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Research Paper

Twenty-Four-Month Longitudinal Study Suggests Little to No Horizontal Gene Transfer In Situ between Third-Generation Cephalosporin-Resistant *Salmonella* and Third-Generation Cephalosporin-Resistant *Escherichia coli* in a Beef Cattle Feedyard

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ABSTRACT

Third-generation cephalosporins (3GCs) are preferred treatments for serious human *Salmonella enterica* infections. Beef cattle are suspected to contribute to human 3GC-resistant *Salmonella* infections. Commensal 3GC-resistant *Escherichia coli* are thought to act as reservoirs of 3GC resistance because these strains are isolated more frequently than are 3GC-resistant *Salmonella* strains at beef cattle feedyards. During each of 24 consecutive months, four samples of pen surface material were obtained from five pens ($N = 480$) at a Nebraska feedyard to determine to the contribution of 3GC-resistant *E. coli* to the occurrence of 3GC-resistant *Salmonella*. Illumina whole genome sequencing was performed, and susceptibility to 14 antimicrobial agents was determined for 121 3GC-susceptible *Salmonella,* 121 3GC-resistant *Salmonella,* and 203 3GC-resistant *E. coli* isolates. 3GC-susceptible *Salmonella* isolates were predominantly from serotypes Muenchen (70.2%) and Montevideo clade 1 (23.1%). 3GC-resistant *Salmonella* isolates were predominantly from serotypes Montevideo clade 2 (84.3%). One *bla* gene type ($bla_{\text{CMY-2}}$) and the IncC plasmid replicon were present in 100 and 97.5% of the 3GC-resistant *Salmonella,* respectively. Eleven *bla* gene types were detected in the 3GC-resistant *E. coli,* which were distributed across 42 multilocus sequence types. The $bla_{\text{CMY-2}}$ gene and IncC plasmid replicon were present in 37.9 and 9.9% of the 3GC-resistant *E. coli,* respectively. These results suggest that 3GC resistance in *Salmonella* was primarily due the persistence of *Salmonella* Montevideo clade 2 with very minimal or no contribution from 3GC-resistant *E. coli* via horizontal gene transfer and that 3GC-resistant *E. coli* may not be a useful indicator for 3GC-resistant *Salmonella* in beef cattle production environments.

HIGHLIGHTS

- Scant evidence supports transfer of 3GC resistance genes between *Salmonella* and *E. coli*.
- Most 3GC-resistant *Salmonella* isolates were from serotype Montevideo clade 2.
- *Salmonella* subtype may differ between pens at the same feedyard.
- 3GC resistance in *E. coli* should not be used to infer 3GC resistance in *Salmonella*.

Key words: Antimicrobial resistance; *Escherichia coli*; Horizontal gene transfer; *Salmonella*; Third-generation cephalosporin resistance; Whole genome sequencing

Third-generation cephalosporins (3GCs) are classified as critically important by the World Health Organization and are among the preferred treatments for serious human *Salmonella enterica* infections *(11, 48)*. Currently, the Centers for Disease Control and Prevention (CDC) estimates that in the United States 1.3 million *Salmonella* infections occur each year, and 9% of foodborne *Salmonella* infections are attributed to beef *(6, 17)*. From 2015 to 2017, an average of 3% of all nontyphoidal *Salmonella* isolates recovered from humans were resistant to ceftriaxone *(6)*. In contrast, of the 12,741 retail ground beef samples tested by the U.S. Food and Drug Administration, National Antimicrobial Resistance Monitoring System between 2002 and 2012, only 140 (1.1%) contained *Salmonella* and only 16 (0.1%) contained 3GC-resistant *Salmonella (42)*. Although the result of this susceptibility testing suggests that beef is not a major source of 3GC-resistant *Salmonella,* the CDC concluded that beef is likely a predominant source of human 3GC-resistant *Salmonella* infections *(18)*. This conclusion

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was based on the finding that the two most dominant serotypes among the 978 3GC-resistant *Salmonella* isolates recovered from humans between 1996 and 2013 were Newport (40.2%) and Typhimurium (25.9%), both of which are cattle associated *(18)*. The third most dominant 3GC-resistant *Salmonella* serotype isolated from humans was Heidelberg, which is traditionally associated with poultry. However, a multistate outbreak (56 cases, 20 hospitalizations, and 0 deaths) of 3GC-resistant *Salmonella* Heidelberg infections that occurred from 2015 to 2017 was associated with contact with dairy cattle from a Wisconsin sale barn *(5)*.

U.S. public health agencies have declared an urgent need to provide better insight into the occurrence of 3GC-resistant *Salmonella* in cattle production environments *(15)*. Despite their extreme importance, factors contributing to the occurrence of 3GC-resistant *Salmonella* throughout the beef production and processing continuum are very poorly understood. Commensal 3GC-resistant *Escherichia coli* are more frequently isolated than are 3GC-resistant *Salmonella* at beef cattle feedyards *(35, 45)* and are have been suggested as reservoirs of 3GC resistance *(13, 47)*. The reservoir designation is primarily based on the isolation of *Salmonella* and *E. coli* harboring incompatibility type A/C_2 ($IncA/C_2$) plasmids with very similar genetic structures, including presence of the bla_{CMY-2} gene, which confers 3GC resistance *(13, 47)*. These $IncA/C_2$ plasmids contain genes that facilitate the exchange of large fragments of genetic material between bacterial species (horizontal gene transfer). However, to our knowledge no published research has been conducted to assess the similarities or differences between comingled 3GC-resistant *Salmonella* and 3GC-resistant *E. coli* populations at a cattle feedyard over time. The goal of this research was to gain insights into the contribution of 3GC-resistant *E. coli* to the occurrence of 3GC-resistant *Salmonella* at a beef cattle feedyard in Nebraska.

To achieve this goal, several studies were conducted. First, monthly levels of *Salmonella* regardless of antimicrobial resistance status, 3GC-resistant *Salmonella,* 3GC-resistant *E. coli,* and *E. coli* regardless of antimicrobial resistance status in pen surface material were determined. Second, 3GC-resistant isolates were screened for the presence of bla_{CMY-2} and bla_{CTX-M}. In the United States, most 3GC-resistant *Salmonella* isolates from human clinical cases harbor the bla_{CMY-2} gene, although the bla_{CTX-M} gene, which also confers 3GC resistance, is occasionally present *(39)*. Nearly all 3GC-resistant *Salmonella* isolated throughout the U.S. beef production continuum harbor the bla_{CMY-2} gene *(35–37, 39, 44, 45)*. In contrast, 3GC-resistant *E. coli* isolated from the beef production system frequently contain either bla_{CMY-2} or bla_{CTX-M} *(35–37, 44, 45)*. 3GC-resistant *Salmonella, Salmonella,* and 3GC-resistant *E. coli* were subjected to whole genome sequencing (WGS) to increase our understanding of these bacterial populations at cattle feedyards and assess the similarity in antimicrobial resistance genes (ARGs) and plasmid replicons between 3GC-resistant *Salmonella* and 3GC-resistant *E. coli* isolates.

MATERIALS AND METHODS

Experimental design and pen surface material sample acquisition. A beef cattle feeding operation in Nebraska was enrolled in the study. Enrollment was contingent upon protection of the feedyard identity. Five nonadjacent pens were enrolled in the study. During each monthly sample period, each of five nonadjacent pens was divided into four quadrants, and a sample of pen surface material (defined as the soil-manure mixture present on the pen surface) was obtained from each quadrant. Thus, 20 samples were obtained each month. Pen surface material was obtained from the same five pens for 24 consecutive months beginning in April 2014 for a total of 480 samples collected. Months were numbered sequentially beginning with April 2014. Pen surface material was collected with gloved hands from multiple locations within the quadrant and placed in a sterile bag. Care was taken to obtain samples from the top 10 cm of surface material while avoiding obvious fecal pats. Gloves were changed between each sample. Approximately 25 g of pen surface material was collected per quadrant with four samples per pen. All samples were held at 4°C during transport to the laboratory and were processed within 24 h of collection.

Each pen surface material sample was hand massaged for 15 s to mix, and a 10-g sample was removed to a filter barrier bag and suspended in 90 mL of phosphate-buffered tryptic soy broth (TSB-PO; 30 g/L tryptic soy broth, 2.31 g/L KH_2PO_4, and 12.54 g/L K_2HPO_4, final pH 7.2) (BD, Sparks, MD). The suspension was hand massaged for 15 s, and a 1-mL aliquot was removed for direct plating. The TSP-PO suspensions were incubated at 25°C for 2 h and then at 42°C for 6 h and then held at 4°C (primary enrichment) until secondary enrichments cultures were made the following day.

Detection of *Salmonella* on XLD plates. For each sample, a 1-mL aliquot of TSB-PO primary enrichment was mixed with 20 μL of *Salmonella*-specific immunomagnetic separation beads (Dynal, Lake Success, NY). *Salmonella* was eluted into 3 mL of Rappaport-Vassiliadis soy peptone broth (RVS; Remel, Lenexa, KS). The RVS secondary enrichment cultures were incubated at 42°C for 18 to 24 h then streaked onto xylose lysine desoxycholate (XLD) agar plates (Remel) supplemented with 4.6 mg/L Tergitol, 15 mg/L novobiocin, and 5 mg/L cefsulodin (TNC). The streaked plates were incubated overnight at 37°C, and black colonies were considered presumptive *Salmonella*. For each plate, two presumptive colonies were inoculated into TSB and incubated overnight at 37°C. An aliquot of each of these overnight cultures was removed, combined with BAX lysis buffer (DuPont Qualicon, Wilmington, DE), and incubated according to the manufacturer's instructions to obtain genomic DNA. Presumptive *Salmonella* colonies were confirmed by using previously described PCR methods *(29, 34)*. TSB cultures of *Salmonella* colonies were combined with glycerol to a final concentration of 10% (v/v) and were stored at –20°C.

Enumeration of *Salmonella* on XLD plates. For each sample, a 10-fold dilution of each direct plating aliquot was made. Then 50 μL of the direct plating aliquot and 50 μL of 10-fold dilution were applied to XLD+TNC plates with a WASP2 spiral plater (Microbiology International, Frederick, MD). Plates were incubated overnight at 37°C, and black colonies were enumerated as presumptive *Salmonella*. For each sample, two presumptive *Salmonella* colonies were inoculated into TSB and incubated overnight at 37°C. Genomic DNA preparation, PCR confirmation of *Salmonella,* and sample preservation were as described above.

Detection of 3GC-resistant *Salmonella* on XLD+CTX plates. For each sample, the RVS secondary enrichment culture was streaked onto an XLD agar plate supplemented with 2 mg/L cefotaxime (XLD+CTX). XLD+CTX plates were incubated overnight at 37°C, and black colonies were considered presumptive 3GC-resistant *Salmonella*. For each plate, two presumptive colonies were inoculated into TSB and incubated overnight at 37°C. Genomic DNA preparation, PCR confirmation of *Salmonella,* and sample preservation were as described above.

Enumeration of 3GC-resistant *Salmonella* on XLD+CTX plates. For each sample, 50 μL of the direct plating aliquot was applied onto an XLD+CTX plate with the spiral plater. Plates were incubated overnight at 37°C, and black colonies were enumerated as presumptive 3GC-resistant *Salmonella*. For each sample, two presumptive colonies were inoculated into TSB and incubated overnight at 37°C. Genomic DNA preparation, PCR confirmation of *Salmonella,* and sample preservation were as described above.

Detection of *E. coli* on CEC plates. For each sample, secondary enrichment cultures were prepared by combining 0.5 mL of TSB-PO primary enrichment culture with 2.5 mL of MacConkey broth (MCB; BD). MCB secondary enrichment cultures were incubated at 42°C for 18 to 24 h and then streaked onto a CHROMagar *E. coli* (CEC) plate (DRG International, Springfield, NJ). Plates were incubated overnight at 37°C, and blue colonies were considered presumptive *E. coli*. For each plate, two presumptive colonies were inoculated into TSB and incubated overnight at 37°C. An aliquot of each overnight TSB culture of a presumptive colony was removed, combined with BAX lysis buffer, and incubated according to the manufacturer's instructions to obtain genomic DNA. Presumptive colonies were confirmed by using previously described PCR methods for *E. coli (16)*. TSB cultures of confirmed *E. coli* colonies were combined with glycerol to a final concentration of 10% (v/v) and stored at –20°C.

Enumeration of *E. coli* on CEC plates. Two serial 10-fold dilutions of each direct plating aliquot were made, and 50 μL of appropriate dilutions were plated with the spiral plater onto CEC plates. Plates were incubated overnight at 37°C, and blue colonies were enumerated as presumptive *E. coli*. For each plate, two presumptive colonies were inoculated into TSB and incubated overnight at 37°C. Genomic DNA preparation, PCR confirmation of *E. coli,* and sample preservation were as described above.

Detection of 3GC-resistant *E. coli* on CEC+CTX plates. For each sample, the MCB secondary enrichment culture was streaked onto a CEC plate supplemented with 2 mg/L CTX. Plates were incubated overnight at 37°C, and blue colonies were considered presumptive 3GC-resistant *E. coli*. For each plate, two presumptive colonies were inoculated into TSB and incubated overnight at 37°C. Genomic DNA preparation, PCR confirmation of *E. coli,* and sample preservation were as described above.

Enumeration of 3GC-resistant *E. coli* on CEC+CTX plates. Two serial 10-fold dilutions of each direct plating aliquot were made, and 50 μL of appropriate dilutions were plated with the spiral plater onto CEC+CTX plates. Plates were incubated overnight at 37°C, and blue colonies were enumerated as presumptive 3GC-resistant *E. coli*. For each plate, two presumptive colonies were inoculated into TSB and incubated overnight at 37°C. Genomic DNA preparation, PCR confirmation of *E. coli,* and sample preservation were as described above.

PCR detection of bla_{CMY-2} and bla_{CTX-M} genes. TSB cultures of confirmed 3GC-resistant *E. coli* and 3GC-resistant *Salmonella* colonies stored at –20°C were thawed, and an aliquot of each culture was removed, combined with BAX lysis buffer, and incubated according to the manufacturer's instructions to obtain template DNA for molecular assays. The bla_{CMY-2} and bla_{CTX-M} genes were detected using previously described primers and conditions *(10, 22)*.

***Salmonella* isolation using XLD plates.** For pens A and D and for each of the 24 months, three *Salmonella* isolates were recovered from independent samples when available (2, 1, or 0 isolates were obtained when 2, 1, or 0 samples were positive during the month, respectively). *Salmonella* colonies were streaked for isolation twice on XLD plates.

3GC-resistant *Salmonella* isolation using XLD+CTX plates. For each pen and for each of the 24 months, three 3GC-resistant *Salmonella* isolates were recovered from independent samples when available and streaked for isolation twice on XLD+CTX plates.

bla_{CMY-2} 3GC-resistant *E. coli* isolation using CEC+CTX plates. For pens A and D and for each of the 24 months, three bla_{CMY-2} 3GC-resistant *E. coli* isolates were recovered from independent samples when available and streaked for isolation twice on CEC+CTX plates.

bla_{CTX-M} 3GC-resistant *E. coli* isolation using CEC+CTX plates. For pens A and D and for each of the 24 months, three bla_{CTX-M} 3GC-resistant *E. coli* isolates were recovered from independent samples when available and streaked for isolation twice on CEC+CTX plates.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed with the Sensititre broth microdilution system and CMV3AGNF plates (TREK Diagnostic Systems, Cleveland, OH). The 14 antimicrobials, antimicrobial classifications, and breakpoints for resistance for the CMV3AGNF plates are listed in Supplemental Table S1. Antimicrobial breakpoints and three-letter abbreviations were as described previously *(7, 43)*. The following organisms were used as quality control strains in the antimicrobial sensitivity assays: *Pseudomonas aeruginosa* ATCC 27853, *E. coli* ATCC 25922, and *Staphylococcus aureus* ATCC 25923.

Genomic DNA isolation and WGS. Each isolate was grown overnight at 37°C in 1 mL of TSB. Genomic DNA was prepared using the QIAamp DNA Mini Kit (cat. no. 51305, Qiagen, Valencia, CA) with a final elution in 200 μL of buffer AE. The quality of each genomic DNA preparation was considered satisfactory when the following criteria were met: (i) double-stranded DNA concentration $\geq$ 40 ng/μL (Quantifluor ONE double-stranded DNA system, cat. no. E4870, Promega, Madison, WI) and (ii) 260/280 ratio of 1.8 to 2.0 (Nanodrop 2000 spectrophotometer, Thermo Fisher Scientific, Waltham, MA). Genomic DNA was fragmented with a focused ultrasonicator (S220, Covaris, Woburn, MA) at the following settings: 5% duty cycle, 175 peak/displayed power, 200 cycles/burst, 50-s duration, frequency sweeping mode, and 5.5 to 6°C. Genomic DNA libraries were prepared using the a PCR-free high throughput library sample preparation kit (TruSeq DNA CD Indexes, Illumina, San Diego, CA), and the manufacturer's protocol for a 550-bp insert size. Genomic DNA libraries were quantified using a

TABLE 1. *Qualitative and quantitative evaluation of bacterial populations at a beef cattle feedyard*

Organism[a]	No. of samples	% detection	No. of samples in each log CFU/g class:						
			<2.30[b]	2.30–2.99	3.00–3.99	4.00–4.99	5.00–5.99	6.00–6.99	7.00–7.99
E. coli	480	100.0	8	24	53	99	168	108	20
3GC^r *E. coli*	480	97.7	251	80	84	38	15	1	0
Salmonella	480	77.9	303	50	19	2	0	0	0
3GC^r *Salmonella*	480	29.0	134	4	1	0	0	0	0

[a] 3GC^r, resistant to third-generation cephalosporins.
[b] Samples detected but not enumerated (lower limit of enumeration = 2.30 log CFU/g).

library quantification kit (Kapa Biosystems, Woburn, MA). Fragment sizes were verified using a fragment kit with a fragment analyzer (HS NGS kit and 5200 analyzer, Agilent Technologies, Santa Clara, CA). Genomic DNA libraries were sequenced (NextSeq 550 platform, Illumina), and a kit (500/550 TG kit version 2, Illumina) was used to generate 150-bp paired end reads.

Genome assembly. Genomes were assembled using the Shovill version 1.0.4 pipeline (https://github.com/tseemann/shovill). Shovill incorporated the following steps: (i) genome size and sequencing coverage estimations with Mash version 2.2 *(33)*; (ii) Illumina adaptor sequence removal using Trimmomatic (version 0.39) *(2)*; (iii) Lighter (version 1.1.2) to correct sequencing read errors *(40)*; (iv) FLASH (version 1.2.11) to merge and extend short reads *(28)*; (v) SPAdes (version 3.14.0) to assemble reads *(1)*; and (vi) minor assembly errors corrected by mapping reads back to contigs, removed short contigs, low coverage contigs, and homopolymers with BWA (version 0.7.17-r1188) *(26),* SAMtools (version 1.10) *(27),* and Pilon (version 1.23) *(46)*.

Genome annotation. Assembled genome sequences were used for the following annotations. *Salmonella* serotypes were determined using SeqSero (version 1.1) *(50),* and SerotypeFinder (version 2018_09_24) was used to determine *E. coli* serotypes *(19)*. *E. coli* and *Salmonella* multilocus sequence types (STs) were determined using the program MLST (version 2019_05_08) *(23)*. Antimicrobial resistance genes and chromosomal mutations known to confer antimicrobial resistance were identified using ResFinder (version 2020_02_06) *(49)*. Plasmid replicons were identified using PlasmidFinder (downloaded 6 April 2021) *(4)*. Local implementations of the Center for Genomic Epidemiology (CGE) "finder" tools utilized Blast+ version 2.10.0 *(3)* to query CGE databases updated 4 April 2021. GC, N50, and L50 statistics were obtained using the stats script from BBMap version 38.79.

Phylogenetic analysis. A *Salmonella* phylogeny was constructed with the two most prevalent serotypes using the U.S. Department of Agriculture (USDA) Scinet Ceres High Performance Computing infrastructure. Reference genomes were identified using Patric 3.6.9 Similar Genome Finder. Assembled genome sequences (*Salmonella* Montevideo, GenBank accession CP017972; *Salmonella* Muenchen GenBank accession CP051389) were aligned using ParSNP 1.2 Linux64, and IQ-Tree version 1.6.12 was subsequently used to determine the most likely phylogenetic tree. The tree was visualized using ITOL version 6 to plot resistance genes, collection month, and pen next to each isolate sequence.

TABLE 2. *Detection of* bla$_{CMY-2}$ *and* bla$_{CTX-M}$ *genes in 3GC^r isolates*[a]

Organism	No. of isolates	% detection			
		bla$_{CMY-2}$ only	*bla*$_{CTX-M}$ only	Both	Neither
E. coli	1,412	31.5	58.4	2.8	7.2
Salmonella	684	100.0	0.0	0.0	0.0

[a] 3GC^r, resistant to third-generation cephalosporins.

RESULTS AND DISCUSSION

Detection and enumeration of *E. coli* and *Salmonella* in pen surface material. Plating on CEC revealed the presence of *E. coli* in 100% of the 480 pen surface material samples, and 98.3% of samples had high enough levels to enumerate (≥2.30 log CFU/g) (Table 1). For these 472 samples, the mean *E. coli* level was 5.22 log CFU/g. Plating on CEC+CTX revealed 3GC-resistant *E. coli* in 97.7% of the pen surface material samples, and 45.4% of samples were enumerable (Table 1). For these 218 samples, the mean 3GC-resistant *E. coli* level was 3.43 log CFU/g. Among the 1,412 3GC-resistant *E. coli* colonies examined (Table 2), the *bla*$_{CMY-2}$ gene (alone or with *bla*$_{CTX-M}$) was detected in 34.2%, and the *bla*$_{CTX-M}$ gene (alone or with *bla*$_{CMY-2}$) was detected in 61.2%.

Plating on XLD revealed the presence of *Salmonella* in 77.9% of the 480 pen surface material samples, and 14.8% of samples had high enough levels to enumerate (≥2.30 log CFU/g) (Table 1). Plating on XLD+CTX revealed 3GC-resistant *Salmonella* in 29.0% of the pen surface material samples, and 1.0% of samples enumerable (Table 1). Among the 684 3GC-resistant *Salmonella* colonies on XLD+CTX plates that were screened, the *bla*$_{CMY-2}$ gene was detected in 100%, but *bla*$_{CTX-M}$ was not detected (Table 2). This result was in stark contrast with the predominant *bla* types harbored by the 3GC-resistant coresident *E. coli* population, indicating that 3GC-resistant *E. coli* was not actively serving as a 3GC resistance reservoir for *Salmonella*. More intensive isolate characterization using WGS was performed to investigate the population dynamics.

***Salmonella* isolation, WGS, and antimicrobial susceptibility testing.** Pen A was selected for intensive characterization of *Salmonella* isolates because 3GC-resistant and nonresistant isolates were detected most frequently from this pen (Table S2). Pen D was selected for intensive isolate characterization because it had the

TABLE 3. *Susceptibility of* Salmonella *isolates from pen A to 14 antimicrobial agents*

Salmonella serotype	No. of isolates	% of isolates resistant to[a]: AUG	AMP	AZI	FOX	TIO	AXO	CHL	CIP	GEN	NAL	STR	FIS	TET	SXT
Muenchen–no CMY	59	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	100.0	0.0
Montevideo–CMY	51	100.0	100.0	0.0	100.0	100.0	100.0	100.0	0.0	0.0	0.0	100.0	100.0	100.0	0.0
Montevideo–no CMY	3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Muenchen–CMY	1	100.0	100.0	0.0	100.0	100.0	100.0	0.0	0.0	0.0	0.0	0.0	100.0	100.0	0.0

[a] AUG, amoxicillin–clavulanic acid; AMP, ampicillin; AZI, azithromycin; FOX, cefoxitin; TIO, ceftiofur; AXO, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; NAL, nalidixic acid; STR, streptomycin; FIS, sulfisoxazole; TET, tetracycline; SXT, sulfamethoxazole-trimethoprim.

second-lowest 3GC-resistant *Salmonella* detection rate but the second-highest nonresistant *Salmonella* detection rate (Table S2). *Salmonella* isolates were recovered from samples from pens A and D using two methods. With the first method, XLD plates were used to isolate and identify the dominant *Salmonella* populations regardless of antimicrobial resistance status. With the second method, XLD+CTX plates were used to isolate the 3GC-resistant *Salmonella* subpopulation previously detected and enumerated by plating (Table 1) to ensure its characterization. The 3GC-resistant *Salmonella* isolates at this feedyard were further characterized by recovering 3GC-resistant *Salmonella* isolates from pens B, C, and E using XLD+CTX plates only. Descriptive statistics for the 242 *Salmonella* isolates that were subjected to WGS are reported in Data File S1. Serotypes, STs, ARGs, and plasmid replicons identified by WGS and antimicrobial resistance phenotypes and MICs for the 242 *Salmonella* isolates are reported in Data File S2.

TABLE 4. *Monthly distribution of* Salmonella *isolates from pen A*

Month	*Salmonella* Muenchen–no CMY	*Salmonella* Montevideo–CMY	*Salmonella* Montevideo–no CMY	*Salmonella* Muenchen–CMY
1	3	0	0	0
2	3	2	0	0
3	3	3	0	0
4	3	0	0	0
5	0	4	1	0
6	3	3	0	0
7	2	4	0	0
8	2	4	0	0
9	3	3	0	0
10	3	1	0	0
11	3	3	0	0
12	1	0	1	0
13	2	1	0	0
14	3	1	0	0
15	3	0	0	0
16	2	1	0	0
17	3	3	0	0
18	2	3	0	0
19	3	3	0	0
20	3	2	0	1
21	2	3	1	0
22	3	3	0	0
23	2	4	0	0
24	2	0	0	0
Total	59	51	3	1

Pen A *Salmonella*. The 114 pen A *Salmonella* isolates were sorted into four groups by serotype and the presence of $bla_{\text{CMY-2}}$: *Salmonella* Muenchen–no CMY (59 isolates), *Salmonella* Montevideo–CMY (51 isolates), *Salmonella* Montevideo–no CMY (3 isolates), and *Salmonella* Muenchen–CMY (1 isolate) (Table 3). All 59 pen A Muenchen–no CMY isolates were resistant to sulfisoxazole and tetracycline (Table 3). The ARGs *aac(6′)-Iaa, aph(3″)-Ib, sul2,* and *tet*(A) and the plasmid replicons Col(pHAD28), ColRNAI, and IncQ1 were detected in all 60 *Salmonella* Muenchen isolates from pen A (Tables S3 and S4). All 51 pen A *Salmonella* Montevideo–CMY isolates were resistant to amoxicillin–clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline (Table 3). The ARGs *aac(6′)-Iaa, aph(3″)-Ib, aph(6)-Id,* $bla_{\text{CMY-2}}$, *floR, sul2,* and *tet*(A) and the IncC plasmid replicon were present in all 51 *Salmonella* Montevideo–CMY isolates from pen A (Tables S3 and S4).

Forty-seven of the 51 pen A *Salmonella* Montevideo–CMY isolates were obtained from XLD+CTX plates (Table S5). The XLD+CTX isolation method was essential to demonstrating the persistence of the *Salmonella* Montevideo–CMY subpopulation isolated from pen A during 19 of the 24 study months (Table 4). The pen A–dominant *Salmonella* Muenchen–no CMY population was isolated during 23 of the 24 study months (Table 4).

Pen D *Salmonella*. The 72 *Salmonella* isolates from pen D were sorted into eight groups by serotype and the presence of $bla_{\text{CMY-2}}$ (Table 5). The four groups with more than two isolates were *Salmonella* Muenchen–no CMY, *Salmonella* Montevideo–no CMY, *Salmonella* Montevideo–CMY, and *Salmonella* Anatum–no CMY (Table 5). All 26 pen D *Salmonella* Muenchen isolates were resistant to sulfisoxazole and tetracycline (Table 5). The ARGs *aac(6′)-Iaa, aph(3″)-Ib, sul2,* and *tet*(A) and the plasmid replicons Col(pHAD28), ColRNAI, and IncQ1 were detected in all 26 *Salmonella* Muenchen isolates from pen D (Tables S6 and S7). Twenty-two of the 25 pen D *Salmonella* Montevideo–no CMY isolates were pansusceptible (Table 5). All pansusceptible pen D *Salmonella* Montevideo–no CMY isolates harbored no ARGs except the cryptic *aac(6′)-Iaa*

TABLE 5. *Susceptibility of* Salmonella *isolates from pen D to 14 antimicrobial agents*

Salmonella serotype	No. of isolates	% of isolates resistant to[a]:													
		AUG	AMP	AZI	FOX	TIO	AXO	CHL	CIP	GEN	NAL	STR	FIS	TET	SXT
Muenchen–no CMY	26	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	100.0	0.0
Montevideo–no CMY	25	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	8.0	0.0	12.0	0.0
Montevideo–CMY	13	100.0	100.0	0.0	100.0	100.0	100.0	100.0	0.0	0.0	0.0	100.0	100.0	100.0	0.0
Anatum–no CMY	3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0
Kiambu–no CMY	2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lubbock–no CMY	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0
I-:d:1,2–no CMY	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	100.0	0.0
I-:g,m,s:−−no CMY	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a] AUG, amoxicillin–clavulanic acid; AMP, ampicillin; AZI, azithromycin; FOX, cefoxitin; TIO, ceftiofur; AXO, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; NAL, nalidixic acid; STR, streptomycin; FIS, sulfisoxazole; TET, tetracycline; SXT, sulfamethoxazole-trimethoprim.

(Table S6). No plasmid replicons were identified for the 22 pansusceptible pen D *Salmonella* Montevideo–no CMY isolates (Table S7). All 13 pen D *Salmonella* Montevideo–CMY isolates were resistant to amoxicillin–clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline (Table 5). The ARGs *aac(6′)-Iaa, aph(3″)-Ib, aph(6)-Id,* $bla_{\text{CMY-2}}$, *floR, sul2,* and *tet*(A) and the IncC plasmid replicon were present in all 13 pen D *Salmonella* Montevideo–CMY isolates (Tables S6 and S7).

Isolation on XLD+CTX plates was essential for identification and characterization of the pen D 3GC-resistant *Salmonella* subpopulation; 12 of the 13 *Salmonella* Montevideo–CMY were isolated by this method (Table S8). *Salmonella* Montevideo–CMY were isolated from pen D during 6 of the 24 study months (Table 6). All 13 3GC-resistant *Salmonella* isolates from pen D were *Salmonella* Montevideo–CMY, which strongly suggests that very low levels of *Salmonella* Montevideo–CMY were the dominant factor contributing to persistence of 3GC-resistant *Salmonella* isolates in this pen. The pen D overall *Salmonella* population was more diverse than that of pen A, with the two predominate subtypes (*Salmonella* Muenchen–no CMY and *Salmonella* Montevideo–no CMY) isolated roughly equally across the 24 study months (Table 6).

3GC-resistant *Salmonella* isolates from pens B, C, and E. To further explore the 3GC-resistant *Salmonella* populations at this feedyard, 56 3GC-resistant *Salmonella* isolates were recovered from samples from pens B, C, and E using XLD+CTX plates only. When categorized by serotype and $bla_{\text{CMY-2}}$ detection, these 56 *Salmonella* isolates sorted into three groups: *Salmonella* Montevideo–CMY (40 isolates), *Salmonella* Reading–CMY (14 isolates), and *Salmonella* Muenchen–CMY (2 isolates) (Table S9).

Similar to the isolates from pens A and D, all 40 *Salmonella* Montevideo–CMY isolates from pens B, C, and E were resistant to amoxicillin–clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline (Table S10) and harbored *aac(6′)-Iaa, aph(3″)-Ib, aph(6)-Id,* $bla_{\text{CMY-2}}$, *floR, sul2,* and *tet*(A) and the IncC plasmid replicon (Tables S11 and S12). These results provided strong evidence that *Salmonella* Montevideo–CMY was the predominant 3GC-resistant *Salmonella* population in four of the five sampled pens at the feedyard during the 24 study months. However, the isolation of 14 *Salmonella* Reading–CMY from 8 months of pen C samples clearly demonstrated that results cannot be extrapolated to unsampled pens. The *Salmonella* Reading–CMY isolates accounted for 14 of the 28 pen C isolates and codominated pen C with *Salmonella* Montevideo–CMY (Table S9). All 14 *Salmonella* Reading–CMY were resistant to amoxicillin–clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline (Table S10). The ARGs *aac(6′)-Iaa, aadA7, aph(3″)-Ib, aph(6)-Id,* $bla_{\text{CMY-2}}$, *floR, fosA7, sul1, sul2,* and *tet*(A) and the IncC plasmid replicon were present in all 14 *Salmonella* Reading–CMY isolates (Tables S11 and S12). Although identification of the dominant *Salmonella* population in each pen would be interesting and possibly insightful, isolation of *Salmonella* from pen C on XLD plates was beyond the goals of this study.

These results indicate that the *Salmonella* populations can differ between pens at a cattle feedyard. This result is consistent with those of other studies in which *Salmonella* serotypes have been found to differ between pens at the same feedyard *(12, 24, 25)*. The results of these studies should discourage researchers from extrapolation of *Salmonella* results to unexamined pens at beef cattle feedyards.

Phylogenetic analysis of *Salmonella* Montevideo isolates. Phylogenetic analysis of the 132 *Salmonella* Montevideo isolates (regardless of isolation method, $bla_{\text{CMY-2}}$ category, or pen) revealed two distinct clades (Fig. 1). *Salmonella* Montevideo clade 1 consisted of 30 isolates and had a total single nucleotide polymorphism (SNP) difference of 314 compared with the reference genome (GenBank accession CP017972). Five *Salmonella* Montevideo clade 1 isolates were identical (0 SNPs) within their core genome. The majority (25 of 30) of *Salmonella* Montevideo clade 1 isolates were pansusceptible, harbored no ARGs other than the cryptic *aac(6′)-Iaa,* and harbored

TABLE 6. *Monthly distribution of* Salmonella *isolates from pen D*

Month	*Salmonella* Muenchen–no CMY	*Salmonella* Montevideo–no CMY	*Salmonella* Montevideo–CMY	*Salmonella* Anatum–no CMY	*Salmonella* Kiambu–no CMY	*Salmonella* Lubbock–no CMY	*Salmonella* I-:d:1,2–no CMY	*Salmonella* I-:g,m,s:−–no CMY
1	3	0	0	0	0	0	0	0
2	2	0	0	0	0	0	0	0
3	2	1	3	0	0	0	1	0
4	0	2	0	0	0	0	0	0
5	0	0	3	2	0	1	0	0
6	3	0	3	0	0	0	0	0
7	2	0	0	0	1	0	0	0
8	2	0	0	0	1	0	0	0
9	1	2	0	0	0	0	0	0
10	1	0	2	1	0	0	0	0
11	2	1	1	0	0	0	0	0
12	0	0	0	0	0	0	0	0
13	0	3	0	0	0	0	0	0
14	3	0	0	0	0	0	0	0
15	1	2	1	0	0	0	0	0
16	0	3	0	0	0	0	0	0
17	0	0	0	0	0	0	0	1
18	2	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0
20	0	2	0	0	0	0	0	0
21	1	2	0	0	0	0	0	0
22	1	2	0	0	0	0	0	0
23	0	3	0	0	0	0	0	0
24	0	2	0	0	0	0	0	0
Total	26	25	13	3	2	1	1	1

no plasmid replicons. Two *Salmonella* Montevideo clade 1 isolates were resistant to multiple antimicrobials (amoxicillin–clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline), and harbored the ARGs *aac(6′)-Iaa, aph(3″)-Ib, aph(6)-Id,* $bla_{\text{CMY-2}}$, *floR, sul2,* and *tet*(A) and the IncC plasmid replicon. *Salmonella* Montevideo clade 1 isolates were recovered during 14 of the 24 study months (months 3 to 5, 9, 11 to 13, 15, 16, and 20 to 24).

Salmonella Montevideo clade 2 consisted of 102 isolates and had a total core genome difference of 433 SNPs compared with the reference genome. The core genomes of 25 *Salmonella* Montevideo clade 2 isolates were identical. All *Salmonella* Montevideo clade 2 isolates were resistant to amoxicillin–clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline. The ARGs *aac(6′)-Iaa, aph(3″)-Ib, aph(6)-Id,* $bla_{\text{CMY-2}}$, *floR, sul2,* and *tet*(A) and the IncC plasmid replicon were detected in all *Salmonella* Montevideo clade 2 isolates. *Salmonella* Montevideo clade 2 were recovered during 20 of the 24 study months (months 2 to 3, 5 to 11, and 13 to 23).

Phylogenetic analysis of *Salmonella* Muenchen isolates. Three clades were identified by phylogenetic analysis (regardless of isolation method, $bla_{\text{CMY-2}}$ category, or pen) among the 88 *Salmonella* Muenchen isolates (Fig. 2). All *Salmonella* Muenchen isolates regardless of clade contained *aac(6′)-Iaa, aph(3″)-Ib, sul2, tet*(A) and the Col (pHAD28), ColRNAI, and IncQ1 replicons. All *Salmonella* Muenchen isolates regardless of clade were resistant to sulfisoxazole and tetracycline.

Salmonella Muenchen clade 1 consisted of 33 isolates, 14 of which were identical within the core genome. Within *Salmonella* Muenchen clade 1 the total core genome difference was 168 SNPs compared with the reference genome (GenBank accession CP051389). *Salmonella* Muenchen clade 1 isolates were recovered during 16 of the 24 study months (months 1 to 3, 6 to 8, 10, 11, 13, and 18 to 24). All 16 *Salmonella* Muenchen clade 1 isolates recovered during months 18 to 24 harbored the Col156 plasmid replicon, but no additional ARGs or phenotypic resistance factors were detected for these isolates compared with all 17 *Salmonella* Muenchen clade 1 isolates recovered before month 18, which did not harbor the Col156 plasmid replicon.

Salmonella Muenchen clade 2 consisted of 25 isolates and had a total core genome difference of 155 SNPs compared with the reference genome. Ten *Salmonella* Muenchen clade 2 isolates had identical core genomes. *Salmonella* Muenchen clade 2 isolates were recovered during 15 of the 24 study months (months 1 to 4, 6 to 11, 14 to 16, 18, and 22). Only one *Salmonella* Muenchen clade 2 isolate had an additional plasmid replicon (Col440I) beyond the replicons present in all *Salmonella* Muenchen isolates in this study. No *Salmonella* Muenchen clade 2 isolate possessed ARGs or antimicrobial resistance beyond those shared by all *Salmonella* Muenchen isolates in this study.

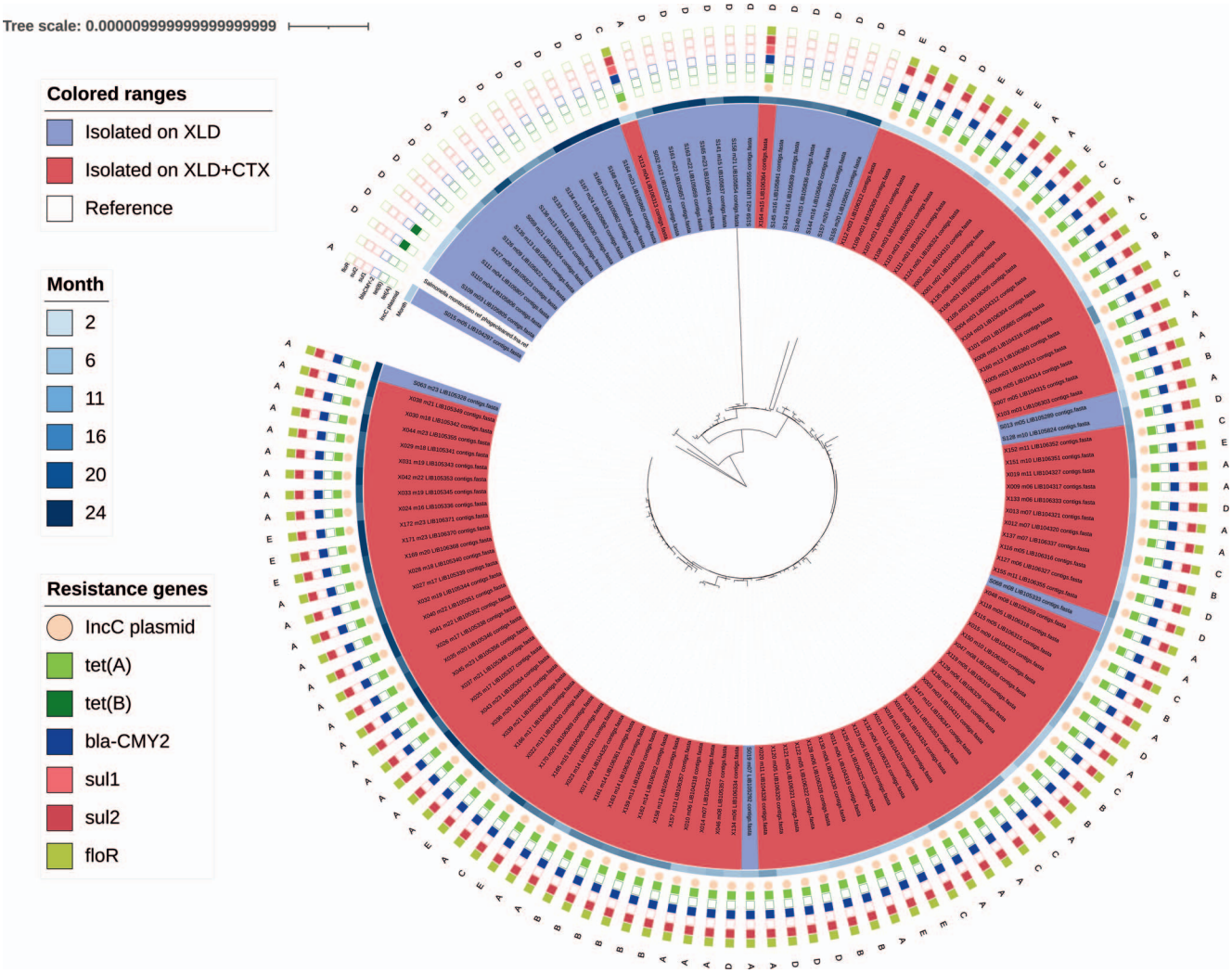

FIGURE 1. *Phylogenetic analysis of* Salmonella *Montevideo isolates. Colored range indicates isolation medium, blue heat map corresponds to month of collection, colored symbols indicate presence of resistance genes, and cattle pens are labeled with uppercase letters (A through E).*

Salmonella Muenchen clade 3 consisted of 30 isolates and had a total core genome difference of 192 SNPs compared with the reference genome. Twelve *Salmonella* Muenchen clade 3 isolates had identical core genomes. *Salmonella* Muenchen clade 3 isolates were recovered during 17 of the 24 study months (months 1, 3, 4, 6 to 15, 17, 18, 20, and 23). Only three *Salmonella* Muenchen clade 3 isolates possessed ARGs, plasmid replicons, or antimicrobial resistance factors beyond those shared by all *Salmonella* Muenchen isolates in this study. All three *Salmonella* Muenchen isolates that carried $bla_{\text{CMY-2}}$ were in clade 3. The three *Salmonella* Muenchen–CMY isolates were multidrug resistant (resistant to amoxicillin–clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, sulfisoxazole, and tetracycline). Two *Salmonella* Muenchen–CMY clade 3 isolates contained the IncX4 plasmid replicon, and one isolate contained the IncI1-Iγ plasmid replicon.

3GC-resistant *E. coli* isolates from pens A and D. To assess the extent that 3GC-resistant *E. coli* was a reservoir contributing to the occurrence of 3GC-resistant *Salmonella*, samples from pens A and D were selected for intensive characterization of 3GC-resistant *E. coli* isolates. Every study month, three $bla_{\text{CTX-M}}$ 3GC-resistant *E. coli* isolates and three $bla_{\text{CMY-2}}$ 3GC-resistant *E. coli* isolates were recovered if possible from pens A and D.

Descriptive statistics for the 203 3GC-resistant *E. coli* isolates subjected to WGS are reported in Data File S3. Serotypes, STs, ARGs, plasmid replicons, antimicrobial resistance phenotypes, and MICs identified for the 203 3GC-resistant *E. coli* isolates are reported in Data File S4. Among the whole genome sequences of the 203 3GC-resistant *E. coli* isolates, 11 β-lactam resistance genes were identified (Table 7). The $bla_{\text{CMY-2}}$ gene was identified in 37.9% of these isolates. Among the 77 3GC-resistant *E. coli* isolates with the $bla_{\text{CMY-2}}$ gene, 21 plasmid replicons were identified (Table 8). IncFIB(AP001918) was the most common plasmid replicon (59.7%), and IncC was the sixth most frequently detected plasmid replicon (26.0%). WGS identified 25 STs among the 77 3GC-resistant *E. coli* isolates with the $bla_{\text{CMY-2}}$ gene (Table 9). ST3018 was the most prevalent ST and accounted for 24.7% (19 of 77) of

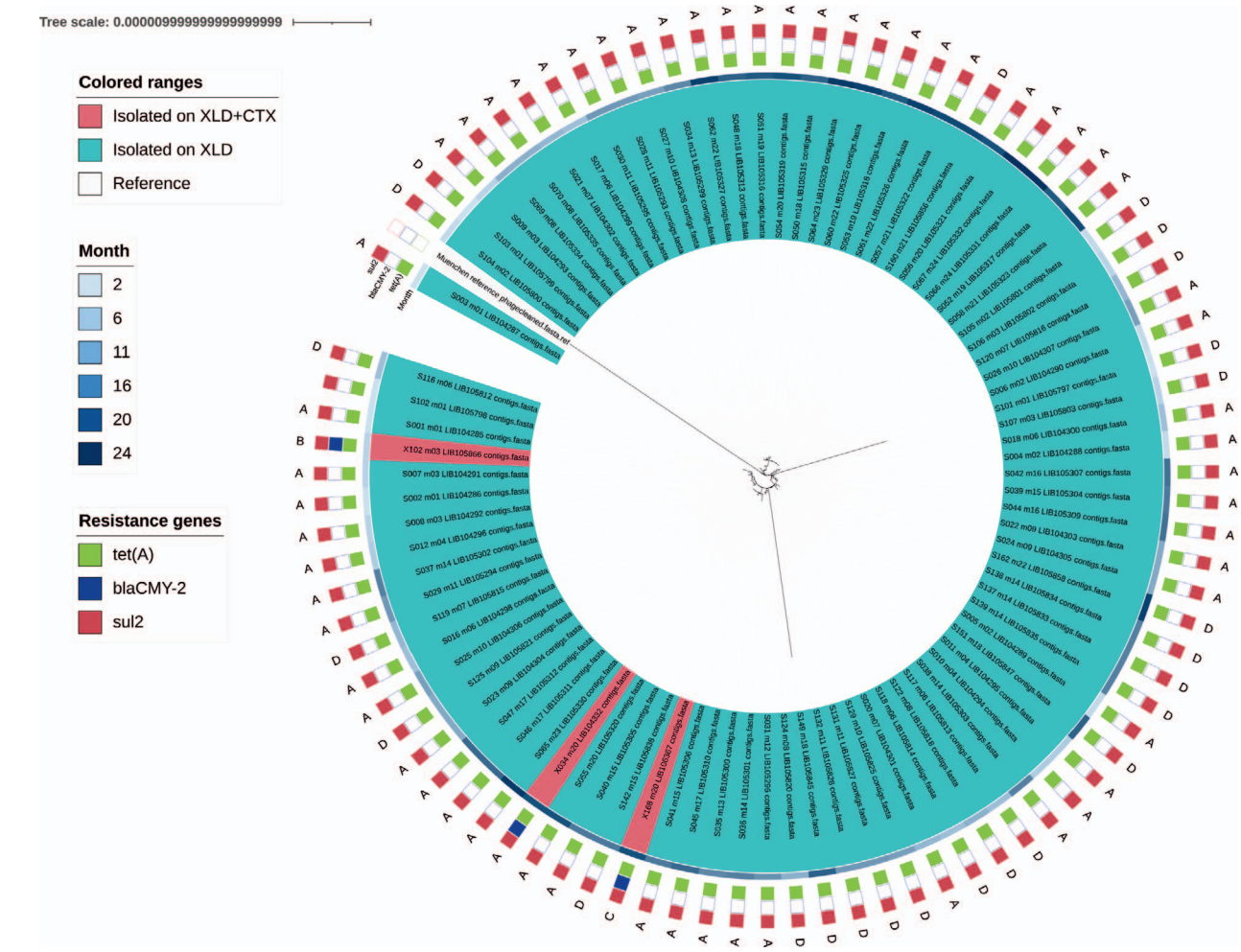

FIGURE 2. *Phylogenetic analysis of* Salmonella *Muenchen isolates. Colored range indicates isolation medium, blue heat map corresponds to month of collection, colored symbols indicate presence of resistance genes, and cattle pens are labeled with uppercase letters (A through E).*

these isolates. The ARGs *aadA2b, aph(3′)-Ia, cmlA1, mdf*(A), *sul3, tet*(A), and *tet*(M) and the plasmid sequences IncFIB (AP001918), IncFIB(H89-PhagePlasmid), and IncFII were identified in all 19 ST3018 $bla_{\text{CMY-2}}$ 3GC-resistant *E. coli* isolates. The 20 3GC-resistant *E. coli* isolates with both the $bla_{\text{CMY-2}}$ gene and the IncC plasmid sequence were distributed across 11 STs, with no ST accounting for >3 isolates (Data File S4).

TABLE 7. *β-Lactam resistance genes identified by whole genome sequencing of 3GC-resistant* E. coli *isolates*

	% of isolates		
bla Gene	Pen A (n = 99)	Pen D (n = 104)	Total
CMY-2	33.3	42.3	37.9
TEM-1A	35.4	38.5	36.9
CTX-M-32	31.3	27.9	29.6
CTX-M-27	24.2	24.0	24.1
TEM-1B	19.2	17.3	18.2
CTX-M-1	11.1	7.7	9.4
TEM-150	2.0	0.0	1.0
CTX-M-15	0.0	1.0	0.5
CTX-M-65	1.0	0.0	0.5
CTX-M-124	1.0	0.0	0.5
CTX-M-174	0.0	1.0	0.5

Although the $bla_{\text{CTX-M}}$ gene was not detected in any *Salmonella* isolates in this study, it was identified in 63.5% of the 3GC-resistant *E. coli* isolates. Seven $bla_{\text{CTX-M}}$ gene variants were present among the 3GC-resistant *E. coli* isolates, but only three of these variants were identified in more than one isolate (Table 7). The most frequent $bla_{\text{CTX-M}}$ gene variant was $bla_{\text{CTX-M-32}}$, identified in 60 isolates (Table 7). Sixteen plasmid replicons were identified among the 60 $bla_{\text{CTX-M-32}}$ 3GC-resistant *E. coli* isolates, with the most common replicon (IncR) identified in 33 of these isolates (Table S13). The $bla_{\text{CTX-M-32}}$ gene was detected in 3GC-resistant *E. coli* isolates assigned to 11 different STs, with the most frequent (ST7588) including 14 isolates (Table S14).

The second most frequent $bla_{\text{CTX-M}}$ gene variant was $bla_{\text{CTX-M-27}}$ (Table 7). Sixteen plasmid replicons were identified among the 49 $bla_{\text{CTX-M-27}}$ 3GC-resistant *E. coli* isolates, with IncFII present in all 49 isolates (Table S15). The $bla_{\text{CTX-M-27}}$ gene was detected in 3GC-resistant *E. coli* isolates assigned to 10 STs, with the most frequent (ST10) including 14 isolates (Table S16).

TABLE 8. *Plasmid replicons of 3GC-resistant* E. coli *isolates with the* bla$_{CMY-2}$ *gene*

Plasmid replicon	% of isolates		
	Pen A ($n = 33$)	Pen D ($n = 44$)	Total
IncFIB(AP001918)	54.5	63.6	59.7
IncFIC(FII)	48.5	50.0	49.4
IncI1-Iγ	33.3	50.0	42.9
IncFIB(H89-PhagePlasmid)	30.3	38.6	35.1
IncX4	33.3	25.0	28.6
IncC	33.3	20.5	26.0
IncFIA	15.2	31.8	24.7
IncFII	24.2	25.0	24.7
IncX1	18.2	20.5	19.5
IncY	0.0	20.5	11.7
Col(pHAD28)	6.1	13.6	10.4
IncR	21.2	2.3	10.4
IncFII(29)	6.1	11.4	9.1
IncFII(pSFO)	18.2	0.0	7.8
IncFII(pRSB107)	15.2	0.0	6.5
IncI2(Delta)	3.0	9.1	6.5
p0111	6.1	0.0	2.6
Col156	3.0	0.0	1.3
ColRNAI	0.0	2.3	1.3
IncFII(pSE11)	0.0	2.3	1.3
IncN	3.0	0.0	1.3

TABLE 9. *Sequence types (STs) of 3GC-resistant* E. coli *isolates with the* bla$_{CMY-2}$ *gene*

ST	% of isolates		
	Pen A ($n = 33$)	Pen D ($n = 44$)	Total
3018	18.2	29.5	24.7
7618	3.0	13.6	9.1
6118	18.2	0.0	7.8
10	6.1	4.5	5.2
3381	3.0	6.8	5.2
3602	6.1	4.5	5.2
1152	9.1	0.0	3.9
162	9.1	0.0	3.9
345	3.0	4.5	3.9
442	3.0	4.5	3.9
1310	0.0	4.5	2.6
20	3.0	2.3	2.6
226	0.0	4.5	2.6
278	0.0	4.5	2.6
58	6.1	0.0	2.6
993	0.0	4.5	2.6
1140	3.0	0.0	1.3
1587	0.0	2.3	1.3
4429	0.0	2.3	1.3
540	3.0	0.0	1.3
6416	0.0	2.3	1.3
6732	0.0	2.3	1.3
746	3.0	0.0	1.3
770	0.0	2.3	1.3
86	3.0	0.0	1.3

The third most frequent *bla*$_{CTX-M}$ gene variant was *bla*$_{CTX-M-1}$ (Table 7). Sixteen plasmid replicons were identified among the 19 *bla*$_{CTX-M-1}$ 3GC-resistant *E. coli* isolates, with IncR present in all 19 isolates. The *bla*$_{CTX-M-1}$ gene was detected in 3GC-resistant *E. coli* isolates assigned to five STs, with the most frequent (ST5727) including 15 isolates.

All 124 3GC-resistant *E. coli* isolates with a *bla*$_{CTX-M}$ gene but lacking the *bla*$_{CMY-2}$ gene were resistant to ampicillin, ceftiofur, and ceftriaxone but were susceptible to amoxicillin–clavulanic acid and cefoxitin (Table S17). All 77 3GC-resistant *E. coli* isolates with the *bla*$_{CMY-2}$ gene were resistant to amoxicillin–clavulanic acid, ampicillin, cefoxitin, ceftiofur, and ceftriaxone (Table S17). The two 3GC-resistant *E. coli* isolates with no β-lactamase genes detected by WGS were resistant to ampicillin, ceftiofur, and ceftriaxone (Table S17).

Different detection rates, dominant β-lactamase genes, dominant plasmid replicons, and population diversities for 3GC-resistant *E. coli* and *Salmonella* isolates. Some researchers have suggested that antimicrobial-resistant *E. coli* is a sentinel or indicator of antimicrobial resistance among *Salmonella* strains and that horizonal gene transfer contributes to the sentinel or indicator status of *E. coli (30)* (https://www.fda.gov/animal-veterinary/national-antimicrobial-resistance-monitoring-system/about-narms#bacteria). Abundant nutrients and high bacterial levels, similar to those present in beef cattle feedyard environments, facilitate horizontal gene transfer in laboratory experiments, leading researchers to theorize that commensal 3GC-resistant *E. coli* in cattle feedyard environments are reservoirs that actively and rapidly transfer genes conferring 3GC resistance to coresident *Salmonella* strains *(13, 41, 47)*.

However, pen surface 3GC-resistant *E. coli* isolates were unrelated to 3GC-resistant *Salmonella* based on either phenotypic detection or molecular characteristics, casting doubt on the strength and relevance of sentinel, indicator, or reservoir relationships between resistant *E. coli* and *Salmonella*. 3GC-resistant *E. coli* were ubiquitous at this feedyard, whereas 3GC-resistant *Salmonella* were far less prevalent. 3GC-resistant *E. coli* were detected in 97.7% (469 of 480) of the samples, but 3GC-resistant *Salmonella* were detected in only 29.0% (139 of 480) of the samples (Table 1). Two *bla* genes, *bla*$_{CMY-2}$ and *bla*$_{CTX-M}$, are predominantly responsible for conferring resistance to 3GCs (ceftriaxone, cefotaxime, and ceftiofur) in *E. coli* and *Salmonella (8, 9, 14, 39)*. In this study, all 684 3GC-resistant *Salmonella* isolates evaluated by PCR harbored *bla*$_{CMY-2}$ but none harbored *bla*$_{CTX-M}$. Conversely, *bla*$_{CMY-2}$ and *bla*$_{CTX-M}$ were detected in 34.3 and 61.2% of the 1,412 3GC-resistant *E. coli* isolates evaluated by PCR (Table 2). WGS further confirmed *bla*$_{CMY-2}$ and *bla*$_{CTX-M}$ discordance between 3GC-resistant *E. coli* and *Salmonella* populations. Among pen A samples, *bla*$_{CMY-2}$ was harbored by 100% of the 52 3GC-resistant *Salmonella* isolates but only 33.3% of the 99 3GC-resistant *E. coli* isolates (Tables S3 and 7). Among pen D samples, *bla*$_{CMY-2}$ was harbored by 100% of

the 13 3GC-resistant *Salmonella* isolates but only 42.3% of the 104 3GC-resistant *E. coli* isolates (Tables S6 and 7).

The IncC plasmid replicon was detected in 97.5% of the 121 3GC-resistant *Salmonella* isolates but was not detected in any of the 121 3GC-susceptible *Salmonella* isolates. In contrast, the IncC plasmid replicon was detected in just 9.9% of the 203 3GC-resistant *E. coli* isolates. All 138 3GC-resistant isolates with the IncC plasmid replicon harbored the $bla_{\text{CMY-2}}$ gene. Overall, the association of the $bla_{\text{CMY-2}}$ ARG and the IncC plasmid replicon was significant ($\chi^2 = 250.3$, df = 445, $P < 0.001$) but was driven by the *Salmonella* isolates. Although these data strongly suggest that the $bla_{\text{CMY-2}}$ gene was located on a plasmid with the IncC replicon, colocation could not be confirmed because for all 138 isolates with IncC and $bla_{\text{CMY-2}}$ these determinants were contained within separate contigs.

The prevalence and molecular characteristics 3GC-resistant *E. coli* and *Salmonella* were clearly different at this feedyard. Thus, *E. coli* was not a reliable indicator or sentinel of 3GC resistance in *Salmonella*. The 3GC-resistant *E. coli* data clearly indicated that the feedlot pen surface environment contained a very diverse array of *bla* genes, and associated plasmid replicons were found in various *E. coli* STs. However, the data strongly suggested that the dominant 3GC-susceptible *Salmonella* populations were not actively acquiring 3GC resistance via horizontal gene transfer. A single clade, *Salmonella* Montevideo clade 2, accounted for 84.3% of the 121 3GC-resistant *Salmonella* isolates recovered during the 24-month study period. This finding suggests that the persistence of a well-adapted 3GC-resistant *Salmonella* subtype was a more significant contributor to the occurrence of 3GC-resistant *Salmonella* than was horizontal gene transfer from an *E. coli* reservoir.

Available evidence suggests that the presence of 3GC-resistant *Salmonella* in cattle feedyards is driven by persistent subtypes. The results of this study clearly indicate that 3GC-resistant *Salmonella* Montevideo clade 2 isolates were a subpopulation within the 3GC-susceptible *Salmonella* Muenchen clades and *Salmonella* Montevideo clade 1 in pens A and D. This persistent 3GC-resistant *Salmonella* Montevideo clade 2 subpopulation would have gone largely undetected if only traditional isolation methods without cefotaxime supplementation had been used in this study. To our knowledge, no directly comparable longitudinal studies have been conducted to examine long-term (>6 months) dynamics of *Salmonella* subtypes at a commercial beef feedyard. However, our results are consistent with those of a short-term study (26 days) in which the impact of ceftiofur crystalline-free acid (CCFA) and chlortetracycline (CTC) on *Salmonella* subtypes was tracked at the West Texas A&M University experimental feedyard *(20, 21, 31, 32, 38)*. Before antimicrobial treatments, pansusceptible *Salmonella* isolates were recovered from 75% of 176 cattle fecal samples *(32)*. These pretreatment *Salmonella* isolates belonged to five serotypes (Anatum, Give, Kentucky, Mbandaka, and Montevideo). CCFA and CTC exposure decreased *Salmonella* prevalence, but the *Salmonella*-positive samples were increasingly dominated by multidrug-resistant *Salmonella* Reading, which was not detected before the antimicrobial treatment *(32)*. These *Salmonella* isolates were recovered from feces on media without supplemental antimicrobials. Ohta et al. *(32)* hypothesized that the multidrug-resistant *Salmonella* Reading was present in cattle feces before CCFA and CTC exposure but was not detected either because of the low level of this subpopulation within the overall fecal *Salmonella* population or because of enrichment bias. In a follow-up experiment, Siceloff et al. *(38)* used tetracycline supplemented enrichment followed by CRISPR-SeroSeq amplification and sequencing to detect multidrug-resistant *Salmonella* Reading in 8 of 55 samples that were preserved at −80°C before exposure to CCFA and CTC. These results and those of our study suggest that persistent 3GC-resistant *Salmonella* subpopulations may expand during antibiotic use and provide no evidence that active and on-going horizontal gene transfer of *bla* genes to *Salmonella* contributes to the presence of 3GC-resistant *Salmonella*. These results are also consistent with those of Levent et al. *(24, 25),* who focused on the *Salmonella* impacts of feedlot cattle exposure to tulathromycin and CCFA and found that the *Salmonella* serotype isolated from feces, hides, and lymph nodes was mostly influenced by the geographical origin, sample day, and pen. Antimicrobial treatment did not influence the *Salmonella* serotype, perhaps because azithromycin- and 3GC-resistant *Salmonella* were not present when these antimicrobials were administered.

To our knowledge, no longitudinal studies >160 days have been conducted to examine the dynamics of *Salmonella* subtypes at a commercial beef feedyard. The absence of longitudinal WGS studies at commercial cattle feedyards has prevented an empirical investigation into theories that 3GC-resistant *E. coli* is an important reservoir of 3GC resistance by transferring *bla* genes to *Salmonella*. However, our findings do not completely refute theories that *E. coli* is a reservoir for potential transfer of ARGs. The 3GC resistant *E. coli* isolates from two pens at a single feedyard contained a remarkable diversity of *bla* genes, plasmid replicons, and STs. Any ARGs identified in *E. coli* have the potential to be transferred to another bacterial species. However, the data collected in this study provided no evidence that 3GC-resistant *Salmonella* at this feedyard was actively acquiring and maintaining *bla* genes by horizonal gene transfer from *E. coli*. The detection rates, *bla* genes, and plasmid replicons were also vastly different between 3GC-resistant *E. coli* and 3GC-resistant *Salmonella* isolates. These results strongly support the conclusion that 3GC resistance in *E. coli* may not be used to infer 3GC resistance in *Salmonella*.

Identification of the dominant preharvest factors contributing to *Salmonella* contamination of final beef products has become an increasing important research gap. The results of this study clearly show that *Salmonella* populations can differ across pens in a feedyard and are consistent with results from shorter studies *(24, 25, 32, 38)*. The dominant factors that contribute to *Salmonella* and antimicrobial-resistant *Salmonella* persistence at cattle

feedyards remain unidentified. Previous *Salmonella* exposure, cattle source, backgrounding processes, feed, and antimicrobial use are among the factors that may influence *Salmonella* occurrence. The impact of these factors in this study is unknown because records of antibiotic use and cattle movement were not available. However, the results of this study do indicate the power of WGS for the rapid acquisition of critical information (serotype, plasmid replicon profile, ARGs present, and evolutionary relationships) about *Salmonella* strains in cattle feedyards. Intensive WGS and longitudinal studies at commercial feedyards that can provide records of population movements and antimicrobial use are urgently needed to gain the knowledge required to design, monitor, and assess preharvest interventions for *Salmonella*.

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SUPPLEMENTAL MATERIAL

Supplemental material associated with article can be found online at: https://doi.org/10.4315/JFP-21-371.s1; https://doi.org/10.4315/JFP-21-371.s2; https://doi.org/10.4315/JFP-21-371.s3; https://doi.org/10.4315/JFP-21-371.s4; https://doi.org/10.4315/JFP-21-371.s5

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