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Nonpathogenic *Escherichia coli* strains as a surrogate for Shiga toxin–producing *E. coli*: Fecal shedding dynamics and pen environmental contamination in weaned calves and feedlot steers

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ABSTRACT

Objective: The purpose of this study was to use nonpathogenic *Escherichia coli* as a surrogate to assess the transmission dynamics of Shiga toxin–producing *E. coli* in cattle.

Materials and Methods: Two studies were performed. For the first study, 3 nonpathogenic *E. coli* strains were orally inoculated into 12 weaned Holsteins calves. Calves were sampled daily the first week and 3 times a week for the following 4 wk. The nonpathogenic *E. coli* strain with the highest average fecal shedding concentration was chosen for use in the second study. The second study was performed twice over consecutive summers. In each summer, 5 steers were inoculated in a pen of 70 feedlot steers and shedding and transmission were monitored.

Results and Discussion: For the first study, all inoculated strains were shed in the feces of the calves and were detected in the hide, oral, and pen surface samples. During both years of the second study, fecal shedding in the steers was established, as well as transmission of the inoculated strain between steers and into the pen environment. The inoculated strain was also detected in hide surface and oral cavity samples, as well as pen surface, water, and feed samples.

Implications and Applications: These studies provide baseline data on shedding and transmission of a nonpathogenic *E. coli* strain in dairy calves and feedlot steers and detection in the environment. These data will be useful for studying host-to-host and host-to-environment contact structures and to develop a modeling framework for *E. coli* transmission in a commercial feedlot setting.

Key words: *Escherichia coli*, inoculation, feedlot, food safety

The authors have not declared any conflicts of interest.
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INTRODUCTION

Cattle have been recognized as a major reservoir of *Escherichia coli* O157:H7 (Ferens and Hoyde, 2011), a Shiga toxin–producing serotype (**STEC**), which has been of concern to the commercial beef industry. Other STEC have been identified, and cattle have been shown to be a reservoir of STEC, with the prevalence of *E. coli* O157 in individual pens of feedlot cattle ranging from 10 to 28% but occasionally as high as 80% in the summer months (Callaway et al., 2003). Shiga toxin–producing *E. coli* have also been found on the pen floors and hides of cattle, as well as cattle feed, making these important potential environmental sources for STEC transmission between animals (Cobbold and Desmarchelier, 2002; Dodd et al., 2003). Water troughs that have been contaminated with feces are another important environmental source that may contribute to the transmission of STEC (Faith et al., 1996; Hancock et al., 1997; Shere et al., 1998; LeJeune et al., 2001).

Though STEC are important human pathogens with significance to the beef cattle industry, the transmission dynamics of STEC in feedlot cattle are not fully understood (Van Donkersgoed et al., 2001; Turner et al., 2003; Vosough Ahmadi et al., 2006). Current understanding of STEC transmission dynamics is dependent on the longitudinal study of naturally occurring STEC infections in cattle. In studies of naturally occurring STEC, the temporal initiation of infection is unknown as is the initial source. Inoculation studies where the temporal initiation and source is known must be done in a biosafety level 2 setting for biosafety reasons. As such, experiments to evaluate transmission in natural settings that account for the time of onset or exposure, environmental factors, and cattle contact and social dynamics have not been done. A well-characterized and easily detected nonpathogenic *E. coli* surrogate would facilitate research on direct and indirect transmission dynamics and potential identification of control methods for STEC in a non-biosafety-level,

commercial feedlot setting. The use of a surrogate strain could also be used to establish baseline data for host-to-host and host-to-environment contact structures to better study pathogens with an environmental transmission component, such as *E. coli* and *Salmonella*. Our data provide a known initial onset and source as well as a known initially negative environment to collect data on transmission.

Our objective was to evaluate the use of nonpathogenic *E. coli* strains as surrogates for STEC shedding and transmission from a known initial exposure to describe the temporal progression of transmission. First, we describe shedding duration and concentrations of 3 nonpathogenic serotypes of *E. coli* in weaned Holstein calves and their pen environment. Second, we describe the shedding and transmission of one nonpathogenic *E. coli* strain in a 70-head pen research feedlot system as a surrogate for studying STEC transmission.

MATERIALS AND METHODS

All procedures performed in studies involving animals were in accordance with the ethical standards of the Kansas State University College of Veterinary Medicine (IACUC #4065, IBC #1327).

Strain Selection and Classification

Wild strains of *E. coli* that were naturally nalidixic acid (50 μg/mL) resistant (**NalR**) were isolated from a group of commercial feedlot cattle in the summer of 2016. Eight isolates were submitted to the *E. coli* Reference Center at Pennsylvania State University to identify the serotype and verify virulence gene negative status for *estA*, *stx1*, *stx2*, *cnf1*, *cnf2*, *eae*, *K99*, *CS31A*, and *F41*. Rifampicin resistance was induced in virulence gene negative strains by incrementally increasing the concentration of rifampicin in Tryptic soy broth (ThermoFisher) over 10 d up to a concentration of 50 μg/mL. To compare the growth of the NalR parent strain and the nalidixic acid (50 μg/mL) and rifampicin (50 μg/mL) resistant strain (**NalR-RifR**), growth curves were done in triplicate (Supplemental Figures 1A–1C; https://doi.org/10.15232/aas.2021-02260). Nalidixic acid is a quinolone antibacterial, and rifampicin is a macrocyclic antibiotic. Growth was quantitated by optical density readings every 15 min for 7.5 h, and the standard growth curves were then plotted. Strains selected for study 1 were chosen based on the similarity in the growth curves between the parent strain and the dual-resistant strain. The single strain selected for studies 2A and 2B was done based on the highest area under the curve concentration of the inoculated strain shed in the feces of the calves in study 1.

Study 1—Dairy Calf

Calf Arrival and Randomization. Twelve weaned Holstein calves were purchased from a commercial dairy producer in September of 2016 and transported to outdoor pens at the Large Animal Research Center at Kansas State University. Mean calf BW was 134 kg (SD = 19.5 kg). Calves were randomized to 1 of 3 pens based on a random number generator, with 4 calves being assigned to each pen and all calves in each pen assigned one strain of *E. coli* for inoculation. Pens were selected so that there would be no fence-line contact between pens. Calves were fed a commercial calf pelleted feed and provided ad libitum access to long-stem hay. All calves were given a single dose of enrofloxacin (Baytril 100, Bayer Corporation) by s.c. injection at 10 mg/kg the on the second day after arrival. Enrofloxacin was chosen to control bovine respiratory disease risk. Furthermore, it can significantly decrease the concentration of *E. coli* in the feces during peak drug concentrations (Ferguson et al., 2019), thereby increasing likelihood of establishing colonization with the inoculated strain.

Inoculations. Calves were inoculated using an esophageal tube with an average of 6.6×10^8 cfu (95% CI 2.8×10^8 to 1.4×10^9 cfu) of their assigned *E. coli* strain each day for 5 consecutive days. Inoculations were done first thing in the morning, before calves were fed. The first 2 d of inoculations used 10 mL of fresh culture inoculum, and the final 3 d of inoculations were with 10 mL of frozen, premade culture that was thawed just before the inoculations. The inoculum was flushed through the esophageal tube with approximately 300 mL of distilled water. To control cross contamination of strains between groups, Tyvek suits and boot covers were worn and changed between each group. Gloves were changed between each calf.

Sample Collection. Fecal and pen surface samples were taken 1 d before the first day of inoculation to establish that all calves and their pen environment were negative for natural nalidixic acid– and rifampicin-resistant *E. coli*. Following the first day of inoculation, calf and pen surface and water tank samples were taken daily for 6 d. For the following 4 wk, samples were taken 3 times a week. Fecal samples were collected from the rectum, and oral and hide swabs were collected using a Speci-Sponge (Nasco) premoistened with 10 mL of buffered peptone water. Hide samples were collected by swabbing an area of approximately 400 cm^2 (20 cm wide × 20 cm long). Due to the frequency of sampling, the hide sampling sites were rotated between the right shoulder, right ribcage, right hip, left shoulder, left ribcage, or left hip. The sites were rotated between each sampling day in the order just listed, and all calves had the same location sampled on the same day. To control cross contamination of strains between groups and calves, Tyvek suits and boot covers were worn and changed between each group and gloves were changed between each calf.

For each pen on each sampling day, pen surface samples and a water sample from the water trough were collected. Pen surface samples were collected in 3 locations around the water tank. Approximately 10 g of soil was collected from each site using plastic spoons that were scooped into sterile Whirl-Pak bags (Nasco). The water tank area was

selected because it was a high traffic area for the calves and to optimize the probability of detecting the presence and survival of the strains in the environment. For each pen, 10 mL of water was collected from the water trough using a screw-top conical tube.

Studies 2A and 2B—Feedlot

Calf Arrival and Randomization. Seventy Angus/Angus-cross steers were purchased through commercial channels in May of each of the years 2018 (study 2A) and 2019 (study 2B). Steers were housed in an outdoor pen at a commercial research feedlot. The pen measured 38 by 30.5 m, providing approximately 17 m^2 of area per steer. The pen had a centrally located water tank, a 24.5-m-long fence-line bunk, and a 3-m-wide concrete apron behind the bunk and around the water tank (Supplemental Figure 2; https://doi.org/10.15232/aas.2021-02260). Steers were fed a high forage–based diet consisting of ground hay, wet distillers grain, and a mineral pack. Steers were fed a measured ration that was increased as needed by monitoring the feed bunk for consumption. All steers were given a single s.c. injection of enrofloxacin (Baytril 100) at 10 mg/kg the day after arrival to control bovine respiratory disease risk and improve inoculation success, as in study 1. All calves received 2 ear tags for visual identification, as well as a real-time location system ear tag. For both studies, pen surface soil samples were taken before steer arrival and fecal samples were collected from each steer on the day after arrival during tagging and metaphylaxis to determine that the pen environment and each steer were negative for *E. coli* resistant to nalidixic acid and rifampicin.

Inoculations. Five steers were randomly selected for inoculation. These steers were identified with a uniquely colored ear tag to facilitate identification and removal from the group on the days of inoculation. Inoculations were done each day for 5 consecutive days using an esophageal tube. For study 2A, an average concentration of freshly prepared culture was 1×10^9 cfu (95% CI 1.2×10^8 to 1.9×10^9). For study 2B, the average concentration of freshly prepared culture was 2.5×10^9 (95% CI 1.5×10^9 to 3.4×10^9). The 10 mL of inoculum culture was flushed through the esophageal tube with approximately 500 mL of distilled water. Gloves were changed between each steer. Inoculations were done at approximately the same time every morning before the calves were fed.

Timeline. Fecal samples of the 5 selected steers used in the inoculation were collected on d 2 through 5 of inoculation. For study 2A, all steers and their pen surface, water tank, and feed were sampled once a week for 10 wk. For study 2B, all steers and their pen surface, water tank, and feed were sampled once a week for 9 wk.

Sample Collection. Following inoculation, all calves were sampled weekly starting on d 7. From each steer on each sampling day, 3 samples were taken: a fecal sample was collected from the rectum, an oral swab was taken using a Speci-Sponge premoistened with 10 mL of buffered peptone water, and a hide swab was taken using a Speci-Sponge premoistened with 10 mL of buffered peptone water. Hide samples were collected on an area of approximately 400 cm^2 (20 cm wide × 20 cm long) on the right upper hip of the steer. This site was the same during each weekly sampling. Gloves were changed between steers.

Environment samples were collected weekly on the same day as steers were sampled. Three types of pen environment samples were taken during each sampling day: pen surface, water, and feed samples. Pen surface samples were collected in 25 locations distributed systematically around the pen. At each pen surface site, approximately 10 g of soil was collected into sterile Whirl-Pak bags using plastic spoons. Ten locations along the concrete feed apron at the front of the pen and 15 samples equally spaced in a grid fashion throughout the rest of the pen were sampled, including around the water tank (Supplemental Figure 2; https://doi.org/10.15232/aas.2021-02260). These areas were selected to cover the high traffic area around the concrete apron, containing the feed bunk and water trough, and to cover the rest of the pen environment to give an estimate of the strain presence and concentration in the whole pen environment. Three 20-mL samples of the water from the water tank were collected into screw-top 50-mL conical tubes. The trough was stirred between samples, mixing up any residue on the sides and bottom of the trough. Five feed samples, evenly spaced along the bunk, were collected approximately a third of the way through the feeding duration of the calves. Gloves were changed between each sample.

Sample Processing

All samples were placed on ice and transported to the Pre-Harvest Food Safety laboratories at the Kansas State University College of Veterinary Medicine for same-day processing. Samples were initially processed for enumeration of NalR-RifR *E. coli*. Samples that were negative by enumeration were subjected to enrichment.

Fecal Sample Processing. Approximately 1 g of feces was transferred to 9 mL of *E. coli* broth (**EC broth**; ThermoFisher). Samples were then vortexed for 30 s, and 1 mL of solution was diluted in another 9 mL of EC broth. Both dilutions were again vortexed for 30 s before being spiral plated for enumeration. All 1:10 dilutions were incubated for enrichment at 37°C for 6 h, after which sample tubes were transferred to a refrigerator for storage.

Hide and Oral Sample Processing. Liquid contents were squeezed from sponges and transferred to sterile 15-mL sterile glass tubes. Samples were then vortexed for 30 s and spiral plated for enumeration. A total of 1 mL of the sample solution was added to 9 mL of EC broth and

incubated at 37°C for 6 h before being transferred to a refrigerator for storage pending enumeration results.

Pen Soil Surface Sample Processing. Approximately 1 g of pen surface was added to 9 mL of EC broth. Samples were vortexed for 30 s and then spiral plated for enumeration. All sample tubes were incubated for enrichment at 37°C for 6 h and then transferred to a refrigerator for storage pending enumeration results.

Water Sample Processing. The screw-cap conical tubes were vortexed for 30 s, and approximately 5 mL was transferred to a 15-mL sterile empty tube. These tubes were then vortexed for 30 s before being plated. A total of 1 mL of the original sample was added to 9 mL of EC broth and incubated for enrichment at 37°C for 6 h before being transferred to a refrigerator for storage pending enumeration results.

Feed Sample Processing (Studies 2A and 2B Only). Approximately 1 g of feed contents was added to 9 mL of EC broth. Samples were vortexed for 30 s and then spiral plated for enumeration. All sample tubes were incubated for enrichment at 37°C for 6 h and then transferred to a refrigerator for storage pending enumeration results.

A total of 100 μL of all sample suspensions was plated for enumeration onto MacConkey agar containing nalidixic acid (50 μg/mL) and rifampicin (50 μg/mL) using a spiral plater (Eddy Jet V. 1.23, IUL S. A.). Plates were incubated at 37°C for 18 to 24 h. After incubation, plates were counted by overlaying a counting grid on the agar plate that relates the colonies on the plate to the volume deposited in that area. Colonies were counted according to the manufacturer's instructions and guidelines outlined in the *Bacteriological Analytical Manual* (Maturin and Peeler, 2001).

After completion of sample enumeration, if the sample was recorded as no growth, the enrichment of that same sample was retrieved from the refrigerator and vortexed for 30 s and then approximately 10 μL was plated onto new MacConkey agar containing nalidixic acid (50 μg/mL) and rifampicin (50 μg/mL). Plates were incubated for 18 to 24 h at 37°C, and plates were recorded as having "growth" or "no growth" of morphological *E. coli*.

Weather Data (Studies 2A and 2B Only)

Daily temperature and precipitation data were downloaded from the National Oceanic and Atmospheric Administration National Centers for Environmental Information (NOAA, 2021). The data set details maximum and minimum 24-h temperature (in °C) and daily precipitation (in cm) in Manhattan, Kansas.

Statistical Methods

Descriptive statistics for all studies were produced using Microsoft Excel. The R package flux 0.3-0 was used to evaluate area under the curve following the trapezoidal rule for fecal shedding of each calf during study 1.

RESULTS AND DISCUSSION

Strain Selection

Six different O groups were represented in the 8 selected isolates that were submitted to the *E. coli* Reference Center at The Pennsylvania State University. Naturally nalidixic acid–resistant strains selected and adapted to rifampicin for study 1 were *E. coli* O28:H43, *E. coli* O19:H$^+$, and *E. coli* O101:H10. Growth curves, maximum optical density, and mean specific growth rate for the 3 selected strains (NalR-RifR) used in study 1 were similar to their parent strains (NalR; Supplemental Table 1 and Figures 1A–1C; https://doi.org/10.15232/aas.2021-02260), suggesting that the dual antibiotic resistance did not change the growth rate of the strain.

Study 1—Dairy Calves

In study 1, all calves had an enumerable level of the inoculated strain in their feces at least one sample day during the study. On the first day after inoculation (d 1), the average pen-level fecal shedding concentrations was 1.8 $\log_{10}$ cfu/g (O19:H$^+$), 2.9 $\log_{10}$ cfu/g (O101:H10), and 2.4 $\log_{10}$ cfu/g (O28:H43; Supplemental Figure 3; https://doi.org/10.15232/aas.2021-02260). The highest pen-level average fecal shedding for each strain was 3.9 $\log_{10}$ cfu/g (O19:H$^+$, d 6), 4.4 $\log_{10}$ cfu/g (O101:H10, d 4), and 5.2 $\log_{10}$ cfu/g (O28:H43, d 32). Strain O28:H43 yielded the highest concentration in the feces by an individual calf at 5.8 $\log_{10}$ cfu/g on study d 32 (Table 1). Strain O19:H$^+$ and strain O101:H10 were detected in the feces of at least one calf by enumeration on 17 (94%) and 16 (89%) of the sampling points, respectively (Table 2). Strain O28:H43 was enumerable in the feces of at least one calf for all sampling points. Strains O19:H$^+$ and O28:H43 were last enumerable on d 34. Strain O101:H10 was last enumerable in calf feces on d 30 but was positive by enrichment in at least one calf through the end of the study. The average area under the curve was greatest for strain O28:H43 (Table 3).

All strains were detected on hide samples either by enumeration or enrichment. Strain O28:H43 had the highest concentration detected (2.9 $\log_{10}$ cfu/400 cm^2, d 3) on an individual hide sample (Table 1). Strain O19:H$^+$ was enumerable on at least one individual calf hide on 6 (33%) sampling days and was last enumerable on d 30. Strain O101:H10 was detected by enumeration on at least one individual calf hide on 10 (56%) sampling days. Strain O28:H43 was enumerable on at least one individual calf hide sample on 8 (44%) sampling days. Strains O101:H10 and O28:H43 were last enumerable on study d 34.

All strains were detected in oral samples either by enumeration or enrichment. The highest oral concentration detected in an individual oral sample was strain O101:H10 (3.0 $\log_{10}$ cfu/mL, d 4). Strain O19:H$^+$ was enumerable in at least on oral sample on 5 (28%) sampling days, and all 4 oral samples were enumerable on d 3 and 4. Strain O101:

Table 1. Study 1 (dairy calf)—Highest enumerable concentration of the inoculated strains detected in fecal, hide, oral, and pen surface soil samples

	Highest enumerable concentration (sampling day)		
Item	**O19:H⁺**	**O101:H10**	**O28:H43**
Feces (log_{10} cfu/g)	4.46 (d 6)	4.97 (d 4)	5.76 (d 32)
Hide (log_{10} cfu/400 cm^2)	1.85 (d 13)	2.86 (d 2)	2.88 (d 3)
Oral (log_{10} cfu/mL)	2.90 (d 3)	2.95 (d 4)	2.91 (d 3)
Pen surface (log_{10} cfu/g)	4.00 (d 27)	4.56 (d 27)	4.55 (d 27)

H10 was enumerable in at least one oral sample on 10 (56%) sampling days, and all 4 oral samples were enumerable on study d 4 and 13. Strain O28:H43 was enumerable in at least one oral sample on 9 (50%) sampling days. The last day strains O19:H$^+$ and O101:H10 were detected in an oral sample by enumeration was d 27. Strain O28:H43 was enumerable in one oral sample and detected by enrichment in another oral sample on d 34.

All strains were detected in the pen surface soil samples by either enumeration or enrichment. For all strains, the highest concentration detected was on study d 27. All 3 strains were enumerable in the soil samples on 13 (72%) sampling days. All soil samples from strain O19:H$^+$ remained enumerable from study d 23 through the end of the study. All 3 soil samples for strain O101:H10 were enumerable on d 30; however, on d 32 and 34, only 2 of the soil samples were enumerable. All soil samples from strain O28:H43 remained enumerable from study d 27 through the end of the study.

Only 2 water samples were positive during the study period. A water sample of strain O19:H$^+$ was detected positive by enrichment on study d 18, and a water sample of strain O28:H43 was enumerable at a concentration of 1.0 log_{10} cfu/mL on study d 27.

In study 1, we established that 3 verified nonpathogenic *E. coli* strains were able to colonize the gastrointestinal tract of weaned Holstein calves, shed in their feces, and spread to their pen environment. Fecal shedding was established in the calves, though shedding was variable between calves and days. In previous studies of STEC, day-to-day fecal shedding has been noted to be variable (Howe et al., 1976; Cray and Moon, 1995; Sanderson et al., 1999).

Table 2. Study 1 (dairy calf)—Count of positive fecal samples by strain, method, and day[1]

	O19:H⁺		O101:H10		O28:H43	
Study day	**Enumeration**	**Enrichment**	**Enumeration**	**Enrichment**	**Enumeration**	**Enrichment**
1	0	4	3	0	3	1
2	4	—	4	—	2	2
3	4	—	4	—	4	—
4	3	1	4	—	4	—
5	4	—	4	—	4	—
6	3	1	3	1	4	—
9	3	0	4	—	3	1
11	4	—	2	2	2	2
13	2	1	1	3	4	—
16	3	0	3	1	1	3
18	1	2	2	2	4	—
20	2	2	4	—	4	—
23	2	1	2	1	2	2
25	3	1	3	0	1	3
27	1	2	1	1	4	—
30	3	1	1	3	4	—
32	1	2	0	1	4	—
34	1	1	0	3	4	—

[1]For each strain, n = 4. Enumerable positive samples are those defined as positive via spiral plating methods. Enrichment positive samples are those defined as negative via spiral plating methods but positive via 6-h incubations in *Escherichia coli* broth.

Table 3. Study 1 (dairy calf)—Total area under the curve (AUC) data for fecal concentrations of each individual calf, as well as a strain average[1]

Strain	Calf tag number	AUC (log_{10} cfu)	Strain average AUC (log_{10} cfu)
O19:H⁺	2	57.75	77.17
	4	92.38	
	11	81.87	
	175	76.68	
O101:H10	9	74.86	79.52
	12	81.57	
	171	108.10	
	173	54.54	
O28:H43	5	86.91	102.67
	6	103.47	
	7	90.47	
	172	129.83	

[1]Results are shown in total log_{10} cfu for the entire study period.

This variability can be due to the range of shedding seen on an individual basis, as well as the transient nature of colonization of *E. coli* in the gut and the potential for reinfection. Though our inoculated strains established shedding in the calves, their shedding curves were more variable than those seen with calves that are experimentally inoculated with *E. coli* O157 (Sanderson et al., 1999). Our inoculated strains lack the *stx*- and LEE-associated gene communities. Though the functional associations between these genes are only speculated to confer adaptation benefits in the bovine intestinal environment (Arimizu et al., 2019), their absence could have disadvantaged our strains in the bovine hindgut. The variability may also be due to the intermittent shedding interacting with the lower limit of detection for both of our detection methods, spiral plating and EC broth enrichment.

For each calf in study 1, the total area under the curve was used to describe the concentration of fecal shedding and potential environmental transmission. This measure has been used previously to describe the concentration of *E. coli* shedding in calves in challenge studies (Naylor et al., 2007). Strain O28:H43 NalR-RifR was chosen for use in studies 2A and 2B based on the greatest average area under the curve for fecal shedding.

Studies 2A and 2B—Feedlot

Study 2A—2018. In study 2A, 70 steers were enrolled in the study, and 5 were inoculated with *E. coli* O28:H43 NalR-RifR. The mean arrival BW of the steers was 271.7 kg (SD = 19.4 kg). Steers were then sampled once a week for 10 wk (70-d study period). One steer was removed from the study on d 35 due to septic laminitis.

Fecal samples from all steers during initial processing (study d 0) were negative for *E. coli* resistant for both nalidixic acid and rifampicin antibiotics. The feces of 5 randomly selected steers used for the initial inoculations were positive by enrichment for *E. coli* O28:H43 NalR-RifR during the inoculation period (d 2 through 5). The highest concentration of the strain detected in the feces of an individual steer was 3.7 log_{10} cfu/g (d 56, Supplemental Table 2; https://doi.org/10.15232/aas.2021-02260). Feces of at least 1 of the 70 steers in the pen was enumerable for 7 (70%) of the sampling weeks (Table 3). The first week (d 7) yielded the highest number of positive fecal samples detected by enumeration or enrichment with 34 positives (49%). No fecal samples were enumerable or positive by enrichment on d 63, and only 5 fecal samples were positive by enrichment on d 70. Mean shedding count of enumerable samples was 2.6 log_{10} cfu/g of feces.

The average number of positive fecal samples detected throughout the entire study period per steer by either method (enumeration or enrichment) was 2 (Supplemental Table 2; https://doi.org/10.15232/aas.2021-02260). Throughout the entire sampling period, 7 steers were positive in their feces for at least 2 consecutive weeks by either detection method (i.e., positive 1 wk via enumeration and positive 1 wk by enrichment or vice versa). Seventeen steers had at least 2 consecutive weeks in which their fecal samples were positive via enrichment. No steers had fecal samples that were enumerable for 2 consecutive sampling weeks. Seven steers were never fecal positive during the entire study period. Six of these 7 negative steers were enrolled in the entire study period, and 1 was removed on study d 35 because of septic laminitis.

The inoculated strain was detected on the hide samples of the individual steers by both enumeration and enrichment methods. The highest concentration detected on the hide of an individual steer was 2.3 log_{10} cfu/400 cm^2 on study d 28. At least one hide sample was enumerable for 5 (50%) of the sampling weeks (Table 4). Twelve hide samples were positive by enrichment on study d 70.

The inoculated strain was detected in the oral cavities of the individual steers by both enumeration and enrichment methods. The highest concentration detected in an individual steer was 3.1 log_{10} cfu/mL on study d 7. At least one oral sample was positive by enrichment on 4 (40%) sampling weeks (Table 4). The last day an oral sample was positive by either enumeration or enrichment was study d 56.

All pen surface samples taken before steer arrival were negative for *E. coli* resistant to both nalidixic acid and rifampicin antibiotics. After steer inoculation, the inoculated strain was detected in the soil of the pen surface at the first sampling time (study d 7). The highest concentration detected in an individual pen surface sample was 3.79 log_{10} cfu/g on study d 49. At least one pen surface sample was enumerable for 5 wk (50%; Table 5). The highest number of pen surface samples positive by either method occurred on d 42 with 23 (92%) positive samples. Day 49 was the last day a pen surface sample was enumerable with 2 (8%) of the samples positive. The last day a pen surface sample was positive by enrichment was d 56 with one (4%) sample

Table 4. Study 2A sample counts for fecal, hide, and oral samples[1]

Study day	Fecal number (% positive)			Hide number (% positive)			Oral number (% positive)		
	Enumeration	Enrichment	Total positive	Enumeration	Enrichment	Total positive	Enumeration	Enrichment	Total positive
7	4 (6)	30 (43)	34 (49)	0	4 (6)	4 (6)	9 (13)	8 (11)	17 (24)
14	2 (3)	10 (14)	12 (17)	0	24 (34)	24 (34)	2 (3)	3 (4)	5 (7)
21	2 (3)	17 (24)	19 (27)	1 (1)	0	1 (1)	0	6 (9)	6 (9)
28	0	4 (6)	4 (6)	7 (10)	4 (6)	11 (16)	5 (7)	0	5 (7)
35	7 (10)	9 (13)	16 (23)	15 (22)	4 (6)	19 (28)	0	0	0
42	2 (3)	29 (42)	31 (45)	2 (3)	19 (28)	21 (31)	0	0	0
49	2 (3)	24 (35)	26 (38)	0	1 (1)	1 (1)	0	0	0
56	13 (19)	0	13 (19)	4 (6)	15 (22)	19 (28)	4 (6)	15 (22)	19 (28)
63	0	0	0	0	8 (12)	8 (12)	0	0	0
70	0	5 (7)	5 (7)	0	12 (17)	12 (17)	0	0	0

[1]Study 2A (feedlot, 2018)—Number positive (%) in the samples taken from the individual steers by day, sample, and method (n = 70 through d 28, then n = 69). Enumerable positive samples are those defined as positive via spiral plating methods. Enrichment positive samples are those defined as negative via spiral plating methods but positive via 6-h incubations in *Escherichia coli* broth. Total positives are the total number of samples positive via enumeration and enrichment.

Table 5. Study 2A sample counts for pen surface soil samples[1]

Study day	Enumeration number (% positive)	Enrichment number (% positive)	Total positive number (% positive)
7	2 (8)	1 (4)	3 (12)
14	0	1 (4)	1 (4)
21	0	3 (12)	3 (12)
28	4 (16)	3 (12)	7 (28)
35	8 (32)	10 (40)	18 (72)
42	2 (8)	21 (84)	23 (92)
49	2 (8)	6 (24)	8 (32)
56	0	1 (4)	1 (4)
63	0	0	0
70	0	0	0

[1]Study 2A (feedlot, 2018)—Number positive (%) in the pen surface soil samples by day and method (n = 25). Enumerable positive samples are those defined as positive via spiral plating methods. Enrichment positive samples are those defined as negative via spiral plating methods but positive via 6-h incubations in *Escherichia coli* broth. Total positives are the total number of samples positive via enumeration and enrichment.

being positive. No samples were positive via enumeration or enrichment on the last 2 wk of the study.

One water sample of the 3 taken on study d 40 was positive by enrichment. No other water samples were positive via enumeration or enrichment throughout the study.

All feed samples (n = 5) were positive by enrichment on study d 42. No other feed samples were positive via enumeration or enrichment throughout the study.

Local weather data obtained from the National Oceanic and Atmospheric Administration was downloaded for each sampling week, with the week starting on Monday (day of sampling) and ending on Sunday (the day before sampling; NOAA, 2021). The highest and lowest temperature recorded during the study period was 40.6°C (wk 6) and 12.2°C (wk 1; Table 6). The weekly total precipitation ranged from 0 cm (wk 3) to 3.9 cm (wk 2). The total precipitation for the study period was 13.6 cm, with an average weekly precipitation of 1.4 cm.

Study 2B—2019. In study 2B, 70 steers were enrolled in the study, and 5 were inoculated with *E. coli* O28:H43 NalR-RifR. The mean arrival BW of the steers in study 2B was 285.2 kg (SD = 26.7 kg). Steers were then sampled once a week for 9 wk (63-d study period). Two steers were removed from the study due to chronic lameness: one on d 35 and another on d 56.

Fecal samples taken from all steers during initial processing (study d 0) were negative for *E. coli* resistant to both nalidixic acid and rifampicin antibiotics. The feces of the 5 randomly selected steers used for the inoculation were all positive for *E. coli* NalR-RifR via enumeration

throughout the entire inoculation period (d 2 through 5). The concentrations detected in the feces of the 5 steers throughout the inoculation period ranged from 3.7 $\log_{10}$ cfu/g to 5.7 $\log_{10}$ cfu/g (Supplemental Table 3; https://doi.org/10.15232/aas.2021-02260). During the study, the highest concentration detected in a fecal sample was 4.9 $\log_{10}$ cfu/g on study d 7. All steers sampled yielded an enumerable count of the inoculated strain in the feces on d 56 (Table 7). On d 14 and 28, 45 and 44% of the steer fecal samples were enumerable, respectively. The last day an enumerable sample was detected in the feces was d 56, though samples remained positive by enrichment through the last week of the study. Mean shedding count of enumerable samples was 3.5 $\log_{10}$ cfu/g of feces.

All steers at some point in the study were positive in their feces for at least 2 consecutive weeks (Supplemental Table 3; https://doi.org/10.15232/aas.2021-02260). The average number of positive fecal samples detected per calf by either enumeration or enrichment was 6. Thirty-three steers were positive by enrichment for at least 2 consecutive sampling weeks, and 21 steers were positive by enumeration for at least 2 consecutive sampling weeks. Fifty-two steers were positive by either method for at least 3 consecutive sampling weeks. Six steers were positive by either method for 6 consecutive sampling weeks.

The inoculated strain, O28:H43 NalR-RifR, was detected by both enumeration and enrichment methods on individual steer hide samples. Positive hide samples were detected during 6 of the 9 study weeks. The highest concentration detected on an individual hide sample was 3.3 $\log_{10}$ cfu/400 cm^2 on d 14. On d 7, 97% of hide samples were positive, with 64% of samples enumerable (Table 7). On d 21 and 56, 90 and 99% of the hide samples, respectively, were quantifiable. The last day an enumerable sample was detected on the hide of the steers was study d 56, though 97% of samples remained positive by enrichment through the end of the study.

The inoculated strain was detected by both enumeration and enrichment in the oral cavities of the steers. The highest concentration detected in a steer oral sample was 3.5 $\log_{10}$ cfu/mL on study d 42. On d 7, 79% of oral samples were positive, with 43% enumerable (Table 7). On d 56, 100% of oral samples were positive and 97% were enumerable. Oral samples were enumerable during 6 of the 9 study weeks. The last study day enumerable samples were detected in the oral swabs of the steers was d 56, though 49% of samples remained positive via enrichment through the end of the study.

All pen surface samples taken before steer arrival were negative for *E. coli* that was resistant to both nalidixic acid and rifampicin antibiotics. After steer inoculation, the inoculated strain was detected by both enumeration and enrichment methods in the pen surface soil samples. The highest concentration detected in an individual pen surface sample was 3.9 $\log_{10}$ cfu/g on both study d 28 and 56. Enumerable levels were found in the pen surface samples during 6 of the 9 wk, with the highest number of enumerable samples being detected on d 14 (96%; Table 8). All soil samples were positive via either enumeration or enrichment during 5 of the 9 wk. The last study day enumerable samples were detected in the pen surface samples was study d 56, though 100% samples remained positive by enrichment through the end of the study.

Table 6. Study 2A weekly temperature range and total precipitation[1]

Study week	Weekly temperature (°C)		Weekly total precipitation (cm)
	High	Low	
1	37.2	12.2	0.1
2	35.0	13.3	3.9
3	35.6	14.4	0.0
4	37.8	20.0	0.8
5	35.6	16.1	0.3
6	40.6	18.3	1.9
7	38.9	16.7	2.9
8	38.3	18.3	2.6
9	34.4	19.4	0.8
10	32.8	16.7	0.5

[1]Study 2A (feedlot, 2018)—Weekly temperature in Celsius (high and low) and total precipitation in centimeters; data obtained from the National Oceanic and Atmospheric Administration for the study period. Study weeks defined as the 7 d before that week's sampling point.

The inoculated strain was found in the feed samples during 5 of the 9 sampling weeks (Table 8). The highest concentration detected in a feed sample was 2.5 $\log_{10}$ cfu/g on study d 14. The last study day enumerable samples were detected in the feed samples was study d 28, though samples were positive via enrichment during d 49, 56, and 63.

The inoculated strain was detected in the water samples taken from the water tank during 5 wk of the study (Table 8). The highest concentration detected in an individual water sample was 2.0 $\log_{10}$ cfu/mL on study d 56. On d 21 and 56, all water samples taken were enumerable. The last day enumerable samples were detected in the water samples was d 56, though samples remained positive via enrichment through the end of the study period.

Weather data obtained from the National Oceanic and Atmospheric Administration was downloaded for each sampling week, with the week starting on Monday (day of sampling) and ending on Sunday (the day before sampling; NOAA, 2021). The highest and lowest temperatures recorded during the study period were 36.1°C (wk 8) and 5°C (wk 1; Table 9). The weekly total precipitation ranged from 0.9 cm (wk 6) to 16.7 cm (wk 1). The total precipitation for the study period was 59 cm, with an average of 6.6 cm per week.

In both studies 2A and 2B, we established that one verified nonpathogenic *E. coli* strain, O28:H43, was able to

Table 7. Study 2B sample counts for fecal, hide, and oral samples[1]

	Fecal number (% positive)			Hide number (% positive)			Oral number (% positive)		
Study day	Enumeration	Enrichment	Total positive	Enumeration	Enrichment	Total positive	Enumeration	Enrichment	Total positive
7	23 (32)	34 (49)	57 (81)	45 (64)	23 (33)	68 (97)	30 (43)	25 (36)	55 (79)
14	38 (54)	2 (3)	40 (57)	54 (77)	0	54 (77)	41 (59)	0	41 (59)
21	0	12 (17)	12 (17)	63 (90)	5 (7)	68 (97)	15 (21)	50 (71)	65 (92)
28	31 (44)	12 (17)	43 (61)	16 (23)	12 (17)	28 (40)	23 (33)	22 (31)	45(64)
35	0	68 (97)	68 (97)	0	19 (27)	19 (27)	0	27 (39)	27 (39)
42	2 (3)	8 (11)	10 (14)	0	1 (1)	1 (1)	32 (46)	1 (1)	33 (47)
49	4 (6)	61 (88)	65 (94)	7 (10)	18 (26)	25 (36)	0	36 (52)	36 (52)
56	68 (100)	0	68 (100)	67 (99)	1 (1)	68 (100)	66 (97)	2 (3)	68 (100)
63	0	43 (63)	43 (63)	0	66 (97)	66 (97)	0	33 (49)	33 (49)

[1]Study 2B (feedlot, 2019)—Number positive (%) in the samples taken from the individual steers by day, sample, and method (n = 70 through d 35, n = 69 on d 42 and 49, n = 68 on d 56 and 63). Enumerable positive samples are those defined as positive via spiral plating methods. Enrichment positive samples are those defined as negative via spiral plating methods but positive via 6-h incubations in *Escherichia coli* broth. Total positives are the total number of samples positive via enumeration and enrichment.

colonize, shed in the feces of feedlot steers, spread to their pen environment, and transmit between steers. For both studies, all fecal and pen surface soil samples taken before each inoculation were negative for any *E. coli* strain resistant to nalidixic acid and rifampicin antibiotics. Thus, any *E. coli* found throughout the study is assumed to be our inoculated strain. Our results also indicated that transmission between steers occurred during the study periods, and the strains persisted in steers and the environment over an extended period. Mean shedding of the enumerable-only positive samples for the feedlot steer fecal samples were 2.6 $\log_{10}$ cfu/g (study 2A) and 3.5 $\log_{10}$ cfu/g (study 2B). These means are comparable to the mean enumerable fecal counts of *E. coli* O157:H7 in the study by Cobbold et al. (2007). As mentioned before, our inoculated strain lacked the *stx*- and LEE-associated gene communities, which are speculated to confer adaptation benefits in the bovine intestinal environment (Arimizu et al., 2019). Their absence, particularly the genes involved in adhesion of *E. coli* to intestinal epithelial cells, could have disadvantaged our strains in the bovine hindgut; however, our mean shedding levels in comparison with longitudinal STEC shedding in Cobbold et al. (2007) suggest this disadvantage was not a factor. In studies 2A and 2B steers that were not initially selected for inoculation became positive with the inoculated strain in their feces. In all studies, there was variability in the fecal shedding of the dairy calves and steers between sampling weeks. The lowest concentration of *E. coli* that quantifiable by spiral plate method was 1.0 $\log_{10}$ cfu/400 cm^2 in hide samples, 1.0 $\log_{10}$ cfu/mL in oral samples, and 2.0 $\log_{10}$ cfu/g in feces. We commonly detected E. *coli* NalR-RifR using enrichment in samples that were negative for enumeration on spiral plates. The enrichment method of detection would have detected samples shedding below the detection limit of the spiral plate method ($<$1.0 $\log_{10}$ cfu/400 cm^2 in hide samples, $<$1.0 $\log_{10}$ cfu/mL in oral samples, and $<$2.0 $\log_{10}$ cfu/g in feces).

Variability in week-to-week detection could be due to individual variability in shedding concentration. The enrichment process in EC broth would improve the ability to detect any *E. coli* compared with direct spiral plating methods. Additionally, the detection of *E. coli* resistant to nalidixic acid and rifampicin in a selective medium containing the antibiotics, to some extent, is affected by the competing bacterial flora. However, the variability in shedding concentration is not surprising because it is well known that fecal shedding of STEC, and possibly any *E. coli*, in cattle is intermittent and highly variable (Berry and Wells, 2010).

In both years, the inoculated strain was detected in the feed, water, and pen surface soil samples, though in 2019 there was a greater proportion of positive samples. The detection of the inoculated strain in these sample types shows the survival of the strain in different environmental samples. Similar to that of STEC O157, which has been isolated from feedlot water tanks and feedlot-cattle feed (Dodd et al., 2003; Sargeant et al., 2004), *E. coli* O157 has

been shown to persist in feedlot soil environments, over a wide range of water and manure contents (Berry and Miller, 2005). Our inoculated strain showed a similar persistence in the pen surface samples. These environmental reservoirs could act as transmission points between animals and should be taken into consideration when modeling *E. coli* transmission within a feedlot setting.

All strains in study 1 and the one strain used in both studies 2A and 2B were detected in the oral samples collected from the calves. STEC O157 has been found in the oral cavity of both naturally colonized (Keen and Elder, 2002) and experimentally inoculated (Buchko et al., 2000) animals. STEC O157 has also been found naturally occurring on the hide surface of beef feedlot cattle (Keen and Elder, 2002). The hide and oral surfaces may serve as ecological reservoirs for STEC O157 that should be accounted for when studying direct or indirect animal-to-animal or animal-to-environment transmission.

Another factor that could be influencing the shedding variability of the calves and shedding differences seen between studies 2A and 2B is the weather. Though both years experienced a similar range of temperatures, study 2B (2019) experienced over 4 times the total precipitation than the study in 2018. It is a generally accepted view that pathogen survival is usually extended in moist soil environments over drier environments (Rudolfs et al., 1950; Van Donsel et al., 1967; Reddy et al., 1981). Study 2B saw a greater proportion of positive strain detection in all sample types taken from the calves (fecal, hide, and oral) and the pen environment (soil, feed, and water) compared with 2018. The extra moisture seen in 2019 compared with 2018 may explain why the inoculated strain survived longer in the environment and was detected at greater concentrations. Nalidixic acid–resistant strains of *E. coli* have been validated as a marker regarding chemical interventions in food safety (Blackburn and Davies, 1994; Taormina and Beuchat, 1999). These studies demonstrated that nalidixic acid–resistant strains had similar growth rates and stress tolerances as nalidixic acid–susceptible parent strains. However, no such validation has been done for the growth and survival of a nalidixic acid–resistant and rifampicin-resistant strain in a feedlot production environment.

APPLICATIONS

These studies describe the shedding duration and concentration of 3 nonpathogenic *E. coli* strains in weaned Holstein calves and the shedding and transmission of one nonpathogenic strain in 2 groups of feedlot steers and their pen environment. Overall, these studies support use of a surrogate strain to establish baseline data for host-to-host and host-to-environment enteric pathogen transmission. The use of a nonpathogenic *E. coli* strain with known temporal onset and source as a surrogate to generate data on shedding and transmission dynamics could help to understand enteric pathogen transmission and to model STEC

Table 8. Study 2B sample counts for pen surface, feed, and water samples[1]

	Pen surface (n = 25), number (% positive)			Feed (n = 5), number (% positive)			Water (n = 3), number (% positive)		
Study day	Enumeration	Enrichment	Total positive	Enumeration	Enrichment	Total positive	Enumeration	Enrichment	Total positive
7	19 (76)	4 (16)	23 (92)	0	0	0	2 (67)	1 (33)	3 (100)
14	24 (96)	0	24 (96)	2 (40)	0	2 (40)	0	0	0
21	23 (92)	2 (8)	25 (100)	0	0	0	3 (100)	—	3 (100)
28	20 (80)	5 (20)	25 (100)	3 (60)	2 (40)	5 (100)	0	0	0
35	0	18 (72)	18 (72)	0	0	0	0	0	0
42	3 (12)	1 (4)	4 (16)	0	0	0	0	0	0
49	0	25 (100)	25 (100)	0	4 (80)	4 (80)	0	2 (67)	2 (67)
56	22 (88)	3 (12)	25 (100)	0	3 (60)	3 (60)	3 (100)	—	3 (100)
63	0	25 (100)	25 (100)	0	5 (100)	5 (100)	0	3 (100)	3 (100)

[1]Study 2B (feedlot, 2019)—Number positive (%) in the environmental pen samples by day, sample, and method. Enumerable positive samples are those defined as positive via spiral plating methods. Enrichment positive samples are those defined as negative via spiral plating methods but positive via 6-h incubations in *Escherichia coli* broth. Total positives are the total number of samples positive via enumeration and enrichment.

Table 9. Study 2B weekly temperature range and total precipitation[1]

Study week	Weekly temperature (°C)		Weekly total precipitation (cm)
	High	Low	
1	25.6	5.0	16.7
2	32.8	7.2	6.2
3	25.6	7.8	7.3
4	30.0	11.1	2.3
5	31.7	13.3	1.7
6	31.1	8.9	0.9
7	32.8	15.6	13.4
8	36.1	12.8	2.1
9	32.8	19.4	8.3

[1]Study 2B (feedlot, 2019)—Weekly temperature in Celsius (high and low) and total precipitation in centimeters; data obtained from the National Oceanic and Atmospheric Administration for the study period. Study weeks are the 7 d before that week's sampling.

transmission in commercial US feedlot systems to explore effective control options in real-world situations.

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