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Emergence of *bla*$_{CTX-M}$ resistance determinants in the bovine *E. coli* reservoir, Northwest United States

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INTRODUCTION

The rapid global spread of bacteria bearing CTX-M extended spectrum beta-lactamases (ESBL) reached the United States much later than most other countries. The earliest detection of CTX-M–type ESBLs in human isolates were reported in *E. coli* and *Salmonella* in the late 1980s in Japan, Europe and South America[1]. By 2007, *bla*$_{CTX-M}$ was detected in 39% of ESBL Enterobacteriaceae of eight U.S. medical centers[2].and the earliest report of *bla*$_{CTX-M}$ in Washington State was in a *Shigella sonnei* isolate cultured from a fecal sample in 2004[3]. Their first detection in US cattle was in fecal *Escherichia coli* collected from Ohio dairy cattle in 2009[4]. Here we report the earliest detection of bovine-source *bla*$_{CTX-M}$-positive *E. coli* in Washington and preliminary results from an ongoing prevalence study.

METHODS

To determine the earliest isolation of *bla*$_{CTX-M}$-positive *E. coli* in Washington dairy cattle we conducted a retrospective survey of banked *E. coli* isolates from calves. These isolates were from several different studies for which culture and isolation protocols differed slightly. In general, fecal samples from individual preweaned Washington State dairy calves were collected and plated either directly onto MacConkey agar supplemented with ceftiofur (2-4 µg/ml) or onto plain MacConkey. In the latter case, 3 or more colonies were picked and resistance to a panel of 12 antibiotics which included ceftiofur was tested using a disk diffusion method according to CLSI standards[5,6]. Isolates that were resistant to ceftiofur were tested for the presence of *bla*$_{CTX-M}$ and *bla*$_{CMY-2}$ using published primers and protocols[7,8]**.** Selected isolates from a single farm were characterized further using pulsed-field gel electrophoresis (PFGE) following digestion with *Xba*I using a standard PulseNet protocol[9]. In addition, the prevalence of *bla*$_{CTX-M}$-positive *E. coli* was determined for prospectively sampled calves. Individual calf fecal samples were enriched in nutrient broth containing cefotaxime (2 µg/ml) by incubation for 18-24 hrs and then plated on MacConkey agar supplemented with cefoxitin (4 µg/ml) and cefepime (4 µg/ml). Growth on cefoxitin is associated with the presence of *bla*$_{CMY-2}$ while growth on cefepime is associated with the presence of *bla*$_{CTX-M}$ [10]. Isolates growing on either media type were transferred to LB broth with glycerol and stored at -80 °C for subsequent detection of genotype.

RESULTS

Among 93 ceftiofur-resistant *E. coli* obtained from 6 farms in 2002, none had *bla*$_{CTX-M}$ and 82 (88.2%) had *bla*$_{CMY-2}$(Table 1). Among ceftiofur-resistant *E. coli* isolates from Farm 60 obtained in 2008, early 2011 and late 2011, 0/18, 0/9 and 5/18 were positive for *bla*$_{CTX-M}$, respectively, while 9/18, 8/8 and 5/18 were positive for *bla*$_{CMY-2}$ (Table 2). Prevalence of *bla*$_{CTX-M}$-positive *E. coli* on four farms sampled in 2011 was 5.3, 30.8, 66.7 and 25.0 % (Table 3). Among 30 *bla*$_{CTX-M}$-positive *E. coli* , 2 had Group 1 *bla*$_{CTX-M}$, 21 had group 9 *bla*$_{CTX-M}$, and 7 were not determined (data not shown). PFGE profiles of *bla*$_{CTX-M}$-positive *E. coli* from a single farm indicated a clonal relationship between isolates from calves in neighboring hutches, and from the same calf sampled 2 weeks apart (Fig. 1). Preliminary results from an ongoing study indicate a high prevalence of phenotypes characteristic of *E. coli* with both *bla*$_{CMY-2}$ *bla*$_{CTX-M}$ genes on 9 farms sampled so far (Table 4).

Table 1. Ceftiofur-resistant *E. coli* isolates collected from individual calf feces on 4 farms in 2002. **All were PCR-negative for *bla*$_{CTX-M}$.**

Farm	N (%) *bla*$_{CMY-2}$ positive	Total tested
47	35 (94.6)	37
46	43 (100.0)	43
371	0	3
913	0	1
1049	0	4
1006	4 (80.0)	5
Total	82 (88.2)	93

Table 2. Number of ceftiofur-resistant *E. coli* isolates from Farm 60 obtained in 2008 and 2011 that were PCR positive for *bla*$_{CMY-2}$ and *bla*$_{CTX-M}$. Each isolate tested was from an independent calf fecal sample.

Year, Month	*bla*$_{CTX-M}$+	*bla*$_{CMY-2}$+	Total
2008			
Feb	0	3	6
Mar	0	6	12
2011			
Feb	0	8	8
Dec	5	5	18
Total	5	22	44

Table 3. Number and percent of *E. coli* isolates from individual calf fecal samples collected in 2011 plated on MacKonkey agar with 2 µg/ml ceftiofur with positive PCR results for *bla*$_{CMY-2}$, *bla*$_{CTX-M}$, or both.

Farm	N (%) *bla*$_{CMY-2}$ positive	N (%) *bla*$_{CTX-M}$ positive	N (%) positive for both genes	Total isolates tested
3	10 (52.6)	1 (5.3)	1 (5.3)	19
46	5 (38.5)	4 (30.8)	3 (23.1)	13
41	3 (16.7)	12 (66.7)	0	18
120	3 (25.0)	3 (25.0)	0	12
Total	21 (33.9)	20 (32.3)	4 (6.5)	62

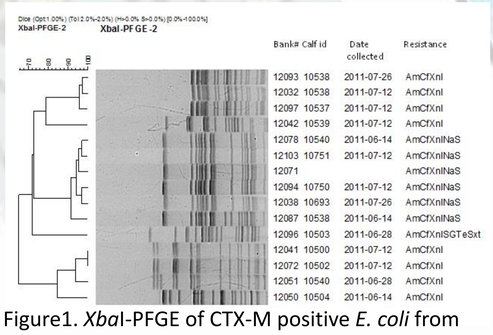

Figure1. *Xba*I-PFGE of CTX-M positive *E. coli* from calves. All were CMY-2 negative, CTX-M positive and CTX-M group 9. Sequential calf ID numbers indicate neighboring calves in a hutch row.

Table 4. Prevalence of *bla*$_{CMY-2}$ phenotype and *bla*$_{CTX-M}$ phenotype among individual dairy calf fecal samples, 2012

Farm	Prevalence (%) of CMY-2 phenotype[a]	Prevalence (%) of CTX-M phenotype[b]	# samples tested
3	100.0	100.0	51
35	100.0	100.0	45
37	100.0	96.0	25
39	95.3	83.7	43
46	67.3	59.6	52
47	98.0	87.8	49
49	55.6	59.3	27
51	87.5	91.7	48
60	100.0	53.2	47
mean:	89.3	81.2	367 total
median:	96.7	87.8	

[a] The percent of individual calf fecal samples that yielded *E. coli* which grew on MacConkey-supplemented cefoxitin.
[b] The percent of individual calf fecal samples that yielded E. coli which grew on MacConkey-supplemented cefepime.

CONCLUSIONS

Before 2010, third-generation cephalosporin resistance in gram-negative bacteria of US dairy cattle was considered to be primarily associated with AmpC enzymes coded by *bla*$_{CMY-2}$. Results of our retrospective testing (Tables 1 and 2) were consistent with that view. Although *E. coli* isolates banked at WSU from previous studies are limited with regard to number of farms and time periods represented, these data suggest that emergence and dissemination of *bla*$_{CTX-M}$-positive *E. coli* may be a recent phenomenon on Washington dairy farms. *bla*$_{CTX-M}$-positive *E. coli* were detected in 2009 at a low prevalence in adult dairy cattle in Ohio[4] and the earliest detection in calves in Washington was in 2011 (this study). On a single, large dairy from which ceftiofur-resistant isolates were available in 2008 and 2011, no evidence of *bla*$_{CTX-M}$-positive *E. coli* was observed until late 2011 (Table 2). Although we cannot rule out an earlier occurrence of *bla*$_{CTX-M}$-positive Enterobacteriaceae in Washington cattle than in human infections, the evidence supports the reverse, that human clinical infections with *bla*$_{CTX-M}$-positive Enterobacteriaceae preceded their occurrence in livestock, possibly by several years. However, whether this represents clonal bacterial or plasmid dissemination, or independent emergence in multiple locations, is as yet unknown. Our limited PFGE results suggest that *bla*$_{CTX-M}$-positive *E. coli* strains in the dairy calf environment can be transmitted between neighboring calves, and can persist in the animals for several weeks.

The rapid emergence and spread of *bla*$_{CTX-M}$-positive *E. coli* currently being observed in Washington dairies suggests that a shift in importance from *bla*$_{CMY-2}$ to *bla*$_{CTX-M}$ may be taking place in the bovine reservoir. To determine risk factors for this shift, our current sampling effort is accompanied by investigations into antibiotic use and animal movements. In addition, further molecular characterizations (plasmid replicon typing and *E. coli* genotyping) will private better insights into determinants of this shift..

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REFERENCES

1. Cantón R., González-Alba, and J.C. Galán. 2012. CTX-M enzymers: origin and diffusion. Front Microbiol 3:110. Epub 2012 Apr 2.
2. Castanheira M, Mendes RE, Rhomberg PR and RN Jones. 2008. Rapid emergence of *bla*$_{CTX-M}$ among Enterobacteriaceae in US Medical Centers: molecular evaluation from the MYSTIC Program (2007). Microb Drug Resist 13:211-6.
3. Kim, S., J. Hu, R. Gautom, J. Kim, B. Lee, and D. S. Boyle. 2007. CTX-M extended-spectrum beta-lactamases, Washington State. Emerg. Infect. Dis. 13:513-514.
4. Wittum TE, Mollenkopf DF, Daniels JB, Parkinson AE, Mathews JL, Fry PR, Abley MJ, Gebreyes WA: CTX-M-type expended-spectrum β -lactamases present in Escherichia coli from the feces of cattle in Ohio, United States. Foodborne Path Dis 2010,7:1575-1579.
5. Bauer, A. W., M. M. W. Kirby, J. C. Sherris, and M. Turck. 1966. Antibiotic susceptibility testing by a standard single disk method. American Journal of Clinical Pathology 45:493-496.
6. CLSI. 2008. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals: approved standard. 3rd ed. CLSI document M31-A3. CLSI document M31-A3 28.
7. Edelstein M, PimkinM, Palagin I, Edelstein I, and Stratchounski L. 2003. Prevalence and molecular epidemiology of CTX-M extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in Russian hospitals. Antimicrob Agents Chemother. 47:3724-3732.
8. Zhao S, White DG, McDermott PF, Friedman S, English L, Ayers S, et al. 2001. Identification and expression of cephamycinase bla (CMY) genes in *Escherichia coli* and *Salmonella* isolates from food animals and ground meat. Antimicrob Agents Chemother. 45:3647–50.
9. Ribot EM, et al. 2006.Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet.Foodborne Pathogen Dis. 3:59-67.
10. Mollenkopf et al. 2012. Variable within- and between-herd diversity of CTX-M cephalosporinase-bearing *Escherichi coli* isolates from dairy cattle. Appl Environ Microbiol 78:4552-60.