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Evidence of sheep and abattoir environment as important reservoirs of multidrug resistant *Salmonella* and extended-spectrum beta-lactamase *Escherichia coli*

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ABSTRACT

The increase in antimicrobial-resistant (AMR) foodborne pathogens, including *E. coli* and *Salmonella* in animals, humans, and the environment, is a growing public health concern. Among animals, cattle, pigs, and chicken are reservoirs of these pathogens worldwide. There is a knowledge gap on the prevalence and AMR of foodborne pathogens in small ruminants (i.e., sheep and goats). This study investigates the prevalence and antimicrobial resistance of extended-spectrum beta-lactamase (ESBL) *E. coli* and *Salmonella* from sheep and their abattoir environment in North Carolina. We conducted a year-round serial cross-sectional study and collected a total of 1128 samples from sheep ($n = 780$) and their abattoir environment ($n = 348$). Sheep samples consisted of feces, cecal contents, carcass swabs, and abattoir resting area feces. Environmental samples consisted of soil samples, lairage swab, animal feed, and drinking water for animals. We used CHROMAgar EEC with 4 μg/ml of Cefotaxime for isolating ESBL *E. coli*, and ESBL production was confirmed by double-disk diffusion test. *Salmonella* was isolated and confirmed using standard methods. All of the confirmed isolates were tested against a panel of 14 antimicrobials to elucidate susceptibility profiles. The prevalence of ESBL *E. coli* and *Salmonella* was significantly higher in environmental samples (47.7% and 65.5%) compared to the sheep samples (19.5% and 17.9%), respectively ($P < 0.0001$). We recovered 318 ESBL *E. coli* and 368 *Salmonella* isolates from sheep and environmental samples. More than 97% (310/318) of ESBL *E. coli* were multidrug-resistant (MDR; resistant to ≥3 classes of antimicrobials). Most *Salmonella* isolates (77.2%, 284/368) were pansusceptible, and 10.1% (37/368) were MDR. We identified a total of 24 different *Salmonella* serotypes by whole genome sequencing (WGS). The most common serotypes were Agona (19.8%), Typhimurium (16.2%), Cannstatt (13.2%), Reading (13.2%), and Anatum (9.6%). Prevalence and percent resistance of ESBL *E. coli* and *Salmonella* isolates varied significantly by season and sample type ($P < 0.0001$). The co-existence of ESBL *E. coli* in the same sample was associated with increased percent resistance of *Salmonella* to Ampicillin, Chloramphenicol, Sulfisoxazole, Streptomycin, and Tetracycline. We presumed that the abattoir environment might have played a great role in the persistence and dissemination of resistant bacteria to sheep as they arrive at the abattoir. In conclusion, our study reaffirms that sheep and their abattoir environment act as important reservoirs of AMR ESBL *E. coli* and MDR *Salmonella* in the U.S. Further studies are required to determine associated public health risks.

1. Introduction

Antimicrobial resistance (AMR) in *Enterobacteriaceae* family members is a significant health threat to animals and humans (CDC, 2019). The environment plays a vital role in the persistence and dissemination of these pathogens between humans and animals (Huijbers et al., 2015; Rostagno et al., 2003). Nontyphoidal *Salmonella* (NTS) infections are the leading cause of hospitalization and deaths in humans in the United States (U.S.). *Salmonella* alone is responsible for 1.35 million illnesses, 26,500 hospitalizations, and 420 deaths annually in the U.S. (CDC,

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2019; Scallan et al., 2011). The number of infections and deaths associated with extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* in the (U.S.) are about 197,400 and 9100 per year, respectively (CDC, 2019). In humans and animals, commensal organisms like *Escherichia coli* (*E. coli*) can serve as reservoirs for AMR. They may also disseminate AMR genes to other *Enterobacteriaceae* family members, including *Salmonella* (WHO, 2017). Among food animals, ruminants, including cattle, sheep, and goats, harbor and transfer *E. coli* and *Salmonella* to other animals and humans (Davidson et al., 2018; Edrington et al., 2009; Lenahan et al., 2007). The National Antimicrobial Resistance Monitoring System (NARMS) conducts routine surveillance of AMR in enteric bacteria from food-producing animals at slaughter and retail meats from cattle, swine, chickens, and turkeys (NARMS, 2015). Previously, meat from small ruminants has not been an important source of foodborne Shiga-toxin producing *E. coli* (STEC) and *Salmonella* infections in the U.S. and Canada (Hoffmann et al., 2017). Although the estimated level of risk for *Salmonella* infections between beef, lamb, poultry, and pork is similar (Hsi et al., 2015), small ruminants were not the focus of NARMS and the USDA's Food Safety and Inspection Service (FSIS) testing in the past. However, currently, the NARMS is in the process of including veal, sheep, lamb, and goats sampling for routine surveillance of critical foodborne pathogens (USDA-FSIS, 2020).

At present, there are about 5.2 million sheep and 2.7 million goats in the U.S., excluding live imports from other countries (USDA-NASS, 2020). Although sheep and goat meat consumption has slightly increased since the last decade (NRC, 2008; USDA-ERS, 2021), there is a significant knowledge gap on AMR of foodborne pathogens from these animals in the U.S. (Dargatz et al., 2015). The majority of past reports were focused on the prevalence of pathogenic Shiga-toxin producing *E. coli* (STEC) and *Salmonella* in feces, carcasses, and hide of sheep and goats (Dargatz et al., 2015; Edrington et al., 2009; Hanlon et al., 2018; Jacob et al., 2013; Kalchayanand et al., 2007; Kilonzo et al., 2011; Kudva et al., 1996; Samadpour et al., 1994). Only a few reports studied AMR in these pathogens. A study by researchers involving sheep operations from 22 states reported that the majority (94.0%, 948/1008) of *Salmonella* isolates recovered from sheep/sheep feces were serotype antigenic formula IIIb 61:k:1,5,(7) and almost all tested isolates (n = 238) were pansusceptible (Dargatz et al., 2015).

Although ESBL genes such as bla_{TEM}, bla_{CMY}, bla_{SHV}, and bla_{PSE} have circulated for a long time in the U.S. (Bradford, 2001; Frye and Jackson, 2013), CTX-M-type ESBLs were uncommon until early 2000. In 2003, the first CTX-M-like ESBL *E. coli* appeared in humans in the U.S. (Moland et al., 2003). Later, transferrable $bla_{CTX\text{-}M}$ genes in *E. coli* were detected in food animals (cattle feces) in the U.S. (Wittum et al., 2010). A recent NARMS report described an abundance of ESBLs in food animal isolates with multiple types of CTX-M type of ESBLs detected in cattle and retail meats, including pork, turkey, chicken, and beef (Tadesse et al., 2018). In addition, investigators recovered ESBL *Enterobacteriaceae* from sheep and their products in other parts of the world (Geser et al., 2012). Abattoir environments, including lairage environments, were identified as an important source of carcass contamination in U.S. beef and pig processing plants (Arthur et al., 2008; Bolton et al., 2013). Moreover, the environment played an important role in disseminating AMR (Berglund, 2015). However, only limited information is available on AMR of *Salmonella* and ESBL *E. coli* from small ruminants in the U.S. To the author's knowledge, no year-round cross-sectional study has been conducted to determine the prevalence and AMR status in sheep and their environment. Therefore, we designed a cross-sectional study to assess the prevalence and antimicrobial susceptibility profile of ESBL *E. coli* and *Salmonella* from sheep and their abattoir environment in North Carolina to address the knowledge gap.

2. Materials and methods

2.1. Sampling

A serial cross-sectional study design was conducted from March 2019 to February 2020. Samples were collected monthly from an abattoir in North Carolina. On average, the abattoir slaughtered 61 (44–70) goats, 37 (20–54) sheep, and 10 cattle (7–15) per day. In the abattoir facility, these animals roamed freely in the abattoir resting area and shared feed and water from the same troughs. A total of 1128 samples were collected from sheep (n = 780) and abattoir environments (n = 348). Sheep samples were comprised of carcass swabs (n = 246), cecal contents (n = 224), feces (n = 220), and abattoir resting area feces (n = 90). Abattoir environmental samples were comprised of lairage swabs (n = 120), animal feed (n = 69), drinking water (n = 69), and soil samples (n = 90). Samples were collected in all seasons, namely spring (March–May 2019), summer (June–August 2019), fall (September – November 2019), and winter (December 2019 to February 2020). The exception was in spring when no samples were collected from the abattoir resting area feces and soil samples. Carcass swabs were collected using sterile sponges (Nasco™) presoaked with buffered peptone water (BPW) (Difco™). Sponging was done by wiping the flank, brisket, and rump (each approximately 100 cm^2 area) of carcasses after evisceration but before carcasses' lactic acid spray. Fecal and cecal content samples were collected by milking about 5 g of rectal feces (pellets) and cecal content into sterile whirl-pack bags (Nasco™) and sterile screw-topped cups, respectively. Sheep feces, cecal contents, and carcass swabs were collected from the same animal immediately after evisceration of sheep carcass. About 5 g of freshly dropped feces (pellets) from sheep were collected from the abattoir resting area where all animals were kept together for few hours to up to three days until slaughtered. Animals were brought for slaughter from North Carolina and neighboring states; however, history on farm level husbandry, health, and antimicrobial use was not made available to us. Lairage swabs were collected from pens that were occupied using sterile sponges (Nasco™) presoaked with BPW (Difco™). About 10 g of feed samples consisting of grass hay were collected in sterile cups from the feeding troughs and storage heaps. Water samples (about 10 ml) were collected from water troughs using sterile screw cups. Soil samples of approximately 10 g of soil were collected with sterile gloves and transported in whirl-pack bags (Nasco™). The collected samples were immediately stored in the icebox and transported to the laboratory for processing within 3 h.

2.2. Bacterial isolation, identification, and confirmation

Salmonella isolation was performed following the previously described protocol (Fedorka-Cray et al., 1997; Wells et al., 2001). Briefly, the samples were pre-enriched in two types of broth media: Tetrathionate (TET) broth (Oxoid™) and Gram-Negative (G.N.) broth (Hajna, Remel™). One gram of sample (fecal and cecal content) was transferred to each tube containing 9 ml G.N. or TET broth and incubated overnight at 37 °C for 24 h and 48 h, respectively. Then, 100 μl of sample from the G.N. broth and TET tubes were transferred to each 9.9 ml of Rappaport Vassiliadis (R.V.) broth and incubated for 20–24 h at 37 °C. From RV, the sample was streaked for isolation on two different media: Brilliant Green Sulfa (BGS) agar and Xylose-Lysine-Tergitol 4 (XLT-4) agar and incubated overnight at 37 °C. All BGS and XLT-4 plates were checked for presumptive positive colonies according to the manufacturer's instructions. Three presumptive isolated colonies were picked per plate and confirmed using biochemical tests by stabbing onto triple sugar iron (TSI) agar and lysine iron agar (LIA) and incubated overnight at 37 °C. Presumptive *Salmonella* isolates (a maximum of 12 isolates/sample) were transferred on to tryptic-soy agar with 5% sheep red blood cells (TSA-SB) added (BBL, MD) and incubated overnight at 37 °C. *Salmonella* was isolated from carcass swabs and environmental samples (lairage swabs, feed, soil, and water) as follows: 90 ml of BPW

was transferred into samples in the whirl-pack bags or cups and mixed well by massaging or shaking and incubated overnight at 37 °C. Then, 1 ml of the sample from each tube was transferred into tubes containing 9 ml of sterile G.N. or TET. Incubations and other procedures were done as stated above for isolation of *Salmonella* from fecal samples.

A colony suspension was used as a template to confirm the presumptive *Salmonella* isolates by *InvA* gene Polymerase Chain Reaction (PCR) (Rahn et al., 1992). PCR mixtures contained 10 μl of PCR Master Mix (Applied Biosystems®), 0.5 μl of each forward primer (5'GTGAAATTATCGCCACGTTCGGGCAA3') and reverse primer (5'TCATCGCA-CCGTCAAAGGAACC3'), 1.0 μl of the template and molecular grade water to a final volume of 20 μl. Amplification was done with initial denaturation at 95 °C for 10 min followed by 34 cycles of denaturation at 96 °C for 3 s, annealing at 54 °C for 3 s and extension at 68 °C for 15 s and final extension at 72 °C for 10 s. Amplified product (about 284 bp) was separated on 1.5% agarose gel.

Presumptive ESBL *E. coli* were isolated on CHROMagar EEC with the addition of 4 μg/ml Cefotaxime (Jacob et al., 2020). Approximately 1 g of sheep feces, cecal contents, and abattoir resting area feces were suspended into 9 ml of sterile phosphate buffered saline and directly streaked onto CHROMagar ECC with 4 μg/ml Cefotaxime and incubated overnight at 37 °C. Likewise, ESBL *E. coli* were isolated from carcass swab and environmental samples using the overnight enrichment culture in BPW. Up to three well isolated presumptive ESBL *E. coli* colonies were picked and streaked onto TSA-SB. Indole test was employed as confirmatory tests, and indole negative isolates were confirmed using Matrix-Assisted Laser Desorption Ionization-Time of Flight mass spectrometry (MALDI-TOF). From presumptive ESBL *E. coli* isolates recovered ($n = 1424$), the first isolates from each positive sample ($n = 516$) were evaluated for ESBL production. We used the Clinical Laboratory Standards Institute (CLSI) double-disc diffusion test using Cefotaxime (30 μg) and Ceftazidime (30 μg) paper disks with and without clavulanic acid (10 μg) on Muller Hinton Agar (CLSI, 2017). *K. pneumoniae* ATCC® 700603 and *E. coli* ATCC® 25922 were used for quality control.

2.3. Antimicrobial susceptibility testing for Salmonella and ESBL E. coli

Minimum inhibitory concentration (MIC), including acquiring antimicrobial susceptibility test (AST) profiles, of one isolate per sample for *Salmonella* ($n = 368$) and ESBL *E. coli* ($n = 318$) were determined using broth microdilution method using the NARMS Sensititre™ gram-negative plate (CMV3AGNF) (Trek Diagnostics, Cleveland, OH). To avoid selection bias, the first *Salmonella* isolate recovered from TET/XLT-4 (96.7%) for each positive sample was selected for further analyses; otherwise, the first isolate from other media were tested. The test panel contained 14 antimicrobials: Ampicillin (AMP), Amoxicillin/Clavulanic acid (AUG), Ceftriaxone (AXO), Azithromycin (AZI), Chloramphenicol (CHL), Ciprofloxacin (CIP), Sulfisoxazole (FIS), Cefoxitin (FOX), Gentamicin (GEN), Nalidixic Acid (NAL), Streptomycin (STR), Trimethoprim/Sulfamethoxazole (SXT), Tetracycline (TET) and Ceftiofur (XNL). Then, plates were inoculated and incubated following the manufacturer's protocol. *Escherichia coli* ATCC®25922 and *Klebsiella pneumoniae* ATCC®700603 were used as ESBL negative and positive quality control strains, respectively. MICs were recorded and breakpoints were used as defined by CLSI (CLSI, 2017) or NARMS (CDC, 2020). Antimicrobial resistant isolates exhibiting resistance to ≥ three classes of antimicrobials were considered as MDR.

2.4. Serotyping of Salmonella isolates

Salmonella isolates ($n = 167$) were selected for whole genome sequencing (WGS) based on their AMR profile, month and season of sampling, and source and type of samples. Nucleic acid (DNA) was extracted from each selected isolate from overnight culture on TSA-SB using Qiagen DNeasy PowerLyser Microbial Kit following the manufacturer's protocol. The purified DNA was quantified using NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA). Sequencing DNA library was prepared using Nextera DNA Flex Library preparation kit (Illumina, San Diego, CA) as previously described (CDC, 2016). WGS was performed on Illumina MiSeq with 250 bp paired-end reads. Sequence reads were submitted to the National Center for Biotechnology Information database (BioProject accession number PRJNA293224). Sequences were assembled using SPAdes 3.14.1 (Bankevich et al., 2012) and annotated with PROKKA (Seemann, 2014) using default parameters. Assembled genomes were uploaded to the in silico SeqSero2 version 1.1.0 database to predict *Salmonella* serotypes (http://www.denglab.info/SeqSero2) (Banerji et al., 2020; Zhang et al., 2015, 2019). Average number of contigs was 128 (range 55 to 421), average genome coverage was 81× (range 22 to 374), and average GC content was 52.1% (range 51.4 to 52.4%). List of sequenced *Salmonella* isolates and number of contigs, length, genome coverage, GC content and AMR profiles are described on Table S1.

3. Statistical analysis

Descriptive statistics, including bar diagrams, contingency tables, and simple proportions, were obtained to describe the frequency of detection of ESBL *E. coli* and *Salmonella* and their antimicrobial susceptibility. Chi-square test or Fisher's exact test were used to determine associations. Univariate and multiple logistic regression were used to determine the effect of season and type of sample on the likelihood of *Salmonella* and ESBL *E. coli* in sheep and environmental samples. Univariate logistic regression was used to determine the effect of co-detection of ESBL *E. coli* on percent resistance of *Salmonella* to antimicrobials. The multivariate models explored associations at the individual level (sheep samples), and at the ecological level (environmental samples). The magnitude of odds ratios (OR) and the corresponding 95% confidence interval (95% CI) were used to indicate the strength of association and its direction. An odds ratio equal to one indicates no association. For other statistical tests, the alpha value was set at ≤ 0.05. Statistical data analyses were carried out using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

4. Results

4.1. Prevalence of ESBL E. coli and seasonal variations in sheep and their abattoir environment

The prevalence of ESBL *E. coli* was significantly higher in environmental samples (47.7%, 166/348) compared to sheep samples (19.5%, 152/780) ($P < 0.0001$) (Table 1). A total of 318 (28.2%) (one isolate per positive sample) ESBL *E. coli* were recovered from 1128 samples divided into 780 sheep samples and 348 environmental samples. In sheep samples, ESBL *E. coli* was detected in 60/220 or 27.3% of sheep feces, 49/224 or 21.9% of cecal contents, 18/90 or 20% of the abattoir resting area feces, and 25/246 or 10.2% of carcass swabs. Among environmental samples, ESBL *E. coli* was detected predominantly in lairage swabs (79/120 or 65.8%) and soil (53/90 or 58.9%), followed by feed (21/69 or 30.4%,) and water (13/69 or 18.8%). ESBL *E. coli* was five times more likely to occur in lairage swabs (OR: 5.6, 95% CI: 3.36–9.20) and soil samples (OR: 5.2, 95% CI: 3.05–9.00) and about four times less likely in carcass swabs (OR: 0.3, 95% CI: 0.15–0.44) compared to sheep feces (Table 2).

The prevalence of ESBL *E. coli* in sheep samples was significantly higher in spring (33.8%) and summer (28.2%) seasons compared to fall (9.1%) and winter seasons (11.0%) ($P < 0.0001$) (Table 3). However, there was no significant seasonal difference in contamination of abattoir resting area feces ($P = 0.4346$) between summer, fall, and winter seasons. The prevalence of ESBL *E. coli* on carcass swabs was significantly higher ($P < 0.0001$) in spring (24.2%, 16/66) followed by summer (11.7%, 7/60). For environmental samples, the prevalence of ESBL *E. coli* was significantly higher in winter (67.7%) and spring (56.7%)

Table 1
Prevalence of ESBL *E. coli* and Salmonella in sheep and environmental samples.

Source of sample	Type of sample	Number of samples	Number positive (%)		Both ESBL *E. coli* and *Salmonella*
			ESBL *E. coli*	*Salmonella*	
Sheep	Feces	220	60 (27.3)	51 (23.2)	18 (8.2)
	Cecal contents	224	49 (21.9)	45 (20.1)	12 (5.4)
	Carcass swab	246	25 (10.2)	6 (2.4)	2 (0.8)
	Abattoir resting area feces	90	18 (20.0)	38 (42.2)	8 (8.9)
Total in sheep source[a]		780	152 (19.5) *	140 (17.9) **	40 (5.1) ***
Environmental samples	Soil samples	90	53 (58.9)	84 (93.3)	53 (58.9)
	Lairage swabs	120	79 (65.8)	103 (85.8)	68 (56.7)
	Animal feed	69	21 (30.4)	28 (40.6)	14 (20.3)
	Water	69	13 (18.8)	13 (18.8)	4 (5.8)
Total in environmental source[b]		348	166 (47.7) *	228 (65.5) **	139 (39.9) ***
Overall		1128	318 (28.2)	368 (32.6)	179 (15.9)

Chi-Square or Fisher's Exact tests were used to compare frequencies.
[a,b]Single, double and triple asterisk indicate statistically significant differences in percent positives between total number of sheep and environmental samples ($P < 0.0001$) for ESBL *E. coli*, *Salmonella,* and co-detection of both ESBL *E. coli* and *Salmonella*, respectively.

Table 2
Multiple logistic regression for predictor variables in the detection of ESBL *E. coli* and Salmonella.

Predictor variable	*ESBL E. coli*		*Salmonella*		Both ESBL *E. coli* and *Salmonella*	
	Odds ratio	95% CI	OR	OR 95% CI	OR	OR 95% CI
Season						
Winter	Reference		Reference		Reference	
Spring	2.4	1.56–3.63	0.4	0.22–0.77	0.6	0.33–1.14
Summer	1.2	0.81–1.75	2.0	1.28–3.11	0.7	0.43–1.17
Fall	0.4	0.26–0.61	2.4	1.53–3.67	0.3	0.18–0.54
Type of sample						
Sheep feces	Reference		Reference		Reference	
Carcass swab	0.3	0.15–0.44	0.1	0.04–0.20	0.1	0.02–0.40
Cecal contents	0.7	0.46–1.14	0.8	0.52–1.32	0.6	0.30–1.35
Abattoir resting area feces	0.8	0.45–1.55	2.0	1.19–3.46	1.1	0.45–2.61
Soil	5.2	3.05–9.00	41.8	17.04–102.71	17.7	9.10–34.35
Lairage swabs	5.6	3.36–9.20	28.7	14.92–55.39	15.9	9.62–29.48
Animal feed	1.1	0.62–2.11	2.5	1.36–4.49	2.9	1.35–6.28
Drinking water (trough)	0.6	0.29–1.16	0.8	0.39–1.56	0.7	0.22–2.12

An odd ratio (OR) is a measure of association indicating the strength and direction of the association, and it is presented with the confidence interval (CI).

seasons than in summer (39.6%) and fall (30.2%) seasons ($P < 0.0001$). There was no significant seasonal variation in contamination of water ($P = 0.3493$) and marginal significance in feed ($P = 0.0501$) (Table 3). The detection of ESBL *E. coli* in any of the samples was more likely in spring (OR: 2.4, 95% CI: 1.56–3.63) compared to winter season (Table 2).

4.2. Prevalence of Salmonella and seasonal variations in sheep and abattoir environment

Out of 1128 samples examined, *Salmonella* was detected in 368 (32.6%) samples (one isolate per positive sample), and prevalence was significantly higher in environmental samples (65.5%, 228/348) than in sheep samples (17.9%, 140/780) ($P < 0.0001$) (Table 1). For the sheep samples, the higher prevalence was recorded in abattoir resting area feces (42.2%, 38/90) followed by sheep feces (23.2%, 51/220), cecal contents (20.1%, 45/224), and carcass swabs (2.4%, 6/246) (Table 1). Among environmental samples, soil samples had the higher prevalence of *Salmonella* (93.3%, 84/90) followed by lairage swabs (85.8%, 103/120), feed (40.6%, 28/69), and water (18.8%, 13/69) (Table 1). The odds for detection of *Salmonella* were much higher in soil (OR: 41.8, 95% CI: 17.04–102.71) and lairage samples (OR: 28.7, 95% CI: 14.92–55.39) compared to sheep feces. The odds for detection of *Salmonella* were about twice in abattoir resting area feces (OR: 2.0, 95% CI: 1.19–3.46) and more than twice as high in feed samples (OR: 2.5, 95% CI: 1.36–4.49) compared to sheep feces. Carcass swabs were about 12 times (OR: 0.1, 95% CI: 0.04–0.20) less likely to be contaminated with *Salmonella* than sheep feces (Table 2).

The seasonal prevalence was consistently lower in sheep samples than in environmental samples for all seasons ($P < 0.0001$) (Table 4). *Salmonella* prevalence in sheep samples was significantly higher in summer (25.7%, 8/154) and fall (26.2%, 55/210) seasons than in winter (11.4%, 24/210) and spring (5.2%, 8/154) ($P < 0.0001$). However, there was no significant difference in the contamination of sheep carcasses with *Salmonella* between seasons ($P = 0.6114$) (Table 4). *Salmonella* prevalence in environmental samples remained significantly higher in summer (67.7%, 65/96), fall (75.0%, 72/96) and winter (69.8%, 67/96) seasons compared to spring (40.0%, 24/60) ($P < 0.0001$). There was no significant seasonal difference in the prevalence of *Salmonella* within environmental samples ($P > 0.05$) except in feed samples where higher contamination was detected in fall (66.7%, 12/18) and summer (50.0%, 9/18) seasons (Table 4) ($P = 0.0041$). The odds for detecting *Salmonella* in any of the samples was 2.4 (OR 95% CI: 1.53–3.67) times higher in fall and about twice (OR 95% CI: 1.28–3.11) in summer compared to the winter season. *Salmonella* was less likely to be detected in spring (OR: 0.4, 95% CI: 0.22–0.77) compared to the winter season (Table 2).

4.3. Co-detection of ESBL E. coli and Salmonella

ESBL *E. coli* and *Salmonella* were co-detected in 15.9% (179/1128) of samples. Percent co-detection was significantly higher in environmental samples (39.9%, 139/348) than in sheep samples (5.1%, 40/780) ($P < 0.0001$) (Table 1). Co-detection of ESBL *E. coli* and *Salmonella* significantly differed among sheep samples with higher percentages detected in abattoir resting area feces (8.9%), sheep feces (8.2%), and cecal

Table 3
Seasonal variation and comparison of prevalence of ESBL *E. coli* in sheep and environmental samples.

Sample type	Number of samples per season (% positives)				*P* value[a]
	Spring	Summer	Fall	Winter	
Sheep feces	43 (39.5)	57 (43.9)	60 (11.7)	60 (18.3)	0.0001*
Cecal content	45 (42.2)	59 (30.5)	60 (13.3)	60 (6.7)	<0.0001*
Carcass swab	66 (24.2)	60 (11.7)	60 (0.0)	60 (3.3)	<0.0001*
Abattoir resting area feces	N	30 (26.7)	30 (13.3)	30 (20.0)	0.4346
All sheep samples	154 (33.8)	206 (28.2)	210 (9.1)	210 (11.0)	<0.0001*
Soil samples	N	30 (40.0)	30 (50.0)	30 (86.7)	0.0006*
Lairage samples	30 (93.3)	30 (50.0)	30 (36.7)	30 (83.3)	<0.0001*
Feed	15 (20.0)	18 (38.9)	18 (11.1)	18 (50.0)	0.0501
Water	15 (20.0)	18 (22.2)	18 (5.6)	18 (27.8)	0.3493
All environmental samples	60 (56.7)	96 (39.6)	96 (30.2)	96 (67.7)	<0.0001*
All samples	214 (40.2)	302 (31.8)	306 (15.7)	306 (28.8)	<0.0001*

Chi-square or Fishers Exact tests were used to compare frequencies.
[a] An asterisk indicates statistically significant seasonal difference in prevalence of ESBL *E. coli* among compared groups using either N indicates that samples were not collected.

Table 4
Seasonal variation and comparison of prevalence of Salmonella in sheep and environmental samples.

Sample type	Number of samples per season (% positives)				*P* value[a]
	Spring	Summer	Fall	Winter	
Sheep feces	43 (9.3)	57 (35.1)	60 (31.7)	60 (13.3)	0.0019*
Cecal content	45 (4.4)	59 (33.9)	60 (25.0)	60 (13.3)	0.0009*
Carcass swab	66 (3.0)	60 (3.3)	60 (3.3)	60 (0.0)	0.6114
Abattoir resting area feces	N	30 (36.7)	30 (63.3)	30 (26.7)	0.0121*
All sheep samples	154 (5.2)	206 (25.7)	210 (26.2)	210 (11.4)	<0.0001*
Soil samples	N	30 (86.7)	30 (93.3)	30 (100.0)	0.1589
Lairage samples	30 (76.7)	30 (86.7)	30 (90.0)	30 (90.0)	0.4389
Feed	15 (6.7)	18 (50.0)	18 (66.7)	18 (33.3)	0.0041*
Water	15 (0.0)	18 (22.2)	18 (27.8)	18 (22.0)	0.1512
All environmental samples	60 (40.0)	96 (67.7)	96 (75.0)	96 (69.8)	<0.0001*
All samples	214 (15.0)	302 (39.1)	306 (41.5)	306 (29.7)	<0.0001*

Chi-square or Fishers Exact tests were used to compare frequencies.
[a] An asterisk indicates statistically significant seasonal difference in prevalence of *Salmonella* among compared groups using either N = samples were not collected.

content (5.4%) compared to carcass swab (0.8%) ($P = 0.001$). Likewise, co-detection of ESBL *E. coli* and *Salmonella* significantly differed in environmental samples with a higher percentage in soil (58.9%) and lairage swabs (56.7%) compared to feed (20.3%) and water samples (5.8%) ($P < 0.0001$). The odds for co-detecting ESBL *E. coli* and *Salmonella* were more likely in soil samples (OR: 17.7, 95% CI: 9.10–34.35), lairage swabs (OR: 15.9, 95% CI: 9.62–29.48), and animal feed (OR: 2.9, 95% CI: 1.35–6.28) and less likely in carcass swabs (OR: 0.1, 95% CI: 0.02–0.4) compared to sheep feces (Table 2). Co-detection was significantly higher in winter (21.2%) and summer (17.9%) seasons compared to spring (12.6%) and fall (10.8%) seasons ($P = 0.0017$) (data not shown). Co-detection was less likely in the fall season (OR: 0.3, 95% CI: 0.18–0.54) compared to the winter season (Table 2).

4.4. Serotypes of Salmonella

Serotypes of 167 selected *Salmonella* isolates were determined using WGS, and a total of 24 different *Salmonella enterica* serovars were identified (Table 5). The five most frequently isolated serotypes among sequenced isolates ($n = 167$) were *S.* Agona (19.8%, $n = 33$), *S. Typhimurium* (16.2%, $n = 27$), *S.* Cannstatt (13.2%, $n = 22$), *S.* Reading (13.2%, n = 22), and *S.* Anatum (9.6%, n = 16). A total of twelve different serotypes were detected both in sheep and abattoir environment samples, including those mentioned above and *S.* Give, *S.* Adelaide, *S. Infantis*, *S.* Newport, *S.* Derby, *S.* I4:f,g,s:1,5 and *S.* Muenster. The monophasic variant of *S. Typhimurium* (I4,[5],12:i:-) was found in two isolates (1.2%) and both were recovered from environmental samples (lairage and soil samples). Details of the breakdown of these and the remaining serotypes are described in Table 5.

4.5. Antimicrobial susceptibility of ESBL E. coli in sheep and the abattoir environment

A total of 44 different AMR profiles were detected in ESBL *E. coli* isolates ($n = 318$) from the sheep and their abattoir environment, and predominantly (97.5%; 310/318) were MDR. The majority (83%, 264/318) of the ESBL *E. coli* was resistant to seven or more antimicrobials. The top five resistance profiles detected are shown in Table 6.

All tested ESBL *E. coli* isolates were resistant to Ampicillin and Ceftriaxone. The higher percentage of resistance was exhibited to Ceftiofur (99.7%, 317/318) followed by Tetracycline (96.2%, 306/318), Sulfisoxazole (85.8%), Streptomycin (79.9%), Chloramphenicol (77.7%), Azithromycin (36.5%), Trimethoprim/Sulfamethoxazole (28.6%), Gentamicin (16.4%), Nalidixic acid (11.0%), Ciprofloxacin (8.5%), Amoxicillin/Clavulanic acid (5.0%) and Cefoxitin (4.7%) (Table 7).

4.6. Sample type and season were associated with AMR in ESBL E. coli

Similar AMR patterns were detected in both sheep and environmental samples (Table 6). Proportions of ESBL *E. coli* isolates from sheep samples ($n = 152$) and environmental samples ($n = 166$) were compared for resistance to antimicrobials. There was no statistically significant difference in percent resistance between sheep, and environmental ESBL *E. coli* isolates to any of the antimicrobials tested ($P > 0.05$) (Table 7). However, a statistically significant difference was detected in percent resistance among isolates from sheep samples to Streptomycin ($P = 0.0379$) with the highest percentage in isolates from carcass swabs (100%) and to Sulfisoxazole ($P = 0.0387$) with the highest percentage in isolates from abattoir resting area (100%) and carcass swabs (96%). Among isolates from the environmental samples, percent resistance to Gentamicin ($P = 0.0275$) was significantly different with higher percentages in isolates from water (30.8%) and soil samples (24.5%) (Table 7).

Eighteen out of the 44 AMR patterns (40.9%) of ESBL *E. coli* were detected in at least two seasons. Seasonal variation in percent resistances of ESBL *E. coli* is shown in Tables 6 and 8. The percent resistance observed for Azithromycin and Trimethoprim/Sulfamethoxazole were significantly higher in spring ($P < 0.05$). Percent resistance was significantly higher in winter for Ciprofloxacin and Nalidixic acid and in summer for Gentamicin than in the rest of the seasons ($P < 0.05$). The percent resistance of ESBL *E. coli* for Sulfisoxazole (76.7%) was significantly lower in spring than in the rest of the seasons ($P = 0.0297$) (Table 8). There was no statistically significant difference between seasons in percent resistance of ESBL *E. coli* isolates for the remaining

Table 5
Percentage of serotypes of *Salmonella* in sheep and environmental samples based on SeqSero2 v.1.1 serotyping using WGS of 167 selected isolates.

Total number of isolates		Number of isolates from sheep samples					Number of isolates from environmental samples				
Salmonella serotypes	N (%)	Carcass swabs	Cecal contents	Sheep feces	Abattoir resting area feces	N (%)	Lairage samples	Soil swabs	Feed samples	Water samples	Total
Agona	33 (19.8)	0	1	2	1	4 (6.3)	13	13	3	0	29 (28.2)
Typhimurium	27 (16.2)	2	5	7	3	17 (26.6)	2	6	1	1	10 (9.7)
Cannstatt	22 (13.2)	0	2	3	1	6 (9.4)	5	5	3	3	16 (15.5)
Reading	22 (13.2)	2	3	3	5	13 (20.3)	3	4	0	2	9 (8.7)
Anatum	16 (9.6)	0	0	0	3	3 (4.7)	7	3	3	0	13 (12.6)
Give	7 (4.2)	0	0	1	0	1 (1.6)	4	2	0	0	6 (5.8)
Adelaide	5 (3.0)	0	0	1	0	1 (1.6)	0	1	3	0	4 (3.9)
Sundsvall	5 (3.0)	1	1	3	0	5 (7.8)	0	0	0	0	0 (0.0)
Infantis	4 (2.4)	0	2	1	0	3 (4.7)	0	0	1	0	1 (1.0)
Newport	4 (2.4)	0	1	2	0	3 (4.7)	0	1	0	0	1 (1.0)
Cerro	2 (1.2)	0	0	0	0	0 (0.0)	2	0	0	0	2 (1.9)
Derby	2 (1.2)	0	1	0	0	1 (1.6)	0	0	1	0	1 (1.0)
I4,[5],12:i:-	2 (1.2)	0	0	0	0	0 (0.0)	1	1	0	0	2 (1.9)
I4:f,g,s:1,5	2 (1.2)	0	0	0	1	1 (1.6)	1	0	0	0	1 (1.0)
IIIb 61:k:1,5,(7)	2 (1.2)	1	1	0	0	2 (3.1)	0	0	0	0	0 (0.0)
Meleagridis	2 (1.2)	0	0	0	0	0 (0.0)	0	2	0	0	2 (1.9)
Muenster	2 (1.2)	0	0	1	0	1 (1.6)	0	1	0	0	1 (1.0)
Senftenberg	2 (1.2)	0	1	1	0	2 (3.1)	0	0	0	0	0 (0.0)
Altona	1 (0.6)	0	0	0	0	0 (0.0)	1	0	0	0	1 (1.0)
Enteritidis	1 (0.6)	0	0	1	0	1 (1.6)	0	0	0	0	0 (0.0)
Havana	1 (0.6)	0	0	0	0	0 (0.0)	1	0	0	0	1 (1.0)
Kentucky	1 (0.6)	0	0	0	0	0 (0.0)	1	0	0	0	1 (1.0)
London	1 (0.6)	0	0	0	0	0 (0.0)	1	0	0	0	1 (1.0)
Mbandaka	1 (0.6)	0	0	0	0	0 (0.0)	0	0	1	0	1 (1.0)
Total	167 (100)	6	18	26	14	64 (100)	42	39	16	6	103 (100.0)

Table 6
Top five AMR profiles of ESBL *E. coli* and *Salmonella* isolates from sheep and environmental samples.

Resistance profiles[a]	n	%	Serotypes (number of isolates)
Salmonella (N = 368)			
TET*	21	5.7	Reading (11), Agona (2), Cerro (1), Mbandaka (1) Meleagridis (1), not-serotyped (5)
FIS-TET**	18	4.9	Agona (14), I4:f,g,s:1,5 (2), not-serotyped (2)
AMP-CHL-FIS-STR-TET**	17	4.6	Anatum (14), Cannstatt (1), not-serotyped (2)
FIS-STR-TET*	13	3.5	Agona (12), not-serotyped (1)
STR*	4	1.1	Give (2), Typhimurium (1), Cannstatt (1)
ESBL *E. coli* (N = 318)			
AM-AXO-CHL-FIS-STR-TET-XNL*	75	23.6	
AMP-AXO-AZI-CHL-FIS-STR-SXT-TET-XNL*	41	12.9	
AMP-AXO-AZI-CHL-FIS- STR-TET-XNL*	37	11.6	
AMP-AXO-FIS-GEN-STR- TET-XNL*	25	7.9	
AMP-AXO-AZI-CHL-FIS- SXT-TET-XNL*	18	5.7	

N = total number of isolates tested, n = number of isolates with the specific type of phenotypic resistance, AMP = Ampicillin, AUG = Amoxicillin/Clavulanic acid, AXO = Ceftriaxone, AZI = Azithromycin, CHL = Chloramphenicol, CIP = Ciprofloxacin, FIS = Sulfisoxazole, FOX = Cefoxitin, GEN = Gentamicin, NAL = Nalidixic Acid, STR = Streptomycin, SXT = Trimethoprim/Sulfamethoxazole, TET = Tetracycline and XNL = Ceftiofur. All displayed resistance profiles were detected both in isolates from sheep and abattoir environment samples. Not-serotyped = *Salmonella* isolates that were not sequenced.

[a] Single and double asterisk indicate resistance profiles detected in at least three seasons and those detected only in fall and winter seasons, respectively.

eight antimicrobials ($P > 0.05$) (Table 8).

4.7. Antimicrobial susceptibility of Salmonella in sheep and the environment

The highest percentage of AMR among all *Salmonella* isolates was observed for Tetracycline (20.9%, 77/368) followed by Sulfisoxazole (15.2%, 56/368), Streptomycin (11.4%, 42/368), Ampicillin (6.0%, 22/368), and Chloramphenicol (5.4%, 20/368) (Table 9).

Percent resistance to each of Tetracycline, Sulfisoxazole, Streptomycin, and Ampicillin was significantly higher for isolates from environmental samples compared to those from sheep source samples ($P < 0.05$) (Table 9). Among the serotyped *Salmonella* isolates ($n = 167$), the pentaresistant pattern (resistance to Ampicillin, Chloramphenicol, Streptomycin, Sulfonamide, and Tetracycline) was observed in 14 (87.5%) *S.* Anatum, one (4.6%) *S.* Cannstatt and two (1.0%) not-serotyped isolates (Table 6). In addition, other phenotypes of MDR were detected in *S.* Agona (39.4%, $n = 13$), *S. Infantis* (75.0%, $n = 3$), monophasic variant of *S. Typhimurium* (*S.* I4,[5],12:i:-) (100.0%, $n = 2$), *S.* Meleagridis (50%, n = 1) and one not-serotyped isolate (0.5%) (Table 10). All of the MDR *Salmonella* were resistant to at least FIS-STR-TET profile (Table 10). All three of the MDR *S. Infantis* isolates were resistant to Nalidixic acid. Two of them were also resistant to Chloramphenicol, and one topped them with resistance to Azithromycin, Gentamicin, and Trimethoprim/Sulfamethoxazole. One *S.* Agona was additionally resistant to Ampicillin, Ceftiofur, Amoxicillin/Clavulanic acid, and Trimethoprim/Sulfamethoxazole. One *S.* Meleagridis and two *S.* I4,[5],12:i:- were additionally resistant to Chloramphenicol and Ampicillin, respectively. All *S. Typhimurium* isolates detected were pansusceptible and the remaining isolates were either pansusceptible or resistant to one or two antimicrobials (Table 10). All isolates were susceptible to Cefoxitin, Ceftriaxone, and Ciprofloxacin (Table 10). Almost all (189/201), 94.0%) of the not-serotyped (not sequenced) isolates were pansusceptible.

Table 7
Percent resistance of ESBL *E. coli* from sheep and environmental samples.

Antimicrobials[a]	Sheep samples					Environmental samples					All samples
	SF	CC	CS	RF	Total[b]	SS	LS	FS	WS	Total[c]	
	n = 60	*n* = 49	n = 25	*n* = 18	n = 152	*n* = 53	*n* = 79	*n* = 21	n = 13	n = 166	N = 318
Amoxicillin/Clavulanic Acid	3.3	4.1	8.0	5.6	4.6	1.9	5.1	9.5	15.4	5.4	5.0
Ampicillin	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Azithromycin	51.7	32.7	32.0	38.9	40.8	20.8	38.0	47.6	23.1	32.5	36.5
Cefoxitin	3.3	4.1	8.0	5.6	4.6	1.9	5.1	4.8	15.4	4.8	4.7
Ceftiofur	100.0	100.0	100.0	100.0	100.0	100.0	98.7	100.0	100.0	99.4	99.7
Ceftriaxone	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Chloramphenicol	80.0	65.3	92.0	83.3	77.6	79.3	74.7	85.7	76.9	77.7	77.7
Ciprofloxacin	6.7	4.1	12.0	0.0	5.9	11.3	8.9	19.1	7.7	10.8	8.5
Gentamicin*	18.3	20.4	8.0	16.7	17.1	24.5	8.9	9.5	30.8	15.7	16.4
Nalidixic acid	11.7	8.2	24.0	0.0	11.2	13.2	7.6	19.1	7.7	10.8	11.0
Streptomycin**	78.3	79.6	100.0	77.8	82.2	71.7	79.8	85.7	76.9	77.7	79.9
Sulfisoxazole**	88.3	77.6	96.0	100.0	87.5	88.7	79.8	95.2	76.9	84.3	85.8
Tetracycline	93.3	89.8	100.0	100.0	94.1	98.1	97.5	100.0	100.0	98.2	96.2
Trimethoprim/Sulfamethoxazole	30.0	20.4	36.0	27.8	27.6	18.9	32.9	38.1	38.5	29.5	28.6

SF = sheep feces, CC = cecal contents, CS = carcass swab, RF = Abattoir resting area feces, SS = soil sample, LS = lairage swab, FS = feed sample, WS = water sample, n = number of samples, N = total number of samples; values presented in the table indicate percent resistance of ESBL *E. coli*.
[a]Antimicrobial with single asterisk indicates statistically significant difference in percent resistance of isolates among environmental samples ($P = 0.0275$); antimicrobial with double asterisk indicates statistically significant difference in percent resistance of isolates among sheep samples ($P < 0.05$). Chi-square or Fishers' exact tests were used to compare frequencies.
[b,c]No statistically significant difference was detected in total percent resistance of isolates between sheep and environmental samples for each antimicrobial ($P > 0.05$). Contingency table of each antimicrobial was used for test of independence in percent resistance of ESBL *E. coli* from sheep and environmental samples using.

Table 8
Seasonal percent resistance of ESBL *E. coli* and Salmonella from sheep and environmental samples.

Type of bacteria (number of isolates tested)	Resistant to antimicrobials[a]	Seasons				*P* value
		Spring	Summer	Fall	Winter	
		n = 86	*n* = 96	*n* = 48	*n* = 88	
ESBL *E. coli* (N = 318)	Azithromycin	57.0	29.2	22.9	31.8	<0.0001
	Ciprofloxacin	1.2	5.2	2.1	22.7	<0.0001
	Gentamicin	3.5	31.3	8.3	17.1	<0.0001
	Nalidixic acid	8.1	6.3	2.1	23.9	<0.0001
	Sulfisoxazole	76.7	87.5	87.5	92.1	0.0297
	Trimethoprim/Sulfamethoxazole	40.7	20.8	16.7	31.8	0.0051
		n = 32	*n* = 118	*n* = 127	*n* = 91	
Salmonella (N = 368)	Ampicillin	6.3	0	3.9	16.5	<0.0001
	Chloramphenicol	3.1	0.9	2.4	16.5	<0.0001
	Nalidixic acid	6.3	1.7	0	0	0.0131
	Streptomycin	25.0	8.5	5.5	18.7	0.0011
	Sulfisoxazole	21.9	5.9	8.7	34.1	<0.0001
	Tetracycline	25.0	6.8	9.5	53.9	<0.0001
	At least to one antimicrobial	37.5	8.5	10.2	53.9	<0.0001
	MDR	21.9	5.9	4.7	18.7	0.0003

Chi-square or Fishers' exact tests were used to compare frequencies presented in the table as percentages.
[a] Only antimicrobials with percent resistance that significantly differed between seasons presented; values in the tables indicate percent resistance; MDR = Multidrug resistant.

A total of 14 different AMR profiles were detected in the 368 *Salmonella* isolates. The top five AMR profiles are shown on Table 6. A majority (77.2%, 284/368) of the isolates were susceptible to all antimicrobials tested. A total of 37(10.1%) *Salmonella* isolates were MDR. The most common MDR resistance profiles were AMP-CHL-FIS-STR-TET (4.6%, 17/368) and FIS-STR-TET (3.5%, 13/368) (Table 6). From these, one isolate from soil (*S.* Agona) and another from cecal content (*S. Infantis*) were resistant to seven and eight antimicrobials, respectively. We observed five (35.7%) similar AMR patterns among isolates from sheep and the environmental samples (Table 6).

4.8. Sample type and season were associated with AMR in Salmonella

Percent resistance detected to antimicrobials was compared for *Salmonella* isolates from sheep samples ($n = 140$) and environmental samples ($n = 228$) (Table 9). Percent resistance to at least one antimicrobial was significantly higher in environmental isolates (28.1%, 64/228) than in sheep isolates (14.3%, 20/140) ($P = 0.0022$). This was particularly observed for Ampicillin (8.8% vs. 1.4%), Streptomycin (15.8% vs. 4.3%), Sulfisoxazole (20.6% vs. 6.4%) and Tetracycline (25.9% vs. 12.9%) ($P < 0.05$), respectively (Table 9). There was no statistically significant difference in percent resistance among isolates from the two sources for the remaining ten antimicrobials in the panel ($P > 0.05$) (Table 9). MDR *Salmonella* was detected in 7.9% and 4.4% of isolates from abattoir resting area feces and cecal contents, respectively. MDR *Salmonella* isolates were not detected in sheep feces. All isolates from carcass swabs ($n = 6$) were pansusceptible. Among environmental *Salmonella* isolates, a higher percentage of MDR was detected in those from feed samples (21.4%), followed by lairage swab (14.6%) and soil (13.1%). No MDR isolates were detected in water samples (Table 9).

Similar AMR profiles were detected among seasons. The AMR profile FIS-STR-TET was detected in all seasons (3.5%, 13/368) (Table 6). Percent resistance of *Salmonella* isolates to at least one antimicrobial was significantly higher in spring (37.5%) and winter (53.9%) seasons than

Table 9
Percent resistance of Salmonella isolates from sheep and environmental samples.

Antimicrobials[a]	Sheep samples					Environmental samples					All samples
	SF	CC	CS	RF	Total	SS	LS	FS	WS	Total	
	n = 51	*n* = 45	n = 6	*n* = 38	n = 140	*n* = 84	*n* = 103	*n* = 28	n = 13	n = 228	N = 368
Amoxicillin/Clavulanic Acid	–	–	–	–	–	1.2	–	–	–	0.4	0.3
Ampicillin*	–	–	–	5.3	1.4	7.1	10.7	10.7	–	8.8	6.0
Azithromycin	–	2.2	–	–	0.7	–	–	–	–	–	0.3
Cefoxitin	–	–	–	–	–	–	–	–	–	–	–
Ceftiofur	–	–	–	–	–	1.2	–	–	–	0.4	0.3
Ceftriaxone	–	–	–	–	–	–	–	–	–	–	–
Chloramphenicol	–	4.4	–	5.3	2.9	6.0	7.8	10.7	–	7.0	5.4
Ciprofloxacin	–	–	–	–	–	–	–	–	–	–	–
Gentamicin	–	2.2	–	–	0.7	–	–	–	–	–	0.3
Nalidixic acid	2.0	4.4	–	–	2.1	–	–	3.6	–	0.4	1.1
Streptomycin*	–	6.7	–	7.9	4.3	15.5	16.5	21.4	–	15.8	11.4
Sulfisoxazole*	3.9	6.7	–	10.5	6.4	21.4	21.4	25.0	–	20.6	15.2
Tetracycline*	11.8	13.3	–	15.8	12.9	25.0	27.2	28.6	15.4	25.9	20.9
Trimethoprim/Sulfamethoxazole	–	2.2	–	–	0.7	1.2	–	–	–	0.4	0.5
Resistant*	13.7	15.6	–	15.8	14.3	27.4	30.1	28.6	15.4	28.1	22.8
MDR*	–	4.4	–	7.9	3.6	13.1	14.6	21.4	–	14.0	10.1

[a] An asterisk indicates statistically significant difference in percent resistance for antimicrobials between isolates from sheep and environmental samples ($P < 0.05$); values in the tables represent percent resistance; zero values are replaced with dashes (–) for clarity. Resistant = resistant to at least one antimicrobial; MDR = Multidrug resistant.

Table 10
Percent resistance of serotypes of Salmonella in sheep and environmental samples.

Salmonella serotypes	Number (% resistant)	AUG	AMP	AZI	FOX	XNL	AXO	CHL	CIP	GEN	NAL	STR	FIS	TET	SXT	MDR
Agona	33 (90.9)	3.0	3.0	–	–	3.0	–	–	–	–	–	42.4	84.9	90.9	3.0	39.4
Typhimurium	27 (3.7)	–	–	–	–	–	–	–	–	–	–	3.7	–	–	–	–
Cannstatt	22 (9.1)	–	4.6	–	–	–	–	4.6	–	–	–	9.1	4.6	4.6	–	4.6
Reading	22 (50.0)	–	–	–	–	–	–	–	–	–	–	–	–	50.0	–	–
Anatum	16 (87.5)	–	87.5	–	–	–	–	87.5	–	–	–	87.5	87.5	87.5	–	87.5
Give	7 (28.6)	–	–	–	–	–	–	–	–	–	–	28.6	–	–	–	–
Adelaide	5 (0.0)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Sundsvall	5 (0.0)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Infantis	4 (75.0)	–	–	25.0	–	–	–	50.0	–	25.0	75.0	75.0	75.0	75.0	25.0	75.0
Newport	4 (0.0)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Cerro	2 (50.0)	–	–	–	–	–	–	–	–	–	–	–	–	50.0	–	–
Derby	2 (0.0)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
I4,[5],12:i:-	2 (100.0)	–	100.0	–	–	–	–	–	–	–	–	100.0	100.0	100.0	–	100.0
I4:f,g,s:1,5	2 (100.0)	–	–	–	–	–	–	–	–	–	–	–	100.0	100.0	–	–
IIIb 61:k:1,5,(7)	2 (0.0)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Meleagridis	2 (100.0)	–	–	–	–	–	–	50.0	–	–	–	50.0	50.0	100.0	–	50.0
Muenster	2 (0.0)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Senftenberg	2 (0.0)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Altona	1 (0.0)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Enteritidis	1 (100.0)	–	–	–	–	–	–	–	–	–	100.0	–	–	–	–	–
Havana	1 (0.0)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Kentucky	1 (0.0)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
London	1 (0.0)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Mbandaka	1 (100.0	–	–	–	–	–	–	–	–	–	–	–	–	100.0	–	–
Not-serotyped	201 (6.0)	–	2.0	–	–	–	–	1.0	–	–	–	1.5	2.5	5.0	–	1.5
Total	368 (22.8)	0.3	6.0	0.3	–	0.3	–	5.4	–	0.3	1.1	11.4	15.2	20.9	0.5	10.1

AMP = Ampicillin, AUG = Amoxicillin/Clavulanic acid, AXO = Ceftriaxone, AZI = Azithromycin, CHL = Chloramphenicol, CIP = Ciprofloxacin, FIS = Sulfisoxazole, FOX = Cefoxitin, GEN = Gentamicin, NAL = Nalidixic Acid, STR = Streptomycin, SXT = Trimethoprim/Sulfamethoxazole, TET = Tetracycline and XNL = Ceftiofur, MDR = Multidrug resistant. Not-serotyped = *Salmonella* isolates that were not sequenced.

in summer (8.5%) and fall (10.2%) seasons ($P < 0.0001$) (Table 8). We detected a significantly higher percent resistance in winter for Ampicillin, Chloramphenicol, Sulfisoxazole, and Tetracycline and in spring and winter for Streptomycin compared to the other seasons ($P < 0.05$). Nalidixic acid resistance was significantly higher in the spring season ($P = 0.0131$) and not detected in the fall and winter seasons (Table 8).

4.9. Co-detection of ESBL E. coli in the same sample was associated with percent resistance of Salmonella isolates to the pentaresistant pattern of antimicrobials

Percent resistance of *Salmonella* (58/179, 32.4%) was significantly higher in isolates recovered from samples from which both ESBL *E. coli* and *Salmonella* co-detected ($P < 0.0001$) compared to those recovered from samples with no detection of ESBL *E. coli* (26/189, 13.8%) (Table 11). The odds of acquiring resistance to at least one antimicrobial by *Salmonella* co-detected with ESBL *E. coli* in same sample was 3 times that of those without co-detection (OR: 3; 95% CI 1.8–5.0). This phenomenon was particularly observed for *Salmonella* isolates resistant to pentaresistant antimicrobials (Ampicillin, Chloramphenicol, Streptomycin, Sulfisoxazole, and Tetracycline) (Table 11). There was no statistically significant difference in percent resistance of *Salmonella* due to the co-existence of ESBL *E. coli* in the same samples for the rest of the antimicrobials tested ($P > 0.05$).

Table 11
Association of percent resistance to antimicrobials in Salmonella with co-detection of ESBL E coli in same sheep and environmental samples.

Antimicrobials	Number (%) of *Salmonella* isolates co-detected with ESBL *E. coli* (n = 179)	Number (%) of *Salmonella* isolates without co-detection of ESBL *E. coli* (n = 189)	*P*-value	OR (OR 95% CI)
Ampicillin	20 (11.2)	2 (1.1)	<0.0001	11.8 (2.7–51.1)
Chloramphenicol	18 (10.1)	2 (1.1)	0.0001	10.4 (2.4–45.7)
Streptomycin	33 (18.4)	9 (4.8)	<0.0001	4.5 (2.1–9.8)
Sulfisoxazole	41 (22.9)	15 (7.9)	<0.0001	3.4 (1.8–6.5)
Tetracycline	53 (29.6)	24 (12.7)	<0.0001	2.9 (1.7–4.9)
Resistant to at least one antimicrobial	58 (32.4)	26 (13.8)	<0.0001	3.0 (1.8–5.0)

Numbers in brackets are percentages of *Salmonella* detected with and without co-detection of ESBL *E. coli* in same sample. Only antimicrobials which showed significant association are listed. *P*-values are either from Chi-square or Fishers exact test.

5. Discussion

There is a paucity of literature on foodborne pathogens from small ruminants in the U.S., and existing information primarily focuses on the fecal prevalence of STEC and *Salmonella*, emphasizing contamination of carcasses and retail meat. MDR and/or ESBL producing *E. coli* are increasingly reported from other parts of the world in sheep reared at rangeland, feedlot, and at slaughter facilities (Dsani et al., 2020; Müller et al., 2016; Pehlivanoglu et al., 2016; Shabana and Al-Enazi, 2020; Snow et al., 2011). In a year-round serial cross-sectional study, we detected wide dissemination of ESBL *E. coli* and *Salmonella* in an abattoir environment and a relatively lower prevalence in sheep samples. The prevalence of both organisms varies by sample type and seasons of the year in our study.

To our knowledge, this is the first report of ESBL *E. coli* from sheep and their abattoir environment in the U.S. In this study, the overall prevalence of ESBL *E. coli* was significantly higher in an abattoir environment than sheep source samples ($P < 0.0001$). Prevalence of ESBL *E. coli* in sheep feces, cecal contents, and abattoir resting area were comparable (20.0% to 27.3%) but higher than previously reported proportions in feces of sheep in Switzerland (6.9%, 4/58) and Tunisia (0%, 0/23) (Ben Sallem et al., 2012; Geser et al., 2012). This was also higher than the fecal prevalence of ESBL *E. coli* in cattle in the U.S. (6%, 3/50) and Switzerland (13.7%, 17/124) (Geser et al., 2012; Wittum et al., 2010). In a recent report in Spain, the fecal prevalence of ESBL-/AmpC-producing *E. coli* (7.0%, 8/114) in healthy sheep flock was comparable to beef cattle herds (9.6%, 10/104) but lower than in dairy cattle herds (32.9%, 27/82) (Tello et al., 2020). These findings indicated that sheep may play a significant role as a reservoir of ESBL *E. coli* as are cattle in the U.S. We detected a relatively lower percent (10.2%) of ESBL *E. coli* in the post-evisceration sheep carcass samples compared to a study reported from Brazil where the ESBL *E. coli* prevalence was 60% in retail sheep meat although it was from a limited number of samples (n = 25) (Gozi et al., 2021). The lower prevalence in our samples may be due to efficient sanitary dressing procedures followed in U.S. abattoirs (Schmidt et al., 2015). Moreover, we noticed the application of diluted lactic acid on carcasses before transferring them to chilling room in the abattoir we studied, which may further reduce bacterial load on carcasses (Loretz et al., 2010).

The overall prevalence of ESBL *E. coli* in sheep samples was significantly higher in warmer seasons (spring and summer) compared to colder seasons (fall and winter). This is in agreement with the study where the *E. coli* O157:H7 prevalence in cattle fecal samples in the U.S. was higher in the warmer season (Barkocy-Gallagher et al., 2003). Seasonal fecal carriage of ESBL pathogens had been reported in the Netherlands, with higher rates in warmer months (Wielders et al., 2020). A persistent and relatively higher percentage of ESBL *E. coli* contamination was observed in environmental samples ranging from about 30% in fall to 68% in winter seasons. Among environmental samples, lairage swabs and soil samples were more contaminated with ESBL *E. coli* compared to water and feed. The higher environmental prevalence may be due to co-existence of other ruminants (cattle and goats) at the same time in the abattoir resting area and grazing lots. The role of cattle and goat in the dissemination of these organisms were not evaluated in this study. However, previously Small et al. (2002) reported that cattle lairage environments were more contaminated than sheep lairage environments (Small et al., 2002). A higher prevalence of *Salmonella*, *E. coli* O157 and *Campylobacter* spp. was reported in fecal samples collected from mixed pens compared to those from species segregated pens (Hanlon et al., 2018).

In this study, detection of *Salmonella* was more likely in environmental samples (such as soil and lairage swabs) compared to sheep source samples. The role of abattoir lairage environments in the dissemination of *Salmonella* and other pathogens had been previously reported (Small et al., 2002). A higher prevalence of *Salmonella* in environmental samples than in pigs was also previously reported (Keelara et al., 2013). Sheep fecal and cecal prevalence of *Salmonella* was 23.2% and 20.1%, respectively. These results were comparable to individual fecal *Salmonella* prevalence of 24.5% (n = 2589) in sheep reported by the National Animal Health Monitoring System (NAHMS) from a survey in 22 states (Dargatz et al., 2015). *Salmonella* was higher in abattoir resting area feces (42.2%) compared to post-evisceration sheep feces in our study. A comparable fecal prevalence of *Salmonella* (42%, n = 50) was reported in mixed pens (sheep and goat), and lower rates (<12%) were reported from individual animals in the same study (Hanlon et al., 2018). This could be due to contamination from lairage and other abattoir environments as previously suggested in pigs (Dorr et al., 2009). Among environmental samples, the soil had a higher percentage of *Salmonella* contamination (93.3%), followed by lairage swab (85.8%). In the studied abattoir, we observed mixing of sheep, goat, and cattle from different sources, lack of decontamination measures at the grazing environment, and long duration of stay in the abattoir resting area (up to three days), all of which may have contributed to the persistence of *Salmonella* in the environment, facilitating exchange of bacteria between the environment and animals. Previous studies observed a relatively higher *Salmonella* prevalence in cattle feces at U.S. processing plants (44.6%) (Schmidt et al., 2015). We found a low prevalence of *Salmonella* (2.4%) in sheep carcasses as other studies reported in the U.S. commercial processing plant (4.3%, n = 851) (Kalchayanand et al., 2007). In the report, they described that an inverted dressing system would help in reducing carcass contamination. However, in the abattoir, we studied carcasses hung by hindlegs during pelt removal to final transfer to the cold room. Hence, the low prevalence detected, and low odds of carcass contamination could be due to hygienic steps observed in the abattoir, which include washing of grossly soiled body parts and limbs before flaying, careful removal of skin, frequent cleaning and washing of the abattoir floor, removal of contaminated pieces, and hot water cleaning of hands and knives between each carcass among other common measures.

Seasonal variations were detected in the prevalence of *Salmonella* in both sheep and environmental samples, with higher prevalence recorded in summer and fall seasons. This is in agreement with a previous report describing highest contamination on cattle hides and pre-evisceration carcasses during those seasons (Barkocy-Gallagher et al., 2003). Seasonality had been previously observed on human salmonellosis, with higher rates recorded from June to September (Lal et al.,

2012).

We detected 24 different serotypes of *Salmonella* in sequenced isolates from sheep and environmental samples. This is in contrast to the previous study that reported fewer serotypes, including *S.* Arizona (87.1%, 27/31), the remaining being one *S. Typhimurium* and three unknown serotypes (Oloya et al., 2007). In the NAHMS sheep study, nine different serotypes were reported in sheep/sheep feces, with nearly all of them being serotype IIIb 61:-:1,5,7 (94%, 948/1008) (Dargatz et al., 2015). In our study, the relatively common serotypes in sheep feces were *S. Typhimurium*, *S*. Reading, *S*. Cannstatt, *S*. Sundsvall and *S*. Agona. *S.* IIIb 61:k:1,5,(7) was detected only in two isolates from cecal content and carcass swab.

We detected co-presence of *Salmonella* and ESBL *E. coli* in the same samples in all sample types, and this was more likely in soil, lairage environment, and animal feed compared to sheep feces and less likely in carcass swabs (<1%). Co-presence of these pathogens was significantly lower in fall compared to winter and summer seasons ($P < 0.05$). However, this could be a preliminary observation and needs further investigation using a large-scale multistate study. No studies have compared ESBL *E. coli* and *Salmonella* co-existence in the same sample from sheep to the author's knowledge.

Infections caused by MDR ESBLs have limited treatment options (CDC, 2019). In our study, highly diversified AMR profiles (44 different profiles) were detected in ESBL *E. coli* recovered from sheep and abattoir environment samples, and nearly all of them (97.5%) were MDR. The majority (83%) of the ESBL *E. coli* were resistant to seven or more antimicrobials. Co-resistance of the ESBL *E. coli* to third-generation Cephalosporin and Quinolones was detected in our study with 8.5% resistant to Ciprofloxacin and 11.0% resistance to Nalidixic acid. This agrees with a recent report of 7.7% quinolone-resistant *E. coli* possessing beta-lactamase genes (CTX-M-2, CTX-M-15, and CMY-2) in feedlot sheep isolates (Gozi et al., 2019). Interestingly, the percent resistance of Ciprofloxacin and Nalidixic acid was highest in feed and carcass swabs, respectively, compared to all other types of samples in our study. Feed could be an important source for spread of Ciprofloxacin resistant ESBL *E. coli* as all animals at the abattoir consume fed from the same feed source supplied on communal troughs. A higher percentage of Ciprofloxacin and Nalidixic acid resistant ESBL *E. coli* in sheep carcasses could be a health risk for workers in the sheep production chain and consumers. Fluoroquinolone-resistant and ESBL producing *E. coli* infections causing pyelonephritis and diarrhea have been reported in the U.S. and Burkina Faso, respectively (Dembélé et al., 2020; Talan et al., 2017).

In this study, there was no significant difference in percent resistance of ESBL *E. coli* based on their source (sheep and environment) ($P > 0.05$), suggesting close interaction between sheep and environmental isolates. This is also supported by the presence of similar AMR patterns between isolates from sheep and environmental samples. A higher proportion of Streptomycin and Sulfisoxazole resistant isolates from carcass compared to sheep samples could be due to dissemination of resistant isolates from cattle or environment that were transferred through sheep pelt or other means. Sheep pelts were previously reported to be more contaminated with STEC and *Salmonella* than pre-evisceration and post-intervention carcasses in the U.S. sheep processing plants (Kalchayanand et al., 2007).

A significant seasonal variation in percent resistance of ESBL *E. coli* was detected to Azithromycin (highest in spring), Ciprofloxacin (highest in winter), Gentamicin (highest in summer), Nalidixic acid (highest in winter), Sulfisoxazole (comparably high in summer, fall and winter) and Trimethoprim/Sulfamethoxazole (highest in spring). Seasonality of AMR has been previously reported and suggested to be linked to antimicrobial use, seasonality of infectious diseases, and geographic areas (Goossens et al., 2005; Martinez et al., 2019; Suda et al., 2014; Sun et al., 2012). Hence, observed seasonal variations of resistance should be interpreted carefully as the sheep and other animals slaughtered in the abattoir are from different farms and exposed to different antimicrobials, husbandry, and geographical region. This information was not made available to us.

Percent resistance of *Salmonella* isolates from environmental samples (28.1%) were significantly higher than those from animal samples (14.3%) ($P < 0.05$). In our study, the majority (86.3%) of *Salmonella* isolates from sheep feces, and cecal contents (84.4%) were pansusceptible. This is in agreement with NAHMS 2011 report that described more than 90% of *Salmonella* isolates from sheep fecal samples were pansusceptible, and resistance was detected only to Tetracycline and Streptomycin (Dargatz et al., 2015). In the NAHMS sheep study, previous use of antimicrobials was reported in most of the farm operations (84.3%, 150/178). In our study, *Salmonella* isolates recovered from sheep feces were resistant to Tetracycline (11.8%), Sulfisoxazole (3.9%), and Nalidixic acid (2.0%). Although the history of antimicrobial use was not obtained in our study, we expect lower exposure in the slaughtered animals as national sale and distribution of medically important antimicrobials declined by about 36% between 2015 and 2019 except for Fluoroquinolones which showed an increase by about 22% (FDA, 2019). Likewise, it was reported that nearly three fourth of *Salmonella* isolates ($n = 716$) recovered from feces of feedlot cattle in the U.S. were pansusceptible, but a higher percent resistance was reported to Tetracycline (21.7%) and Sulfisoxazole (12.4%) and low (<10%) or no resistance to other antimicrobials (Dargatz et al., 2016).

MDR *Salmonella* isolates were not detected in sheep feces, and only two MDR isolates (4.4%) were detected in cecal contents. However, six (15.8%) isolates from abattoir resting area feces were resistant to at least one antimicrobial, and half of them were MDR. This may complement the notion that abattoir resting area feces might be contaminated with resistant isolates in dust particles from the soil (dust), goat feces, and/or cattle feces, or the isolates might have rapidly acquired resistance determinants from the environment (Keelara and Thakur, 2014). In this study, all *Salmonella* isolates recovered from carcass swabs were pansusceptible. A slightly higher but still low *Salmonella* prevalence (3.3%, 95%CI: 0.51–6.05%) was reported in ground beef from a region that included North Carolina (Bosilevac et al., 2009). However, retail meats including sheep, could be contaminated with resistant *Salmonella* in other countries such as China (Yang et al., 2010).

Twelve out of the twenty-four identified serotypes were detected in both sheep and environmental isolates. This might be due to continuous interaction between sheep and environmental isolates as well as the role of the environment in the persistence and dissemination of *Salmonella*, as previously suggested (Keelara et al., 2013). Among these serotypes, four of them (*S. Typhimurium*, *S*. Newport, *S. Enteritidis*, and a monophasic variant of *S. Typhimurium*) were among the five commonly reported serotypes resistance to antimicrobials in the U.S. (CDC, 2021a). Outbreaks associated with *S. Typhimurium* in sheep products were reported in the U.K. (Evans et al., 1999; Perkins, 2018). Only one (3.7%) *S. Typhimurium* isolate was resistant to an antimicrobial (Streptomycin) in this study. However, both isolates of *S*. I4,[5],12:i:- were resistant to four antimicrobials (AMP-FIS-STR-TET). In addition, the pentaresistance phenotype (AMP-CHL-FIS-STR-TET) was exhibited by almost all *S.* Anatum (87.5%) and one *S*. Cannstatt isolates among serotypes isolates. This MDR pattern was previously detected among various serotypes of *Salmonella* that carry Class 1 integrons (Gebreyes et al., 2004) and predominantly reported in *S. Typhimurium* DT104 strains from clinical human and cattle samples (Afema et al., 2015; NARMS, 2014). However, the decline in the prevalence of *S. Typhimurium* with this pentaresistant pattern had been reported (NARMS, 2014). Other MDR *Salmonella* detected in our study included *S.* Agona, *S.* Meleagridis and *S. Infantis*. Among these resistant isolates, *S. Typhimurium*, *S.* Agona, *S.* I4,[5],12:i:-, *S. Infantis*, and *S.* Anatum were reported as a cause of foodborne human salmonellosis in the U.S. (CDC, 2021b).

Co-presence of ESBL *E. coli* with *Salmonella* in the same samples was associated with increased percent resistance in *Salmonella* isolates. This was particularly more evident in isolates resistant to the pentaresistant (AMP-CHL-FIS-STR-TET) antimicrobials. This could be due to horizontal gene transfer between the two pathogens. Previous reports indicated

that many of the AMR genes and mobile genetic elements found in *E. coli* were similar to those found in *Salmonella* (Frye and Jackson, 2013).

The information on farm and market-level management history, geographic source of the animals, health history, prophylactic and therapeutic antimicrobial use, and other feed and water additives were not obtained and are therefore a limitation of the study. Other limitations may include the lack of information on the history of individual animal's health even though USDA experts conducted pre-and post-slaughter inspections and only apparently healthy animals were slaughtered. Husbandry management and duration of stay at the abattoir environment were also not acquired.

In conclusion, our study elaborated that sheep acts as an important reservoir for ESBL *E. coli* and *Salmonella*. ESBL *E. coli* and *Salmonella* were widely disseminated in the abattoir environment, which might play a significant role in the persistence and spread of these organisms. Further molecular analyses of isolates are required to determine the existence of clinically important AMR determinants and clonality between sheep and environmental isolates. Although reports of outbreaks associated with sheep were rare in the past, the gradually increasing demand for their meat products in the U.S. and widespread presence of MDR ESBL *E. coli* and resistant *Salmonella* in sheep and abattoir environment may demand routine surveillance to ensure there is no public health risk.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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