Short communication: Extended-spectrum cephalosporin-resistant *Escherichia coli* in colostrum from New Brunswick, Canada, dairy cows harbor *bla*_{CTX-M} and *bla*_{TEM} resistance genes

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ABSTRACT

Dairy calves are colonized shortly after birth by multidrug resistant (MDR) bacteria, including *Escherichia coli*. The role of dairy colostrum fed to calves as a potential source of MDR bacteria resistance genes has not been investigated. This study determined the recovery rate of extended-spectrum cephalosporin-resistant (ESC-R) *E. coli* in colostrum from cows. The ESC-R *E. coli* isolates were further investigated to determine their phenotypic antimicrobial resistance pattern and the genes conferring ESC-R. Fresh colostrum was collected from 452 cows from 8 dairy herds in New Brunswick, Canada. The ESC-R *E. coli* was isolated from the colostrum by using the VACC agar, a selective media for extended-spectrum β -lactamase producing *Enterobacteriaceae*. Minimum inhibitory concentration was determined for all the suspected ESC-R *E. coli* isolates using a commercial gram-negative broth microdilution method. Two multiplex PCR were conducted on all the suspected ESC-R *E. coli* isolates to determine the presence of the *bla*_{CTX-M} (groups 1, 2, 9, and 8/25) *bla*_{CMY-2}, *bla*_{SHV}, and *bla*_{TEM} resistance genes. The ESC-R *E. coli* were detected in 20 (4.43%) of the colostrum samples. At least 1 ESC-R *E. coli* isolate was detected in 6 (75%) of the dairy herds. All ESC-R *E. coli* had MDR profiles based on minimum inhibitory concentration testing. No *bla*_{CTX-M} groups genes were detected; however, the *bla*_{CMY-2} gene was detected in 9 or 20 (45%) and *bla*_{TEM} was detected in 7 of 20 (35%) of the ESC-R *E. coli*. No ESC-R *E. coli* had both *bla*_{CMY-2} and *bla*_{TEM} resistance genes. This is the first report of *bla*_{CMY-2} and *bla*_{TEM} genes found in *E. coli* isolates cultured from dairy colostrum to our knowledge.

Key words: dairy colostrum, β -lactamases, extended-spectrum cephalosporins, dairy calves, *Escherichia coli*

Short Communication

Rapid gastrointestinal colonization and development of a dense, diverse, and complex microbial population occur soon after birth in newborn animals. This is influenced by external factors, such as postpartum calf management practices and the calf's environment (Heavey and Rowland, 1999; Mackie et al., 1999). In dairy calves, microbial colonization by multidrug resistant (MDR) bacteria, such as *Escherichia coli*, shortly after birth has been linked to certain risk factors, including farm types, type of calf housing, and other management practices (Pereira et al., 2014a). Exposure of preweaned calves to antimicrobial drugs has also been reported to be responsible for the increased colonization and shedding of MDR bacteria (Berge et al., 2006). However, some studies have also reported colonization of MDR bacteria in the gut of calves unrelated to recent use of antimicrobials (Khachatryan et al., 2004; de Verdier et al., 2012). Feeding of nonsaleable milk to preweaned calves is a common practice in dairy herds (Duse et al., 2013; Randall et al., 2014), and this milk often contain antimicrobial residues (Pereira et al., 2011; Pereira et al., 2014b) and MDR bacteria (Randall et al., 2014).

Preweaned calves may also be exposed to antimicrobial residues, MDR bacteria, and antimicrobial resistance (AMR) genes via feeding colostrum from cows. Dairy colostrum is a very complex fluid secretion that is rich in nutrients, immunoglobulins, and growth and antimicrobial factors essential for specific nutritional and immunologic functions (Pakkanen and Aalto, 1997). However, the role of colostrum as a source of MDR bacteria and AMR genes has not been widely investigated. In the current study, we hypothesized that dairy cow colostrum would be a source of extended-spectrum cephalosporin-resistant (ESC-R) *Escherichia coli* to dairy calves. Our study aims were to determine the potential role of colostrum as source of resistant bacteria through the recovery rate of ESC-R *E. coli* using a selective culture method, to describe the phe-

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notypic AMR patterns of ESC-R *E. coli* isolates, and to identify the AMR genes conferring ESC resistance in these isolates.

Colostrum samples were collected from 452 Holstein dairy cows during the first 24 h postpartum (110, 32, 61, 49, 33, 50, 26, and 91 samples were collected, respectively, from 8 separate farms, determined by the number of calves born into the herd during the study period). The samples were collected from 8 commercial dairy herds in New Brunswick, Canada. Colostrum samples were collected between July, 2014 and February 2015. For every newly calved cow during this time frame, the owner collected 50 mL of colostrum into a vial properly labeled with cow identification number, farm source, and date of sample collection. This collection was from a routine sample collection toward a larger research study investigating failure of transfer of passive immunity in dairy calves as part of MILK 2020 project of New Brunswick, Canada. The samples were then stored in the farm freezer until they were transported to the Maritime Quality Milk Laboratory, University of Prince Edward Island, where samples were stored at -80°C for subsequent bacteriological isolation.

Thawed colostrum samples were initially pre-enriched in buffered peptone water, as previously described (Randall et al., 2014); briefly, 1 mL of colostrum sample was diluted in 9 mL of buffered peptone water before an overnight incubation at 37°C for 18 to 24 h. Selective enrichment and isolation were further carried out as described by CIPARS (Government of Canada, 2010). An equal volume of the inoculated pre-enriched broth was added to *E. coli* broth for selective enrichment, and then samples in broth were incubated at 45°C for 18 to 24 h. The VACC agar (tryptic soy agar plate with 5% sheep blood containing 6 $\mu\text{g}/\text{mL}$ of vancomycin, 2 $\mu\text{g}/\text{mL}$ of amphotericin B, 2 $\mu\text{g}/\text{mL}$ of ceftazidime, and 1 $\mu\text{g}/\text{mL}$ of clindamycin) a selective media for extended-spectrum β -lactamase-producing *Enterobacteriaceae* was used for selective culture (Singh et al., 2012). The VACC agar plates were inoculated with 50 μL of enrichment broth and incubated at 37°C for 18 to 24 h. Presumptive *E. coli* were subcultured to eosin-methylene blue agar and then purified on tryptic soy agar plates supplemented with 5% sheep blood. *Escherichia coli* were further identified by lactose-fermentation, utilization of citrate, and urease production. All isolates were frozen in *Brucella* broth with 15% glycerol at -80°C for further laboratory analysis.

For all recovered isolates, MIC antimicrobial susceptibility testing was performed using a commercial broth microdilution method following the Clinical and Laboratory Standards Institute guidelines (CLSI, 2013). We used CMV2AGNF (Sensititer, Trek Diagnostic Sys-

tems, Westlake, OH) gram-negative MIC susceptibility plates of the National Antimicrobial Monitoring System containing 15 antimicrobials in this study; however, azithromycin was excluded due to intrinsic resistance in *E. coli*. The following antimicrobial agents were tested (with resistant breakpoints in parentheses): ampicillin (≥ 32 $\mu\text{g}/\text{mL}$), amoxicillin-clavulanic acid ($\geq 32/16$ $\mu\text{g}/\text{mL}$), chloramphenicol (≥ 32 $\mu\text{g}/\text{mL}$), ceftriaxone (≥ 4 $\mu\text{g}/\text{mL}$), ceftiofur (≥ 8 $\mu\text{g}/\text{mL}$), ciprofloxacin (≥ 1 $\mu\text{g}/\text{mL}$), ceftiofur (≥ 32 $\mu\text{g}/\text{mL}$), gentamicin (≥ 16 $\mu\text{g}/\text{mL}$), kanamycin (≥ 64 $\mu\text{g}/\text{mL}$), nalidixic acid (≥ 32 $\mu\text{g}/\text{mL}$), streptomycin (≥ 64 $\mu\text{g}/\text{mL}$), sulfisoxazole (≥ 512 $\mu\text{g}/\text{mL}$), trimethoprim-sulfamethoxazole ($\geq 4/76$ $\mu\text{g}/\text{mL}$), and tetracycline (≥ 16 $\mu\text{g}/\text{mL}$). Multidrug resistance was based on World Health Organization's definition of resistance to at least 1 antimicrobial drug in 3 or more antimicrobial categories (Magiorakos et al., 2012).

All the ESC-R *E. coli* isolates detected by the selective culture method were screened for β -lactamase resistance genes. Genomic DNA of isolates was extracted following the procedures provided by a commercial kit (InstaGene Matrix Bio-Rad, Montreal Canada). Two multiplex PCR were performed for the molecular characterization of β -lactamase genes. Multiplex PCR was used for the detection of *bla*_{CMY-2}, *bla*_{SHV}, and *bla*_{TEM} using the consensus primers and protocol (Table 1) previously described by Kozak et al. (2009). The other multiplex PCR was carried out to determine the *bla*_{CTX-M} phylogenetic groups 1, 2, 9, and 8/25 using the consensus primers and protocol (Table 1) previously described (Dallenne et al., 2010). For both multiplex PCR, a Qiagen multiplex PCR kit (Qiagen, Mississauga, Ontario, Canada) with 1 $\times$ Qiagen multiplex PCR master mixture, 1 $\times$ Q-solution, molecular-grade water, 1 $\times$ consensus primer pair mixture, and 2 μL of isolate DNA template were included in the final 25- μL mixtures, according to the manufacturer's instructions (www.qiagen.com). Amplification was carried out in a PCR thermocycler (Techne TC-412, Thermo Fisher Scientific, Mississauga, Ontario, Canada) using the following PCR conditions for the first multiplex PCR: 1 cycle of 15 min at 94°C , 30 cycles of 1 min at 94°C , 1 min at 55°C , and 1 min at 72°C , and 1 cycle of final extension of 10 min at 72°C . For the second multiplex PCR, we used an initial denaturation at 94°C for 10 min; 30 cycles of 94°C for 40 s, 60°C for 40 s, and 72°C for 1 min; and a final elongation step at 72°C for 7 min. Following the PCR reaction, DNA fragments were analyzed by electrophoresis in a 1% agarose gel at 100 V for 1 h in 1 $\times$ Tris boric acid EDTA stained with 1 $\mu\text{g}/\text{mL}$ of ethidium bromide.

The ESC-R *E. coli* isolates were recovered from 20 (4.43%) of the 452 colostrum samples, which came from the 6 of the 8 dairy herds enrolled in the study.

Table 1. Multiplex PCR primer sequences used for the detection of β -lactamase resistance genes in *Escherichia coli* isolates

Gene	Primer ¹	Primer sequence ²	Amplicon size (bp)
<i>TEM</i>	F	TTAACTGGCGAACTACTTAC	247
	R	GTCTATTTTCGTTTCATCCATA	
<i>SHV</i>	F	AGGATTGACTGCCTTTTGT	393
	R	ATTTGCTGATTTTCGCTCG	
<i>CMY-2</i>	F	GACAGCCTCTTTCTCCACA	1,000
	R	TGGACACGAAGGCTACGTA	
<i>CTXM-1</i> group	F	TTAGGAARTGTGCCGCTGYA	688
	R	CGATATCGTTGGTGGTRCCAT	
<i>CTXM-2</i> group	F	CGTTAACGGCACGATGAC	404
	R	CGATATCGTTGGTGGTRCCAT	
<i>CTXM-9</i> group	F	TCAAGCCTGCCGATCTGGT	561
	R	TGATTCTCGCCGCTGAAG	
<i>CTXM-8/25</i> group	F	AACRCRCAGACGCTCTAC	326
	R	TCGAGCCGGAASGTGTGYAT	

¹F = forward; R = reverse.²Y = T or C; R = A or G.

All the isolates ($n = 20$) were resistant to ampicillin, amoxicillin-clavulanic acid, and cefoxitin, whereas all the isolates were susceptible to ciprofloxacin and gentamicin (Table 2). Multidrug resistance was observed in all the ESC-R *E. coli* isolates whereas co-resistance to amoxicillin-clavulanate-chloramphenicol-cephalosporin-penicillin-streptomycin-sulfisoxazole-trimethoprim/sulfamethoxazole-tetracycline was the most prevalent resistance type (Table 2). Polymerase chain reaction screening did not identify more than 1 ESC gene in the ESC-R *E. coli* isolated. No *bla*_{CTX-M} or *bla*_{SHV} genes were detected in the ESC-R *E. coli*. The *bla*_{CMY-2} gene was detected in 9 (45%) of the 20 ESC-R *E. coli*, whereas *bla*_{TEM} was detected in 7 (35%) of the 20 ESC-R *E. coli* (Table 3). Beta-lactamase genes were detected from 6 herds, with *bla*_{CMY-2} gene isolates clustered in 2 dairy herds, whereas *bla*_{TEM} was detected in 5 of the 6 dairy herds in which ESC-R *E. coli* was isolated from the colostrum samples. The 4 isolates without detected ESC-R genes were MIC-susceptible to both ceftriaxone and ceftiofur. From the 6 herds positive for ESC-R *E. coli*, 1 herd used ceftiofur for blanket dry cow treatment; another herd used cloxacillin and cephalixin for blanket dry cow treatment whereas the other 4 herds used novobiocin-procaine penicillin combination, with 1 herd using it for selective treatment and the 3 herds for blanket treatment. For the other 2 herds negative for ESC-R *E. coli*, 1 herd used novobiocin-procaine penicillin combination for blanket dry cow treatment whereas the other used ceftiofur and novobiocin-procaine penicillin combination for blanket dry cow treatment. However, all the 8 herds reported the used of injectable ceftiofur for the treatment of other diseases and health conditions unrelated to dry cow treatment or mastitis.

Colostrum is the first source of both passive immunity and food for newborn dairy calves. We demonstrated

that colostrum is also a possible source of ESC-R *E. coli* exposure to newborn dairy calves. Recovered isolates may have originated from either direct shedding in the mammary gland or from environmental contamination of colostrum, and even from farmers during sample collection; however, the frequency of recovered ESC-R *E. coli* isolates in colostrum samples was low. This low frequency of ESC-R *E. coli* from colostrum may suggest other sources of exposure to calves, such as consuming nonsaleable milk, antimicrobial treatment, environment contamination, feeding practices, and other management influences (Berge et al., 2006; Edrington et al., 2012; Aust et al., 2013; Duse et al., 2015). In addition, evidence of vertical transmission of bacteria from cow to calves has recently been reported, demonstrating the existence of transmission without requiring fecal-oral exposure (Hanson et al., 2016). Considering the convenience sampling in the current study, we chose to sample a large number of cows on a small number

Table 2. Antimicrobial resistance frequency in extended-spectrum cephalosporin resistant *Escherichia coli* ($n = 20$) isolated from colostrum from 8 farms in New Brunswick, Canada

Antimicrobial	Resistance, no. (%)
Ampicillin	20 (100)
Amoxicillin-clavulanic acid	20 (100)
Chloramphenicol	15 (75)
Ceftriaxone	16 (80)
Ceftiofur	16 (80)
Cefoxitin	20 (100)
Gentamicin	0 (0)
Ciprofloxacin	0 (0)
Nalidixic acid	1 (5)
Kanamycin	3 (15)
Streptomycin	18 (90)
Sulfisoxazole	17 (85)
Trimethoprim-sulfamethoxazole	17 (85)
Tetracycline	18 (90)

Table 3. Antimicrobial resistance pattern and β -lactamase frequencies in extended-spectrum cephalosporin-resistant *Escherichia coli* isolated from dairy colostrum in New Brunswick, Canada

Item	Antimicrobial resistance pattern ¹	Frequency	<i>bla</i> _{CMY-2}	<i>bla</i> _{TEM}
1	AUG-C-ESC-NAL-PEN-STR-SUL-TS-TET	1	—	1
2	AUG-C-ESC-PEN-STR-SUL-TS-TET	13	8	3
3	AUG-ESC-PEN-STR-SUL-TS-TET	3	—	3
4	AUG-ESC-PEN-STR-TET	1	1	—
5	AUG-C-ESC-PEN	1	—	—
6	AUG-ESC-PEN	1	—	—

¹AUG = amoxicillin-clavulanic acid; C = chloramphenicol; ESC = cephalosporin; NAL = nalidixic acid; PEN = penicillin; STR = streptomycin; SUL = sulfisoxazole; TS = trimethoprim-sulfamethoxazole; TET = tetracycline.

of farms to maximize the chance of finding ESC-R *E. coli* in the dairy colostrum on those farms. However, with only 8 farms tested, it remains unclear how common MDR ESC-R *E. coli* bacteria are in the colostrum among all the dairy farms in this region. The 6 herds that were positive for ESC-R *E. coli* isolates used antimicrobials belonging to the β -lactam class, including penicillin, cloxacillin, cephalixin, and ceftiofur; these antimicrobials have been reported to be the frequent veterinary residues in waste milk (Brunton et al., 2014; Randall et al., 2014). Residues in the colostrum are also likely to be present when dry cow therapy is practiced.

All the ESC-R *E. coli* isolates recovered from dairy colostrum in our study were MDR, with over 85% of isolates resistant to at least 7 classes of antimicrobials. This may be due to the β -lactamase genes, as these genes are reportedly able to confer multidrug resistance (Martin et al., 2012). To our knowledge, the current study is the first to report the detection of *bla*_{CMY-2} and *bla*_{TEM} genes from *E. coli* isolates from dairy cow colostrum; however, *bla*_{CMY-2} has been reported from bovine mastitic milk from Switzerland (Endimiani et al., 2012). Although none of the *bla*_{CTX-M} groups were detected in dairy colostrum in our study, previous studies have reported the detection of all the *bla*_{CTX-M} groups from bulk tank milk (Odenthal et al., 2016), whereas *bla*_{CTX-M} group 1 has also been reported from raw cow milk (Tekiner and Özpınar, 2016), waste milk (Randall et al., 2014), and mastitic milk (Locatelli et al., 2009). Four isolates were negative for the β -lactamase genes tested and also were susceptible to ceftiofur and ceftriaxone on the MIC test. This would suggest that these isolates were not extended-spectrum β -lactamase-producing *E. coli*. Phenotypically, these 4 isolates were resistant to ceftiofur and amoxicillin-clavulanate, which may indicate the presence of other mechanisms of resistance, such as a chromosomally mediated AmpC, or another plasmid-mediated β -lactamase not screened for with the PCR assays used in our study.

Although the importance of dairy colostrum in conferring passive immunity and nutrition to newborn

calves is critical, the risk of exposure to MDR bacteria and other infectious agents, either from direct shedding in the mammary gland or from postharvest contamination of colostrum before feeding to calves, need to be recognized (Meas et al., 2002; Godden et al., 2005; Nielsen et al., 2008). Feeding of pasteurized colostrum as a means of controlling disease-causing pathogen transmission has been recommended (Godden, 2007), as heat-treated colostrum not only provides the advantages of colostrum with lower bacterial counts but also increases immunoglobulin absorption by calves (Elizondo-Salazar and Heinrichs, 2009).

In the current study, no specific protocol was provided to the farmers on sample collection, which may have resulted in environmental contamination during sample collection influencing the recovery of ESC-R *E. coli* from colostrum. However, this may be a more accurate reflection of the colostrum, and bacterial contamination, that is fed to the calves on those farms. In conclusion, colostrum from dairy cows used in this study contained MDR ESC-R *E. coli*, potentially exposing newborn calves ingesting this colostrum to these bacteria. The recovery rate was relatively low with all isolates being MDR. Our analysis of ESC resistance genes revealed that either *bla*_{CMY-2} or *bla*_{TEM} were present in 16 of the 20 ESC-R *E. coli* isolates, but no *bla*_{CTX-M} or *bla*_{SHV} genes were detected in this convenience sample.

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