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Recent Emergence of *Escherichia coli* with Cephalosporin Resistance Conferred by bla_{CTX-M} on Washington State Dairy Farms

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***Enterobacteriaceae*-associated bla_{CTX-M} genes have become globally widespread within the past 30 years. Among isolates from Washington State cattle, *Escherichia coli* strains carrying bla_{CTX-M} (CTX-M *E. coli* strains) were absent from a set of 2008 isolates but present in a set of isolates from 2011. On 30 Washington State dairy farms sampled in 2012, CTX-M *E. coli* prevalence was significantly higher on eastern than on northwestern Washington farms, on farms with more than 3,000 adult cows, and on farms that recently received new animals. The addition of fresh bedding to calf hutches at least weekly and use of residual fly sprays were associated with lower prevalence of CTX-M *E. coli*. In Washington State, the occurrence of human pathogens carrying bla_{CTX-M} genes preceded the emergence of bla_{CTX-M}-associated *E. coli* in cattle, indicating that these resistance determinants and/or their bacterial hosts may have emerged in human populations prior to their dissemination to cattle populations.**

Emergence and dissemination of antibiotic resistance traits in Gram-negative bacteria can occur rapidly and across wide geographic distances (1). Bacterial strains carrying the bla_{CTX-M} family of extended-spectrum β-lactamase (ESBL) genes have spread globally in less than 30 years (2, 3). Unlike other families of ESBL genes which result from point mutations in preexisting, narrower-spectrum β-lactamase genes (bla_{TEM-1} and bla_{SHV}) (4), bla_{CTX-M} β-lactamase genes originated from chromosomal genes of *Kluyvera* species (2). The earliest *Escherichia coli* plasmid-associated bla_{CTX-M} was detected from laboratory dogs used in pharmaceutical research in Japan in 1986, followed by isolations from human patients in Munich, Germany (1989), Argentina (1989), France (1989), and Poland (1996) (2). In North America, bla_{CTX-M}-bearing *Escherichia coli* strains (CTX-M *E. coli* strains) were detected in humans in Canada in 2000 (5) and in the United States between 2001 and 2002 (6). By 2007 in the United States, 80% of 15 geographically dispersed medical centers reported *E. coli* or *Klebsiella pneumoniae* infections with associated bla_{CTX-M} genes (7). In Washington State, the earliest reported clinical human *E. coli* strain carrying bla_{CTX-M} was isolated in 2001 in Seattle (6). Currently, CTX-M enzymes are considered the most prevalent ESBLs in isolates of *E. coli*, *K. pneumoniae*, and *Proteus mirabilis* associated with human infections globally (8).

In the United States, dairy cattle-associated CTX-M *E. coli* strains were first reported by Wittum and others in Ohio in 2009 (9). This Ohio research group later reported CTX-M in *Salmonella enterica* isolates from equine, swine, and turkey sources from six different states (10) and from swine finishing barns in 5 states (11). In the current study, we first tested *E. coli* isolates from Washington State dairy cattle banked from several earlier research projects to determine the time window of CTX-M *E. coli* emergence in this region and host species. The results of this retrospective study indicated recent emergence of CTX-M *E. coli* in Washington State; in order to determine factors associated with CTX-M *E. coli* prevalence, we conducted a study of management and other factors influencing the prevalence of this novel bacterial resistance determinant among dairy farms.

(Preliminary results from this study were presented at the 113th General Meeting of the American Society for Microbiology, 18 to 21 May 2013, Denver, CO, USA.)

MATERIALS AND METHODS

Retrospective analysis. Ceftiofur, a veterinary third-generation cephalosporin, is used commonly to treat infectious diseases in dairy cattle (12). Resistance to ceftiofur is correlated with clinically relevant drug resistance in human medicine (13) and has been used as a phenotypic marker for targeted research of third-generation cephalosporin resistance genes in bovine bacterial flora (14–16). The *E. coli* isolates used in the retrospective study were obtained during previous studies using various selection methods focused primarily on ceftiofur resistance.

Isolates from the years 2002 and 2003 were obtained from a study in which 3,673 fecal *E. coli* isolates were collected from dairy cattle and calves in Washington State (17) and then tested for resistance to a panel of 12 antibiotics (see Table S1 in the supplemental material) using breakpoint agar dilution (17). From the ceftiofur-resistant *E. coli* isolates ($n = 478$ from 162 individual animals) detected in that study, we tested one isolate per animal by PCR for the presence of bla_{CMY-2} and bla_{CTX-M}. Five isolates were not available, leaving 157 isolates from different animals on 14 different farms for testing.

Isolates from 2008 originated from a study of the dynamics of antibiotic-resistant *E. coli* in dairy calves in which individual dairy calves were sampled repeatedly over time (W. M. Sischo, unpublished data). In that study, fecal samples were plated onto either unsupplemented MacConkey agar or MacConkey agar supplemented with ceftiofur (8 μg/ml) (Pfizer, New York, NY, USA). Lactose-positive (pink) colonies were transferred to

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TABLE 1 PCR primer sequences used in the study

Gene	Primer name	Sequence (5′ to 3′)	Reference
bla_{CTX-M}	Pan CTX-M.F	TTTGCGATGTGCAGTACCAGTAA	21
	Pan CTX-M.R	CGATATCGTTGGTGGTGCCATA	
bla_{CMY-2}	CMY-F	GACAGCCTCTTTCTCCACA	15
	CMY-R	TGGAACGAAGGCTACGTA	
*uid*A	UAL-754	AAAACGGCAAGAAAAAGCAG	18
	UAR-900	ACGCGTGGTTACAGTCTTGCG	

bank tubes containing brain heart infusion (BHI) agar (Hardy Diagnostics, Santa Maria, CA, USA) and incubated overnight at 37°C and later confirmed as *E. coli* by PCR detection of *uidA* (18). *E. coli* isolates were tested for resistance to a panel of 12 antibiotics (see Table S2 in the supplemental material) by agar diffusion assay (19, 20). Isolates with reduced susceptibility to ceftiofur (inhibition zone size of ≤22 mm) were systematically chosen to represent different animals, farms, and collection dates, yielding 65 isolates that were tested by PCR for the presence of bla_{CMY-2} and bla_{CTX-M}.

Isolates from 2011 originated from a project that sampled calves on four dairy farms visited five times at 2-week intervals. Approximately 50 individual calf fecal samples were obtained at each visit and were processed as described for isolates from 2008, except that selective MacConkey plates were supplemented with 2 μg/ml ceftiofur. To capture isolates with intermediate susceptibility, the ceftiofur concentration of 2 μl/ml was chosen, because it is the breakpoint between susceptibility and intermediate susceptibility (19). This resulted in a total of 882 samples which generated 863 *E. coli* isolates from unsupplemented MacConkey and 755 isolates from MacConkey supplemented with 2 μg/ml ceftiofur. From those isolates, a subset was chosen by random selection of up to three isolates per farm per date. This subset included 44 *E. coli* isolates from unsupplemented MacConkey medium and 45 isolates picked from ceftiofur-supplemented MacConkey medium. The resulting 89 isolates were tested by PCR for the presence of bla_{CMY-2} and bla_{CTX-M} and were tested for resistance to ceftiofur using a disk agar diffusion assay as described above (19, 20). Forty-seven of these isolates had reduced susceptibility to ceftiofur (inhibition zone size of ≤22 mm), and their PCR results are reported in Table 2.

PCR protocols. We detected the presence of bla_{CTX-M} and bla_{CMY-2} genes using previously published protocols (8, 21, 22). Boiled cell lysate was used (5 μl in a 25-μl PCR mixture) for the reaction template. Standard amplification reaction volumes were 25-μl final concentrations of 1× buffer, 100 μM deoxynucleoside triphosphate (dNTP), 1.5 μM $MgCl_2$, 1 to 0.1 pmol/μl primer, and 0.5 to 1 U Taq. Cycling conditions for bla_{CMY-2} PCR included an initial denaturation of 95°C for 3 min and 30 cycles of 94°C for 30 s, 55°C for 30 s,72°C for 30 s, and 72°C for 10 min. Cycling conditions for the global bla_{CTX-M} included initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 15 s, annealing at 52°C for 30 s, extensions at 72°C for 30 s, and then a final extension at 72°C for 7 min. Primer sequences are as shown in Table 1.

Prevalence risk factor study. During the summer and fall of 2012, a convenience sample of 30 Washington State dairy farms was identified, and each farm was visited once. The 30 farms were distributed throughout the dairy-intensive agricultural areas of Washington State. For the analysis, we divided the state into two regions, northwestern Washington (region 1; 9 farms) and eastern Washington (region 2; 21 farms) (Fig. 1). Samples collected at each farm included individual animal fecal samples from up to 5 preweaned dairy calves per age group (0 to 6 days old, 7 to 13 days old, 14 to 20 days old, 21 to 27 days old, 28 to 34 days old, 35 to 41 days old, 42 to 48 days old, 49 to 55 days old, 56 to 62 days old, and >62 days old), 5 pooled fecal pat samples from each of the lactation pen, fresh cow pen, hospital pen, and maternity or closeup pens, a milk filter, and one wastewater lagoon sample. Approximately 5 g of fecal material and 5 ml lagoon water were collected for each sample. Individual calf fecal samples were obtained aseptically *per rectum*. These procedures were approved by the Washington State University Institutional Animal Care and Use Committee. Fecal samples were held on ice during transport to the laboratory.

Microbiological assessments. To select for bla_{CTX-M}- and bla_{CMY-2}-associated phenotypes, samples from all farms were processed as previously described (22). Briefly, feces (5 g) were incubated in 45 ml nutrient broth (Hardy Diagnostics, Santa Maria, CA, USA) supplemented with

FIG 1 County boundary map of Washington State. Nine dairies were visited in the northwestern region (region 1), and 21 dairies were visited in the eastern region (region 2) for the bla_{CTX-M} prevalence risk factor study. (Map adapted from the 2012 USDA Census of Agriculture.)

TABLE 2 Results of bla_{CMY-2} and bla_{CTX-M} PCR of *E. coli* isolates from cattle feces in the Pacific Northwest

Time period	Isolate selection or ceftiofur concn in MacConkey medium	Total no. of isolates tested	No. of farms	bla_{CMY-2} positive		bla_{CTX-M} positive	
				No.	% (95% CI)[a]	No.	% (95% CI)[a]
2002-2003	Reduced susceptibility to ceftiofur per breakpoint agar test (17)	157	14	137	87.3 (80.8–91.9)	0	0 (0–3.0)
2008	Reduced susceptibility to ceftiofur per breakpoint agar test (17)	41	4	40	97.6 (85.6–99.9)	0	0 (0–10.7)
2008	8 μg/ml ceftiofur	24	4	23	95.8 (76.9–99.8)	0	0 (0–17.2)
2008 total		65	4	63	96.9 (88.4–99.5)	0	0 (0–7.0)
2011	0 μg/ml ceftiofur	45	4	14	31.1 (18.6–46.8)	2	4.4 (0.1–16.4)
2011	2 μg/ml ceftiofur	44	4	24	54.5 (39.0–69.3)	5	11.4 (3.0–20.2)
2011 total		89	4	38	42.7 (32.4–53.6)	7	7.9 (3.5–16.1)
2011	Reduced susceptibility to ceftiofur according to disk diffusion results (inhibition zone size of ≤22 mm) (19, 20)	47	4	37	78.7 (63.9–88.8)	7	14.9 (6.7–28.9)

[a] CI, confidence interval (23).

cefotaxime (2 μg/ml) at 37°C for 18 to 24 h without shaking. Following incubation, 0.1 ml of broth was independently plated onto MacConkey agar supplemented with cefepime (4 μg/ml) or cefoxitin (4 μg/ml). Cefepime was used to screen for a bla_{CTX-M} phenotype and cefoxitin for a bla_{CMY-2} phenotype (22). Bacterial isolates grown on cefepime- and cefoxitin-supplemented media were tested for the presence of bla_{CTX-M} and bla_{CMY-2}, respectively. Three to five isolated colonies with morphology consistent with *E. coli* were picked from each plate for banking and further characterization. Isolates were confirmed as *E. coli* by PCR detection of *uidA* (18). In addition to selective plating and in order to estimate the prevalence of bla_{CTX-M}-and bla_{CMY-2}-bearing *E. coli* in unselected populations of fecal *E. coli*, samples from 21 farms (9 in region 1 and 12 in region 2) were directly swabbed onto MacConkey agar without antibiotic supplement and incubated overnight at 37°C. Two isolates were picked from each of these plates and characterized by PCR for *uidA*, bla_{CTX-M}, and bla_{CMY-2} as described above.

Questionnaires. At each farm visit, a questionnaire was administered to the farm herd manager, calf manager, or owner. The questionnaire elicited information about the number of animals (adult cows, preweaned calves, heifers, and bull calves), the number of physical locations occupied by the farm, whether heifer calves were raised elsewhere and returned to the dairy, recent animal purchases from other premises, whether feed was raised on site or purchased elsewhere, calving pen practices, frequency and method of cleaning maternity pens and calf hutches, calf feeding practices, protocols for cleaning bottles and buckets used for feeding milk and dry feeds, calf housing practices, fly control, calf treatment crew hygiene, the number of employees and visitors and frequency of visits to the farm, and antibiotics used for calf and cow diseases (see the supplemental material).

Data analysis. To calculate farm-level prevalence of bla_{CTX-M}- and bla_{CMY-2}-positive samples, a sample producing one or more *E. coli* isolates PCR positive for bla_{CTX-M} and/or bla_{CMY-2} on any media was counted as positive for the relevant gene. Farm-level bacterial data were joined with the questionnaire data and analyzed using SAS v. 9.2 for Windows (SAS Institute Corp., Cary, NC, USA). To describe the distribution of prevalence by farm, all sample types were included in the analysis. For comparisons between proportions, a Mantel-Haenszel chi-square test was computed using Proc Freq in SAS, and 95% confidence intervals surrounding proportions were calculated according to Fleiss et al. (23) by using WinPepi (24). For risk factor assessment, farm prevalence of calves positive for bla_{CTX-M} was the outcome variable, and risk factors from the questionnaire data were independent variables. Each potential risk factor was assessed for a univariable association with the outcome of interest using Wilcoxon's two-sample test calculated using Proc Univariate in SAS. A *P* value of ≤0.05 was considered significant.

RESULTS AND DISCUSSION

Retrospective analysis. All *E. coli* isolates obtained from studies conducted prior to 2011 were PCR negative for bla_{CTX-M}. In isolates obtained in the 2011 study, bla_{CTX-M} was detected in 2/45 (4.4%) isolates derived from nonselective (unsupplemented MacConkey agar plates) and in 5/44 (11.4%) isolates derived from ceftiofur-supplemented plates (Table 2).

Risk factor study. Each of 30 Washington State farms was visited once between June and October 2012. The herd size distribution in the northwestern part of Washington was significantly smaller than that in eastern Washington (Kruskal-Wallis *P* value = 0.003) (see Fig. S1 in the supplemental material). An average of 45 fecal samples were collected from each farm for a total of 1,351 samples. The majority of fecal samples were from preweaned calves (see Table S3 in the supplemental material). The numbers of samples collected were uniform across the 10 age interval categories (average number per age category, 4.2; standard deviation [SD], 0.73). Growth on cefepime-supplemented MacConkey plates was highly correlated with bla_{CTX-M}: of 2,785 isolates obtained from cefepime-supplemented plates, bla_{CTX-M} was detected in 2,508 (90.1%). Similarly, growth on cefoxitin-supplemented plates correlated strongly with the presence of bla_{CMY-2}: of 3,914 isolates from cefoxitin-supplemented plates, bla_{CMY-2} was detected in 3,080 (78.7%). Carriage of both beta-lactamase genes in single isolates was also common: bla_{CMY-2} was detected in 339 of 2,169 (13.5%) CTX-M *E. coli* isolates isolated from cefepime-supplemented plates (see Tables S4 and S5 in the supplemental material).

On the basis of selective media and PCR testing, 70.0% (21/30) of farms had a prevalence of *E. coli* isolates carrying bla_{CMY-2} of greater than 80%, and 33.3% (10/30) of farms had a CTX-M *E. coli* prevalence greater than 80% (Table 3). Samples from 21 farms plated onto unsupplemented MacConkey agar showed an overall prevalence of CTX-M *E. coli* of 4.4% (95% confidence interval [CI] of 3.0 to 6.5) and an overall prevalence of bla_{CMY-2}-positive *E. coli* isolates of 32.1% (95% CI of 28.3 to 35.3). On 20/21 farms, the prevalence of bla_{CMY-2}-positive *E. coli* isolates was higher than the prevalence of CTX-M isolates based on nonselective culture, and overall this difference was significant (Wilcoxon signed-rank test *P* value of <0.0001) (Fig. 2). *E. coli* isolates carrying bla_{CMY-2} had

TABLE 3 Number of farms by prevalence of *E. coli* strains with bla_{CMY-2} and bla_{CTX-M} genes

Prevalence range[a]	No. (%) of farms in prevalence category for *E. coli* with:	
	bla_{CMY-2}	bla_{CTX-M}
0–0.2	0 (0)	6 (20.0)
0.2–0.4	0 (0)	1 (3.3)
0.4–0.6	4 (13.3)	5 (16.7)
0.6–0.8	5 (16.7)	8 (26.7)
0.8–1.0	21 (70.0)	10 (33.3)

[a] Proportion of samples plated on selective media that yielded at least one *E. coli* isolate positive for bla_{CMY-2} or bla_{CTX-M}.

consistently higher prevalence than CTX-M *E. coli* isolates in preweaned calves across all calf age groups (Wilcoxon signed-rank test *P* value of 0.002) (see Fig. S2 in the supplemental material), and the prevalence of *E. coli* isolates carrying bla_{CMY-2} did not differ between regions (data not shown), suggesting that *E. coli* isolates carrying bla_{CMY-2} are well established at a population level across dairy farms in Washington State. CTX-M *E. coli* strains were present in 62/116 (53.5% [95% CI, 44.0 to 62.7%]) pooled adult cow fecal samples from 30 farms, 16 (57.1% [95% CI, 37.4 to 75.0]) lagoon samples from 28 farms, and 14 (50.0% [95% CI, 31.1 to 67.0]) milk filter samples from 28 farms.

Fourteen antibiotics were reportedly used to treat calf and cow diseases across all farms. Ceftiofur was reported to be used for treating all surveyed cattle diseases. Other antibiotics reported to be commonly used on study farms included penicillin, ampicillin, enrofloxacin, and florfenicol. The most frequently used antibiotic for intramammary treatment of mastitis was ceftiofur, followed by cephapirin and pirlimycin. Nearly all the antibiotics that were available on study farms were reportedly used to treat calf diarrhea, with enrofloxacin and ceftiofur the most frequently used. Florfenicol and enrofloxacin were the antibiotics most frequently used to treat calf respiratory disease (Table 4).

Univariable nonparametric analysis results indicated that a northwestern Washington location, smaller herd size, adding fresh bedding to calf hutches weekly or more frequently, and use of residual fly sprays for insect control were associated with significantly lower prevalence of CTX-M *E. coli* in calves (exact Wilcoxon two-sample test *P* value of <0.05). Additionally, recent animal movements onto the farm were significantly associated with higher CTX-M *E. coli* prevalence in calves (exact Wilcoxon two-sample test *P* value of 0.03) (Table 5). Management factors for which no associations with CTX-M *E. coli* prevalence were detected included reported use of antibiotics (either any use or the number of diseases for which the antibiotic was used), addition of antibiotics to grain, milk, or milk replacers, frequency of cleaning feeding containers (milk and water containers) or esophageal feeders, type of calf hutch, method of calf hutch cleaning, frequency of hutch cleaning, and type of bedding used in calf hutches. Other variables for which no associations with CTX-M *E. coli* prevalence in calves were detected included calf treaters' hand hygiene, direction of feeding in the calf rows (youngest to oldest, oldest to youngest, or both directions), frequency of visits by feed delivery trucks, veterinarians, nutritionists, hoof trimmers, and breeders, and the number of people on the farm on any given day.

CTX-M *E. coli* strains were not detected among isolates obtained from Washington State dairy cattle prior to 2009 but were detected among isolates from 2011. By 2012, CTX-M *E. coli* strains were widespread among dairy farms throughout Washington

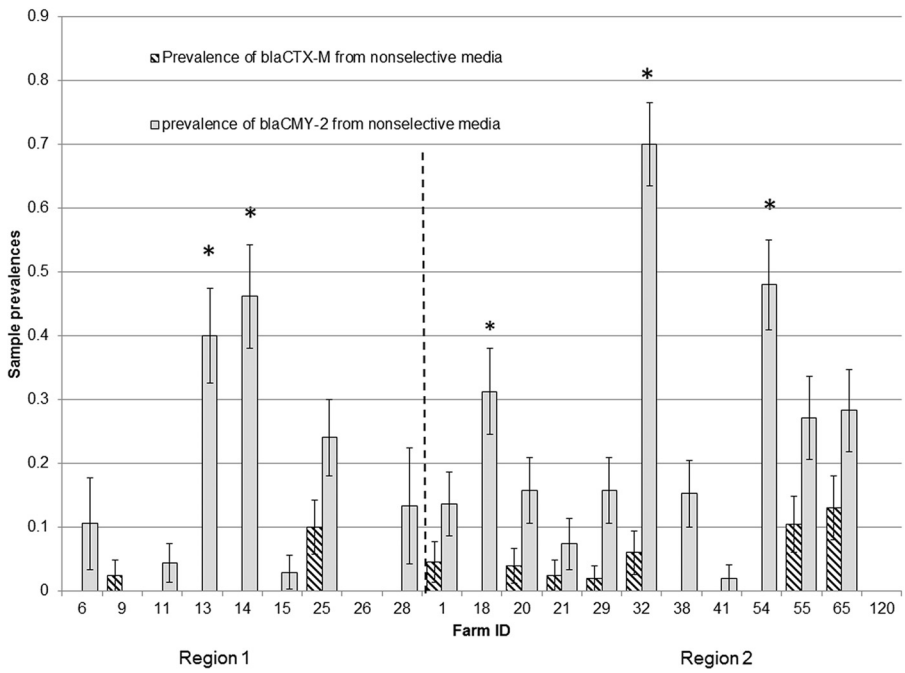

FIG 2 Prevalence of samples that yielded *E. coli* isolates positive for bla_{CTX-M} and bla_{CMY-2} from nonselective media from 21 dairy farms. Error bars represent ±standard errors of the mean (SEM). Farms to the left of the vertical dotted line are located in region 1 (northwestern Washington), and farms to the right of the dotted line are located in region 2 (eastern Washington). Mean farm prevalence of bla_{CMY-2} was higher than the prevalence of bla_{CTX-M} (Wilcoxon signed-rank test *P* value of <0.0001).

TABLE 4 Number of farms out of 30 reporting specific antibiotics used for cow and calf diseases

Antibiotic	No. of farms reporting diseases												
	Calf diseases						Cow diseases						
	Diarrhea	Mycoplasma (ears)	Pneumonia	Sepsis	Swollen joint	Umbilical infection	Displaced abomasum	Dystocia	Foot rot	Intramammary treatment	Metritis	Pneumonia	Retained placenta
Ampicillin	3	7	1	2	8	6	6	5	11	0	9	13	6
Ceftiofur	11	4	3	8	4	5	14	15	8	21	22	14	20
Cephapirin	0	0	0	0	0	0	0	0	0	14	0	0	0
Enrofloxacin	15	0	13	2	1	0	0	0	0	0	0	0	0
Florfenicol	4	1	22	2	0	0	0	0	0	0	0	0	0
Gentamicin	7	0	5	0	1	0	0	0	0	0	0	0	0
Oxytetracycline	3	0	2	0	1	0	1	1	6	0	1	3	1
Penicillin	4	11	3	3	17	20	4	3	14	0	2	4	3
Pirlimycin	0	0	0	0	0	0	0	0	0	10	0	0	0
Sulfamethazine	7	0	2	1	0	0	0	0	0	0	0	1	0
Tilmicosin	1	1	2	1	0	0	0	0	0	0	0	0	0
Tilosin	2	0	1	1	1	0	0	0	3	0	0	0	0
Sulfa-trimethoprim	0	0	0	1	0	0	0	0	0	0	0	0	0
Tulathromycin	3	1	5	1	0	0	0	0	0	0	0	0	0

State. Although the number of isolates in the retrospective set was limited, they were screened for resistance to ceftiofur, which would increase the probability of detecting *E. coli* strains carrying β-lactamase genes, including bla_{CTX-M} (25). Our estimate of the timing of the appearance of CTX-M *E. coli* in Washington State dairy cattle is consistent with findings from dairy studies in Ohio (9, 10) and feedlot cattle in Texas (26), where CTX-M *E. coli* strains were detected in cattle in 2009.

Human infections with *Enterobacteriaceae* strains carrying bla_{CTX-M} were reported from Europe, Asia, Africa, and South America during the late 1980s and 1990s, while the earliest reports of bla_{CTX-M} *Enterobacteriaceae* infections in human patients from North America were in the early 2000s (2, 6, 27–29). Between 2000 and 2006, bla_{CTX-M} became the predominant ESBL determinant among *E. coli* strains in a U.S. hospital (30). By 2010, CTX-M genes were the most frequent ESBL determinants among *E. coli*, *Klebsiella* sp., and *Proteus mirabilis* strains from 72 hospitals in the United States (31). *E. coli* and *Klebsiella* sp. strains carrying bla_{CTX-M} in Seattle hospitals were reported between 2003 and 2007 (7, 32).

A frequently cited concern is that antimicrobial use in food-producing animals generates a population of resistant bacteria that will serve as a reservoir of resistance genes for human enteric pathogens (33–37). Our data show that CTX-M *E. coli* strains first emerged as frequently occurring members of the bovine enteric *E. coli* community only considerably after its emergence as a significant cause of human clinical infection (6, 31, 32) (Table 2). It is possible that these strains were present during earlier years, but if so at most they represented uncommon or rare members of the cattle commensal *E. coli* populations. This observed order of emergence is clearly inconsistent with the hypothesis that emergence in livestock commensal *E. coli* populations is a necessary precursor to emergence in human-pathogenic *E. coli* (35, 38–41).

The results of culture and isolation from fecal and environ-

TABLE 5 Mean and median farm prevalence of bla_{CTX-M}-positive *E. coli* strains according to farm-level characteristics

Characteristic	No. of farms	Farm prevalence of bla_{CTX-M}-positive *E. coli* strains		Wilcoxon 2-sample *P* value
		Mean (SEM)	Median (range)	
Region				
Northwest Washington	9	0.439 (0.121)	0.487 (0.822)	
Eastern Washington	21	0.718 (0.063)	0.822 (0.930)	0.03
No. of adult cows in the herd				
<3,000	19	0.551 (0.081)	0.714 (1.0)	
≥3,000	11	0.779 (0.076)	0.860 (0.822)	0.04
Any animal movements onto the farm				
Yes	11	0.824 (0.046)	0.86 (0.442)	
No	19	0.525 (0.083)	0.714 (1.0)	0.03
Frequency of adding fresh bedding to calf hutches				
≥weekly	5	0.295 (0.703)	0.178 (0.714)	
<weekly	25	0.703 (0.059)	0.8 (1.0)	0.02
Use of residual fly sprays				
No	17	0.774 (0.045)	0.822 (0.692)	
Yes	13	0.452 (0.111)	0.511 (1.0)	0.04

mental samples in 2012 confirmed that CTX-M *E. coli* strains have become widespread on dairies around the state; however, the distribution of CTX-M *E. coli* prevalence on farms was skewed, limiting analysis of risk factors to nonparametric tests (Table 3). Single-variable comparisons indicated associations between high prevalence of CTX-M *E. coli* and eastern Washington location, large herd size, and recent animal movements onto the farm (Table 5). These variables are correlated, as large herds were more likely to be in eastern Washington and to have recent animal movements onto the farm. Large herd size is a plausible contributor to CTX-M *E. coli* prevalence, because large farms are associated with a high frequency of visits by feed trucks and other services, such as veterinarians and hoof trimmers, high volumes of feed imported to the farm, and a high probability of animal movements onto the farm.

Two surprising findings in the current study were the association of frequency of adding fresh bedding to calf hutches and the use of residual fly sprays with reduced CTX-M *E. coli* prevalence (Table 5). Lack of fly control is an established risk factor for infections on dairy farms (42, 43), and residual sprays provide long-lasting insecticide activity. Relatively few farms (5 of 30 [16.7%]) reported adding fresh bedding to calf hutches weekly or more frequently, but the difference in prevalence was large enough to be statistically significant, even with this relatively small sample size. Although the frequency of adding bedding may be a proxy for general good calf management on a farm, other "good management" farm-level management factors, such as frequency of cleaning water and milk bottles and buckets, hand hygiene of treatment crews, and how hutches were cleaned between calves, were not associated with CTX-M *E. coli* prevalence. Fresh bedding may provide a barrier between calves and old bedding that is contaminated with feces and urine.

We did not detect any association between farm-level antibiotic use (including ceftiofur) and farm-level CTX-M *E. coli* prevalence, a finding that is consistent with previous observations (44, 45). A study of 65 dairy farms in the United Kingdom found a significant association between the use of a third- or fourth-generation cephalosporin in the previous 12 months and the presence of CTX-M *E. coli* on the farm (46). Therefore, an expanded Washington State study in which cephalosporin use is specifically targeted will be required to address this question locally. The United Kingdom study also found risk factors similar to ours, specifically an association between CTX-M *E. coli* and recent animal purchases or being an open herd. They also found disinfecting calf feeding equipment and rodent control to be protective against the presence of CTX-M *E. coli*, so low prevalence of CTX-M *E. coli* associated with reduced animal movements between farms, improving calf hygiene, and maintaining pest control were consistent findings of these two studies.

Between 2000 and 2010, third-generation cephalosporin resistance in *E. coli* and *Salmonella enterica* from food-producing animals in the United States was predominantly associated with plasmid-borne bla_{CMY-2} (44, 47, 48). In the current study, bla_{CMY-2} continued to be highly prevalent on most dairies (Table 2, Fig. 2). Although we identified management factors that were associated with lower CTX-M *E. coli* prevalence, we could not identify management changes to which we could attribute the emergence and dissemination of CTX-M *E. coli* strains between 2002 and 2011. A survey of antibiotic use in Washington dairies that was carried out in 2003 found that in 18% of herds, ceftiofur was the most commonly used antibiotic to treat calf respiratory disease (12). In the current study, in 3 of 30 herds (10.0% [95% CI, 2.6 to 27.7%]), ceftiofur was used for calf respiratory disease, suggesting that this use of ceftiofur was similar or even reduced since 2003. These data did not capture possible changes in dosing regimens or frequency of use, however, and the results of our antibiotic use survey indicate that ceftiofur was used for every indication listed. The use of multiple classes of antibiotics in calves may select for CTX-M *E. coli* strains carrying resistance determinants relevant to those other antibiotics.

Although differences in sampling and isolate selection between studies for the retrospective analyses represent a limitation of this study and we cannot rule out the presence of CTX-M *E. coli* in dairy cattle feces prior to 2008, we documented a significant increase in the prevalence of CTX-M *E. coli* between 2008 and 2011. Rapid dissemination of bla_{CTX-M} genes may be in part attributable to their association with mobile elements (49, 50) and the use of specific antibiotics that select for CTX-M *E. coli*. Future characterization of bla_{CTX-M}-associated mobile genetic elements will allow a high-resolution epidemiological investigation into patterns of dissemination.

We observed the recent emergence of CTX-M *E. coli* in dairies. This represents a rare opportunity to explore the determinants of population-level change in antibiotic resistance and to inform research efforts regarding the factors that affect resistance emergence and transmission. The temporal relationship between the emergence of Gram-negative human pathogens carrying bla_{CTX-M} genes and the emergence of CTX-M *E. coli* in Washington dairies does not support the conventional hypothesis that cattle *E. coli* strains donate resistance genes to human bacterial populations in a unidirectional manner (37).

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