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Rectoanal Junction Colonization of Feedlot Cattle by *Escherichia coli* O157:H7 and Its Association with Supershedders and Excretion Dynamics▽

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Feedlot cattle were observed for fecal excretion of and rectoanal junction (RAJ) colonization with *Escherichia coli* O157:H7 to identify potential "supershedders." RAJ colonization and fecal excretion prevalences were correlated, and *E. coli* O157:H7 prevalences and counts were significantly greater for RAJ samples. Based on a comparison of RAJ and fecal ratios of *E. coli* O157:H7/*E. coli* counts, the RAJ appears to be preferentially colonized by the O157:H7 serotype. Five supershedders were identified based on persistent colonization with high concentrations of *E. coli* O157:H7. Cattle copenned with supershedders had significantly greater mean pen *E. coli* O157:H7 RAJ and fecal prevalences than noncopenned cattle. Cumulative fecal *E. coli* O157:H7 excretion was also significantly higher for pens housing a supershedder. *E. coli* O157:H7/*E. coli* count ratios were higher for supershedders than for other cattle, indicating greater proportional colonization. Pulsed-field gel electrophoresis analysis demonstrated that isolates from supershedders and copenned cattle were highly related. Cattle that remained negative for *E. coli* O157:H7 throughout sampling were five times more likely to have been in a pen that did not house a supershedder. The data from this study support an association between levels of fecal excretion of *E. coli* O157:H7 and RAJ colonization in pens of feedlot cattle and suggest that the presence of supershedders influences group-level excretion parameters. An improved understanding of individual and population transmission dynamics of *E. coli* O157:H7 can be used to develop preslaughter- and slaughter-level interventions that reduce contamination of the food chain.

Enterohemorrhagic *E. coli* strains are a recently emerged group of food-borne pathogens that are a significant public health threat, due mainly to the severity of clinical outcomes (15, 26). *E. coli* O157:H7 is the most clinically relevant serotype of enterohemorrhagic *E. coli* strains in most industrialized countries, including the United States (1, 15). Cattle are the primary reservoir of *E. coli* O157:H7, and cattle-derived foods, particularly ground beef, have principally been associated with human morbidity (1, 14, 26). *E. coli* O157:H7 impacts beef production security, trade, and consumer confidence. The employment of stringent measures to exclude this pathogen from retail beef means that costs associated with surveillance for and control of *E. coli* O157:H7 within beef production processes, as well as those associated with recalls of contaminated beef, have become a substantial problem for the beef industry (5, 20).

Minimizing *E. coli* O157:H7 entry into slaughter establishments is a recognized reduction strategy for carcass contamination (13, 26). Reducing the prevalence of *E. coli* O157:H7 excretion by market-ready cattle has so far been the mainstay of preslaughter approaches to risk mitigation. A number of epidemiological studies have investigated factors that are associated with higher shedding prevalence within populations of cattle (1, 14, 16). Yet, gaps in our knowledge of how or why cattle excrete *E. coli* O157:H7 to various degrees remain. A critical aspect of bovine *E. coli* O157:H7 epidemiology that needs to be addressed is the large amount of variation in prevalence between different groups of cattle, e.g., between herds, saleyard lots, or feedlot pens (16, 18, 20). Studies on the patterns of individual animal excretion of *E. coli* O157:H7 and subsequent population transmission dynamics are needed in order to design more-effective control strategies.

Conjecture exists as to the relevance and nature of gastrointestinal colonization by *E. coli* O157:H7 (7, 19). Recently, an anatomical area within the terminal rectum of cattle known as the rectoanal junction (RAJ) was proposed to be a site of preferential colonization (21). The research that followed aimed to confirm the phenomenon of RAJ colonization, investigate its relationship with fecal excretion, and explore RAJ swabbing as a method of testing for *E. coli* O157:H7 (8, 17, 25). Further work is needed to explore the potential importance of RAJ colonization for excretion and transmission dynamics in cattle populations. Several studies have noted wide variation in *E. coli* O157:H7 fecal concentrations between animals (2, 4, 9, 17, 22, 24). Some authors have suggested that certain cattle, designated "supershedders," have greater *E. coli* O157:H7 transmission potential than other cattle, whether through greater incidence or persistence of excretion, excretion of

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greater concentrations of *E. coli* O157:H7, or a combination of these factors (18, 21). We hypothesize that this concept of supershedders may be related to the phenomenon of RAJ colonization and that, together, these are at least partly responsible for the large intergroup variation in bovine *E. coli* O157:H7 prevalence. The aims of the current study were to (i) compare *E. coli* O157:H7 RAJ colonization prevalence, count, and persistence data between individual feedlot cattle and pens of cattle in order to identify potential supershedders, (ii) determine whether the *E. coli* O157:H7 status of cattle within a feedlot pen is associated with the presence of a supershedder within that pen, and (iii) examine RAJ colonization relative to fecal excretion and compare sensitivities of RAJ swabbing and fecal sampling for *E. coli* O157:H7 status determination.

MATERIALS AND METHODS

Experimental approach. A cross-sectional survey of natural RAJ colonization with and fecal excretion of *E. coli* O157:H7 in feedlot cattle was conducted. Cattle were maintained and sampled within experimental research pens located at Lakeside Feeders, Alberta, Canada, a commercial feedlot and slaughter operation. Mixed-breed and mixed-sex cattle were introduced into the feedlot at approximately 350 kg in weight and randomly assigned to 20 pens, with eight head of cattle per pen. Cattle in different pens could not directly contact each other, and there was no sharing of feed or water sources between pens. Apart from the sampling procedures, cattle were fed, managed, and slaughtered in a manner typical to commercial feedlot practice.

Sample collection. Cattle were individually sampled twice per week, commencing 3 days following pen assignment, over a 14-week period from 21 July to 27 October 2003. Over the course of the sampling period, four cattle were withdrawn from the study due to physical or behavioral problems in repeat handling. Two samples were collected from each subject at each sampling date: a 10-g fecal sample collected into Whirl-Pak bags (Nasco, Fort Atkinson, WI) from freshly passed manure and recto-anal mucosal swabs (RAMS). The collection of RAMS has previously been described (8, 25) but briefly involves vigorous mucosal surface swabbing of the rectal area 5 to 10 cm cranial to the anus by using sponge-tipped swabs (VWR International, Buffalo Grove, IL). Samples were transported at 4°C to Washington State University laboratories. Personnel sampling cattle and processing samples were blinded to results throughout the study.

***E. coli* O157:H7 and *E. coli* enumeration.** Fecal samples were decimally diluted in tryptone soy broth (Difco, BD, Franklin Lakes, NJ) and 100-μl aliquots plated onto sorbitol MacConkey agar plates (Remel, Lenexa, KS) containing cefixime (50 ng/ml) and potassium tellurite (2.5 μg/ml) (SMAC-CT; Sigma Chemical Co.). After incubation at 37°C for 18 to 24 h, sorbitol-negative colonies were enumerated. Final *E. coli* O157:H7 counts were calculated based on the sorbitol-negative colony count multiplied by the proportion of sorbitol-negative colonies confirmed as *E. coli* O157:H7 multiplied by the dilution factor. RAMS were placed in 100 ml tryptone soy broth and vortexed for 60 s before being diluted, plated, and counted as per fecal samples. *E. coli* counts were performed by direct plating of serially diluted samples onto violet-red bile agar containing 4-methylumbelliferyl-β-D-glucuronide (VRB-MUG; VWR International). All *E. coli* O157:H7 isolates were banked at −80°C in 30% phosphate-buffered glycerol. Limits of detection for direct plating of RAMS and fecal samples were approximately 10^2 CFU/g.

***E. coli* O157:H7 confirmation.** Subsets of sorbitol-negative colonies (five per plate) were confirmed as *E. coli* O157:H7 through typical colonial morphology on VRB-MUG (VWR International), O- and H-antigen latex agglutination (Oxoid, Ogdensberg, NY), and PCR. The PCR used multiplexed primers directed against markers stx_1 (23), stx_2, *eae*, and $fliC_{H7}$ (10). Reaction mixtures (50 μl) comprised 0.02 nmol/μl primer solutions, 0.02 mM deoxynucleoside triphosphates, 2 mM $MgCl_2$, 2.5 U *Taq* polymerase (Invitrogen, Carlsbad, CA), and 2 μl of boiled cell lysate template. The cycling conditions were denaturation at 95°C for 3 min, amplification for 35 cycles at 94°C for 1 min, 58°C for 1.5 min, and 72°C for 2.5 min, and elongation at 72°C for 10 min. PCR was performed using an iCycler thermal cycler (Bio-Rad, Hercules, CA) and products visualized in ethidium bromide-stained 1% agarose electrophoresis gels.

PFGE. Isolates were chosen for pulsed-field gel electrophoresis (PFGE) analysis on the following bases: from supershedders; other cattle within the same three pens as supershedders; cattle within three other pens, chosen randomly; multiple isolates from each animal isolated over the feeding period; and RAJ and fecal isolates from the same animal on the same sampling date. PFGE was based on XbaI restriction using PulseNet (CDC) standard marker strains and restriction parameters (11). Bands were resolved using a CHEF-DR II system (Bio-Rad) and gel images digitized (Syngene Gene Genius; Synoptics, Cambridge, United Kingdom). PFGE profiles were analyzed using Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium), with both position tolerance and optimization parameters set at 1%. Cluster analyses were performed and dendrograms created with an unweighted-pair group method using arithmetic means with the Dice similarity coefficient.

Data analysis. Data were analyzed using SAS 8.02 (SAS Institute, Cary, NC). Comparative statistics used pens as the unit of analysis rather than individual cattle, as a substantial pen effect was anticipated. Cattle were defined as supershedders on the basis of both high mean RAJ concentration ($\geq 10^4$ CFU/RAMS) and persistent RAJ colonization (≥4 consecutive positive RAMS samples). Analysis of pen *E. coli* O157:H7 parameters excluded supershedder data, i.e., for pens that housed a supershedder, analysis included data only for supershedders' pen mates. For comparisons of RAMS to fecal counts, CFU/RAMS counts were converted to CFU/g by multiplying by 0.242 g, the mean weight of material absorbed by RAMS. Mean and cumulative count data excluded *E. coli* O157:H7-negative results. Statistical differences were considered significant when P was <0.05.

RESULTS

RAJ and fecal *E. coli* O157:H7 prevalence. The mean prevalences for each date over the sampling period (Fig. 1) and the mean prevalences for each feedlot pen (Fig. 2) demonstrated similar general trends for both RAMS and fecal samples. Pen-to-pen variations in both RAMS and fecal prevalences were significant ($P < 0.001$) (general linear model procedure). Mean pen RAMS and fecal prevalences were highly correlated (Pearson's correlation coefficient = 0.9708; $P < 0.001$). The mean RAMS prevalence for *E. coli* O157:H7 was 11.0%, with a 95% confidence interval (CI) of 9.9% to 12.1%, which was significantly higher than overall fecal prevalence (6.6%; CI, 5.6% to 7.6%) ($P < 0.001$ by the Wilcoxon signed-rank test).

RAJ and fecal *E. coli* O157:H7 counts. The mean RAMS *E. coli* O157:H7 count (4.06 $\log_{10}$ CFU/g; CI, 3.94 to 4.17) was significantly higher than the mean fecal count (3.21 $\log_{10}$ CFU/g; CI, 3.08 to 3.33) over the feeding period ($P < 0.001$ by the Wilcoxon signed-rank test). Although these parameters fluctuated over the sampling period (data not shown), the RAMS count was always higher than the fecal count. Mean *E. coli* O157:H7 counts varied between pens, but this variation was not significant when considered for *E. coli* O157:H7-positive animals alone. The frequency distributions of *E. coli* O157:H7 counts for each animal on each sampling date (cattle day) also differed for RAMS and fecal samples (Fig. 3). The RAJ colonization count followed a log-normal distribution, and the fecal count followed a declining log-linear distribution. RAMS and fecal sample mean counts were not correlated.

Ratios of *E. coli* O157:H7 to *E. coli*. Pen day RAMS and fecal ratios (mean pen *E. coli* O157:H7/*E. coli* ratio for each pen on each sampling date) were correlated (Spearman's ρ, 0.479; $P < 0.001$). Median pen day ratios were significantly higher for RAMS (−2.22 $\log_{10}$) than for fecal samples (−3.14 $\log_{10}$) ($P < 0.001$ by the Wilcoxon signed-rank test). Based on median ratios, for every *E. coli* O157:H7 cell detected in RAMS, there were 168 *E. coli* cells. The median proportion of *E. coli* cells that were *E. coli* O157:H7 for fecal samples was >8 times less (for each *E. coli* O157:H7 cell, there were 1,373 *E. coli* cells). Temporal trends for RAMS and fecal sample ratios over the sampling period are demonstrated in Fig. 4. Median pen date *E. coli* O157:H7/*E. coli* ratios for RAMS were significantly

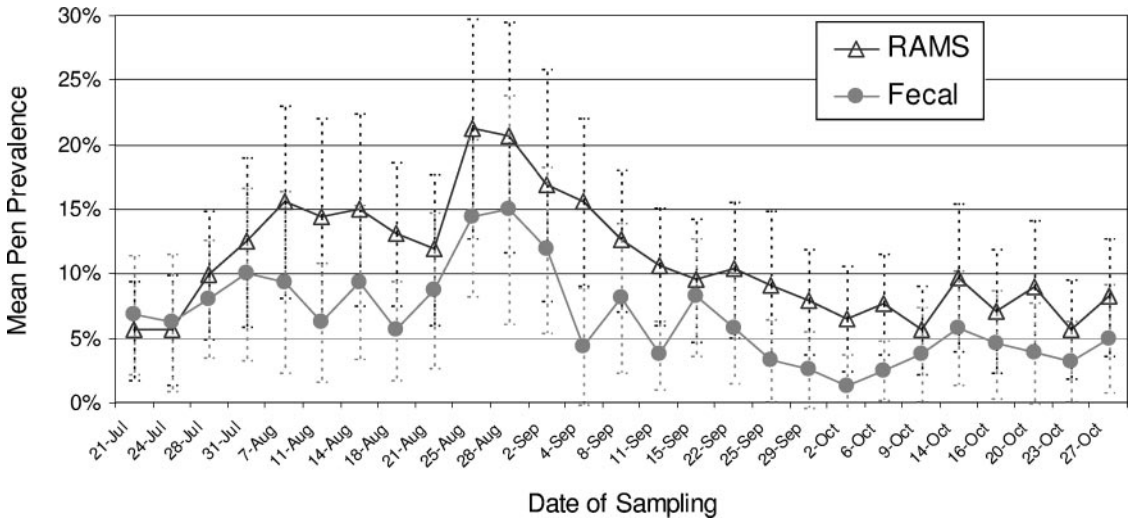

FIG. 1. Mean pen RAMS and fecal *E. coli* O157:H7 prevalences for feedlot cattle over the sampling period. Error bars represent 95% confidence intervals.

($P < 0.05$ by the Wilcoxon signed-rank test) higher for supershedders (-1.93 $\log_{10}$) than for nonsupershedders (-2.89 $\log_{10}$). There was no such significant difference for fecal samples (supershedder and nonsupershedder ratios were -3.33 $\log_{10}$ and -3.17 $\log_{10}$, respectively).

Pen-level *E. coli* O157:H7 associations with supershedders. Based on the defining criteria, five supershedders from three pens were identified. *E. coli* O157:H7 RAJ colonization and fecal excretion parameters with respect to contact (i.e., sharing a pen) with a supershedder are summarized in Table 1. Cattle that were housed in pens with no supershedder present had significantly lower RAMS and fecal prevalences than those that were copenned with supershedders. The mean RAMS and fecal counts for supershedder-present pens were higher than those in which no supershedders were identified, though this difference was not significant. When cumulative fecal counts were calculated (by multiplying mean pen prevalence with mean pen count), however, the presence of a supershedder in pens was significantly associated with higher fecal outputs of *E. coli* O157:H7 over the sampling period. Odds ratio calculations indicate that cattle negative for *E. coli* O157:H7 fecal excretion throughout the sampling period were five times more likely to have been housed in a pen without a supershedder than one with a supershedder.

PFGE results. A total of 112 isolates were subjected to PFGE analysis. Based on isolate matching at the ≤1 band (i.e., ≥97% similarity) level, 20 individual PFGE patterns were identified. Pen clustering was noted (i.e., isolates from within the same pen were more similar to each other than to those from other pens), although some pattern types were noted across many pens. RAMS and fecal isolates were generally highly related; of 30 comparisons for animal- and date-matched RAMS and fecal isolates, 26 (87%) were ≤1 band different. RAMS isolate pattern type tended to be stable over

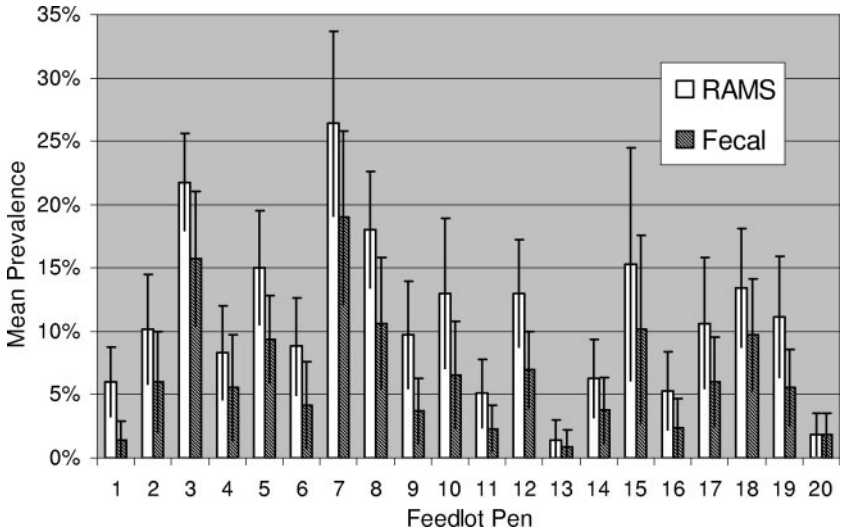

FIG. 2. Mean RAMS and fecal *E. coli* O157:H7 prevalences for feedlot pens. Error bars represent 95% confidence intervals.

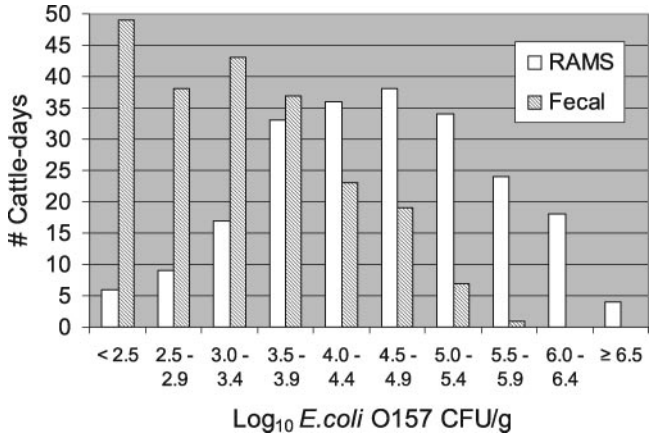

FIG. 3. Distribution of RAMS and fecal *E. coli* O157:H7 counts among feedlot cattle. Bars represent the frequency of cattle days (number of cattle on each sampling date) within each count interval. The data included only cattle days for which both RAMS and fecal samples were simultaneously positive for *E. coli* O157:H7.

time; of 20 longitudinal comparisons of isolates from the same animal, 16 (80%) were within one band similarity. Isolates from supershedders were compared to those from other cattle in their pens and in other pens, as summarized in Table 2. The proportion of matching comparisons between supershedders and copenned cattle was significantly greater than for supershedders and cattle from different pens.

DISCUSSION

Since the emergence of *E. coli* O157:H7, many surveys have examined its epidemiology within cattle populations. Although fecal excretion prevalence data have traditionally been used as the principal basis for such studies, it is important to also recognize the relevance of concentrations of *E. coli* O157:H7 excreted. An individual animal excreting large numbers of *E. coli* O157:H7 will arguably pose a greater risk than the combined output of many animals excreting at low levels, with respect to both potential food contamination and transmission to other cattle (3). This is substantiated by data generated within the relatively small number of studies that have quantified *E. coli* O157:H7 shedding. Omisakin et al. (24) determined that >96% of all *E. coli* O157:H7 cells shed by slaughter cattle were done so by a minority of individuals demonstrating high fecal concentrations. Other authors have similarly noted large variations in fecal *E. coli* O157:H7 concentrations, for both natural (9, 17, 22, 24) and experimental (2, 4) excretions, with a minority of individuals demonstrating uncommonly high levels of excretion. Matthews et al. (18) modeled *E. coli* O157:H7 transmission dynamics and concluded that a model that incorporated *E. coli* O157:H7 "supershedders" best represented cross-sectional epidemiological data. The current study aimed to examine the potential for some cattle to be *E. coli* O157:H7 supershedders, how these supershedders may be associated with *E. coli* O157:H7 feedlot cattle epidemiology, and whether they are related to the phenomenon of RAJ colonization (21).

The presence of supershedders in feedlot pens was associated with higher prevalences of *E. coli* O157:H7 colonization and excretion among copenned cattle. Though a similar association was not found for mean pen RAMS or fecal counts, there was a significant difference with respect to cumulative counts. Cumulative counts factored both mean pen prevalence and count parameters and represented the overall *E. coli* O157:H7 "load" that was being excreted within respective pens over the feeding period. Cattle that were exposed to supershedders excreted median *E. coli* O157:H7 counts 6 orders of magnitude greater than those excreted by non-supershedder-exposed cattle, with maximal shedding rate differences exceeding 25 orders of magnitude. The data excluded the *E. coli* O157:H7 prevalences and counts for the supershedders themselves, which would have again added to the cumulative output for these "superpens" of cattle. Such high-excreting cattle rep-

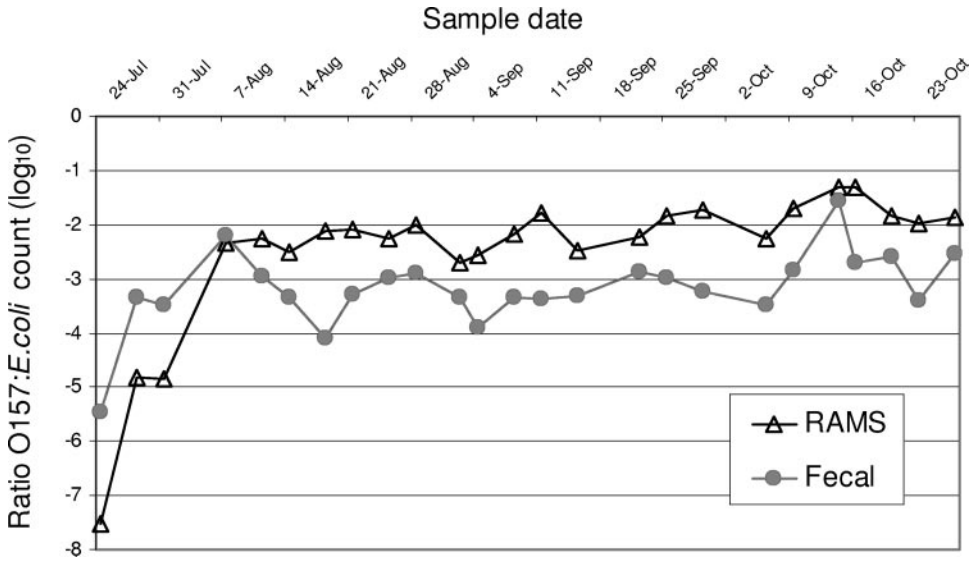

FIG. 4. Mean pen ratios of *E. coli* O157:H7 count to *E. coli* count over the sampling period. The data included only pen days for which RAMS and fecal *E. coli* O157:H7 and *E. coli* counts were all available.

TABLE 1. RAMS and fecal *E. coli* O157:H7 parameters for pens of cattle with or without supershedders[a]

Parameter	Lower quartile		Median		Mean		Upper quartile		Maximum		*P* value
	SS+	SS−	SS+	SS−	SS+	SS−	SS+	SS−	SS+	SS−	
RAMS prevalence (%)	11	0	19	7.4	22	9.0	26	15	78	33	0.0002
Fecal prevalence (%)	7.4	0	11	3.7	14	5.0	19	7.4	52	22	0.0001
RAMS count ($\log_{10}$ CFU/g)	2.67	0	3.33	3.00	3.28	2.41	4.06	3.75	5.27	6.18	0.08
Fecal count ($\log_{10}$ CFU/g)	2.82	0	3.03	2.69	2.88	2.08	3.33	3.39	5.02	5.19	0.08
Cumulative count ($\log_{10}$ CFU/g)	6.02	0	9.06	2.91	11.2	4.44	14.93	7.56	44.28	19.29	0.0002

[a] The results for supershedder-present pens exclude supershedder data (i.e., they represent copenned cattle only). *P* values were derived from Wilcoxon rank sum tests. SS+, with supershedders; SS−, without supershedders.

resent the greatest risks with respect to contamination of the food chain and the maintenance of *E. coli* O157:H7 within bovine populations (18, 24). Although no direction for the association could be demonstrated in this study, it could be assumed that the supershedders were responsible for the higher pen-level *E. coli* O157:H7 parameters through greater degrees of transmission of their *E. coli* O157:H7 cells to pen mates. This hypothesis is supported by the high degree of PFGE pattern identity between supershedder and pen mate isolates, although this finding is biased by pen clustering of *E. coli* O157:H7 PFGE types (data not shown).

In concurrence with other studies (8, 12, 25), RAJ swabbing is a more sensitive means of detecting *E. coli* O157:H7 in cattle than fecal sampling. Sampling individual cattle requires direct livestock handling and is more technically and practically challenging than fecal pat sampling. However, it allows the nomination of *E. coli* O157:H7 status to individual animals and overcomes many of the sampling biases and uncertainties inherent to using fecal pat sampling to estimate animal- or lot-level prevalence (6). The use of *E. coli* O157:H7 concentration data, generated in the current study, has the added utility of incorporation within quantitative risk assessments. As well as demonstrating higher cattle prevalences and concentrations for *E. coli* O157:H7, pen prevalence (i.e., the proportion of pens with at least one *E. coli* O157:H7-positive animal) was higher for RAMS samples than for fecal samples (data not shown). RAMS sampling of a proportion of the pen or herd would therefore potentially represent a more sensitive means of determining group-level *E. coli* O157:H7 status.

E. coli O157:H7/*E. coli* ratios are an indicator of the specificity of the RAJ for colonization by the O157:H7 serotype. Higher RAMS *E. coli* O157:H7/*E. coli* ratios for supershedders suggest that they are proportionally colonized with *E. coli* O157:H7 to a greater degree than most cattle. This has been demonstrated elsewhere (21) and may be part of the mechanism responsible for the greater *E. coli* O157:H7 count and persistence that confer supershedder status. Ratio differences between supershedders and other cattle were not reflected in fecal samples, however, suggesting that while supershedders are excreting larger amounts of *E. coli* O157:H7 overall, they are concomitantly excreting large amounts of generic *E. coli*. Notable changes in RAMS and fecal ratios in the early feeding period suggest complexity in *E. coli* O157:H7 colonization and transmission dynamics during this production phase, which may relate to climatic changes, dietary modifications, or mixing of cattle and the effects of stress.

TABLE 2. PFGE comparisons between supershedder *E. coli* O157:H7 isolates and those from other cattle within their pen (intrapen) or from other pens (interpen)[a]

Pen	Intrapen		Interpen		*P* value
	Total no. of comparisons made	% Similar	Total no. of comparisons made	% Similar	
3	13	69	43	37	0.04
7	14	71	42	21	0.002
8	18	83	38	0	<0.0001

[a] Isolates from six pens were compared. % Similar denotes the proportion of comparisons in which isolates had ≤1 band difference. *P* values were derived from chi-square tests.

The results for the current study were influenced by how supershedders were defined. Other groups that have quantified *E. coli* O157:H7 excretion or described "supershedders" or "high-level shedders" have proposed their own definitions. Low et al. (17) defined supershedders on the basis of either RAMS or fecal *E. coli* O157:H7 counts of ≥3 $\log_{10}$ CFU/g. Another group from the United Kingdom (24) used ≥4 $\log_{10}$ CFU/g for fecal samples. Other researchers have more loosely nominated cattle as supershedders by using prevalence parameters or the simple identification of outlying counts (2, 9, 18). For the purposes of the current study, supershedders were identified on the basis of RAJ rather than fecal parameters, as colonization rather than excretion was the focus. *E. coli* O157:H7 concentration and persistence data derived from longitudinal samples were used rather than point prevalence or concentration data, as these data are likely to be a better indicator of true colonization, through the reduction of time-dependent influences such as transient colonization and passive enteric passage (16, 18). During data analysis, a variety of definitions combining prevalence, count, and persistence data were trialed. Although some differences in results were evident, overall outcomes (as per Table 1) remained essentially the same. The current study differed from most others in that direct plating was used for *E. coli* O157:H7 enumeration and detection, without more-sensitive detection methods such as enrichment and immunomagnetic separation. The results, therefore, may have been biased by a failure to identify low-level colonized/excreting cattle. However, the significance of such low-level shedders to public health risk or cattle transmission dynamics is likely to be minimal (19, 24). Further research into the phenomena of RAJ colonization and supershedders requires the standardization of methods and supershedder definition.

The current study supports the hypothesis that the RAJ

represents an important colonization site for *E. coli* O157:H7 and suggests that supershedders represent cattle that have persistent colonization of the RAJ with high concentrations of *E. coli* O157:H7. The data presented also provide evidence that the presence of supershedders within a pen of feedlot cattle is associated with higher shedding of *E. coli* O157:H7 by pen-cohorted cattle. Ongoing research addresses whether supershedders are the cause of this group-level excretion pattern or whether the supershedders' output is influenced by group-level *E. coli* O157:H7 status and transmission dynamics. Other studies on the epidemiological implications of RAJ colonization and supershedders, e.g., its relevance to other forms of livestock production, other factors mediating pen-pen or herd-herd variation in *E. coli* O157:H7 prevalence, and implications for practical risk management strategies for market-ready cattle, are needed. Preslaughter interventions based on the presence of supershedders are envisaged. These might include targeting of supershedders for treatment to eliminate colonizing *E. coli* O157:H7 immediately prior to slaughter, the use of vaccines or competitive exclusion products specifically on supershedders, and logistic slaughter for supershedders or cattle within superpens. These rely on devising practical and cost-effective ways of identifying supershedders and may need to occur earlier in the production process (i.e., on the farm and at sale) rather than in lairage.

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