Age-Related Shifts in the Density and Distribution of Genetic Marker Water Quality Indicators in Cow and Calf Feces

Orin C. Shanks,^a Catherine A. Kelty,^a Lindsay Peed,^a Mano Sivaganesan,^a Thomas Mooney,^b Michael Jenkins^c

U.S. Environmental Protection Agency, Office of Research and Development, National Risk Management Research Laboratory, Cincinnati, Ohio, USA^a; University of Cincinnati, Cincinnati, Ohio, USA^b; U.S. Department of Agriculture, Agricultural Research Service, National Sedimentation Laboratory, Water Quality and Ecology Research Unit, Oxford, Mississippi, USA^c

Calves make up about 16% of the current bovine population in the United States and can excrete high levels of human pathogens in their feces. We describe the density and distribution of genetic markers from 9 PCR- and real-time quantitative PCR-based assays, including CF128, CF193, CowM2, CowM3, GenBac3, Enterol, EC23S857, CampF2, and ttr-6, commonly used to help assess ambient surface water quality. Each assay was tested against a collection of 381 individual bovine fecal samples representing 31 mother and calf pairings collected over a 10-month time period from time of birth through weaning. Genetic markers reported to be associated with ruminant and/or bovine fecal pollution were virtually undetected in calves for up to 115 days from birth, suggesting that physiological changes in calf ruminant function impact host-associated genetic marker shedding. In addition, general fecal indicator markers for *Bacteroidales*, *Escherichia coli*, and *Enterococcus* spp. exhibited three separate trends across time, indicating that these bacteria respond differently to age-related physiological and dietary changes during calf development. The results of this study suggest that currently available PCR-based water quality indicator technologies can under- or overestimate fecal pollution originating from calves and identify a need for novel calf-associated source identification methods.

A recent study reports that the estimated public health risk associated with recreational waters polluted with fresh cattle feces may not be substantially different from that associated with waters impacted by human sources (1). Identification of bovine fecal contamination in recreational waters is often confounded by the presence of fecal material from other animal sources such as humans and local wildlife. Thus, methods that can accurately discriminate between bovine and other sources of fecal pollution are vital to help assess potential health risks, monitor for the presence of zoonotic infectious pathogens, and effectively manage watersheds. Although studies have shown that different animal feeding practices can dramatically influence host-associated indicator bacteria shedding (2) and fecal microbial community structure (3) in adult cattle, very little is known about calves. Physiological and dietary changes in the first 3 to 8 months of life may influence the shedding of fecal indicators. As a result, some water quality assessment methods could be more suitable than others where fecal contamination originating from juvenile cattle may be present.

Calves (with body mass of ≤ 226 kg) make up about 16% of the current bovine population in the United States (4). Calf production is critical not only for the beef industry but also for dairy agriculture. Adult dairy cattle can produce milk only after calving. As a result, it is common practice to allow dairy cattle to produce one calf each year. Some calves are reared as replacement animals; however, most are used for slaughter to generate a variety of consumer goods. Based on dairy calf manure production rate predictions (5), it is estimated that approximately 1.7×10^8 kg/day (1.24 kg/day $\times$ 18.3 million head) of excrement is produced by calves in the United States. Because calf feces can contain human pathogens such as *Escherichia coli* O157:H7, *Campylobacter jejuni*, *Salmonella* spp., and *Cryptosporidium parvum* (6–10), this demographic represents an important potential public health risk when microorganisms originating from these sources reach ambient surface waters. *Cryptosporidium*

spp. and *Campylobacter* spp. are of particular concern because there is evidence that suggests that preweaned calves (<2 months) are more prone to infections from these pathogens and thus more likely to shed higher levels in manure (11, 12). This age-related trend in pathogen shedding may be important when considering which fecal indicator methods to use to assess fecal pollution in surface waters impacted by juvenile cattle.

The goal of this study was to determine the density and distribution of general fecal indicators, host-associated genetic markers, and fecal pathogens measured by endpoint PCR and quantitative real-time PCR (qPCR) in fecal samples collected from mother and calf pairings from the time of birth through weaning. The results of this study suggest that currently available PCR-based water quality indicator technologies can under- or overestimate fecal pollution originating from calves, provide new insights about the relationship between indicators and pathogens, and identify a need for calf-associated fecal source identification methods.

MATERIALS AND METHODS

Fecal sample collection. Fecal samples ($n = 381$) were collected from 62 individual dairy animals for analysis as previously described (2). Fecal samples were collected approximately every 30 days over a 10-month period from 31 mother/calf pairings housed at a U.S. Department of Agriculture Agricultural Research Service location in Georgia (March through October 2010). Sampling began 14 days after calf birth, with calves and mothering cows sampled on the same days. Animals were housed together throughout the study period. For the first 5 months, pairs

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Address correspondence to Orin C. Shanks, shanks.orin@epa.gov.

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TABLE 1 Primers, probes, and plasmid standards for PCR-based assays

Assay ^a	Platform	Reported target	Primers and probe (5' → 3')	Reference(s)
CF128	PCR	Ruminants	CF128, CCAACYTTCCCGWTAATC; 708R, CAATCGGAGTTCTTCGTG	21
CF193	PCR	Ruminants	CF193, TATGAAAGCTCCGGCG; 708R, CAATCGGAGTTCTTCGTG	21
CowM2	qPCR	Ruminants	CowM2F, CGGCCAAATACTCTGATCGT; CowM2R, GCTTGTTCGCTTCCTTG AGATAAT; CowM2P, [FAM]AGGCACCTATGTCCTTTACCTCATCAAC TACAGACA[TAMRA]	18
CowM3	qPCR	Ruminants	CowM3F, CCTCTAATGGAAAATGGATGGTATCT; CowM3R, CCATACTTCGCC TGCTAATACCTT CowM3P, [FAM]TTATGCATTGAGCATCGAGGCC [TAMRA]	18
GenBac3	qPCR	<i>Bacteroidales</i>	GenBactF3, GGGGTTCTGAGAGGAAGGT; GenBactR4, CCGTCATCCTTCACGCT ACT; GenBactP2, [FAM]CAATATTCCTCACTGCTGCTCCCGTA[TAMRA]	15, 17
EC23S857	qPCR	<i>E. coli</i>	EC23SF2, GGTAGAGCACTGTTTGGCA; EC23SR2, TGTCTCCCGTGATAACTTT CTC; EC23SP2b, [FAM]TCATCCGACTTACCAACCCG-[TAMRA]	14
Entero1	qPCR	<i>Enterococcus</i> spp.	ECST748F, AGAAATTCAAACGAACCTG; ENC854R, CAGTGCTCTACCTCCAT CATT; GPL813TQ, [FAM]TGGTTCTCTCCGAAATAGCTTTAGGGCTA [TAMRA]	16, 17
CampF2	qPCR	<i>Campylobacter</i> spp.	CampF2, CACGTGCTACAATGGCATAT; CampR2, GGCTTCATGCTCTCGAGTT; CampP2, [FAM]CAGAGAACAAATCCGAAGTGGGACA[BHQ1]	19
ttr-6	qPCR	<i>Salmonella</i> spp.	ttr-6, CTCACAGGAGATTACAACATGG; ttr-4, AGCTCAGACCAAAAGTGACCA TC; ttr-5, [FAM]CACCAGCGCGAGACCGACTTT[TAMRA]	20
CowM2 and CowM3	qPCR	Standard construct I	ATCGCGGCCAAATACTCTGATCGTACTCGAGATAGGCACCTATGTCCTTT ACCTCATCAACTACAGACAAAATTATCTCAAGGAACGCAACAGCATCG CCTCTAATGGAAAATGGATGGTATCTTTGGAGCCTTTGAAAGCACTCGA GCCTTATGCATTGAGCATCGAGGCCGGAAGCAAGCAACTATATATAAT AAGGTATTAGCAGCGCAAGTATGGATCG	This study
GenBac3, EC23S857, and Entero1	qPCR	Standard construct II	GGTAGAGCACTGTTTCGGCAAGGGGGTCATCCCGACTTACCAACCCGACT CGAGCTGCCAATACCGGAGAATGTTATCACGGGAGACAATCGGGGGTT CTGAGAGGAAGGTCCCCACATTGGAAGTGAACACGGTCTCGAGCC TACGGGAGGCAGCAGTGAAGGAATATTGGTCAATGGGCGCAGGCCTGAA CCAGCCAAGTAGCGTGAAGGATGACTGATCGGAGAAATTCAAACGAA CTTGGAGATAGCTGTTCTCTCCGAAATAGCTTTAGGGGTAGCTCGAG AATTGGAATGATGGAGGTAGAGCACTGTTTATGTCG	This study
CampF2 and ttr-6	qPCR	Standard construct III	ATCGCTCACCAGGAGATTACAACATGGCTAATGGATCCCGTCGTCAGTGGC TAAAGTCGGTCTCCCGTCCGTCGGTGGGATGGTCACTTTGGTCTGAGCTA TCGCACGTGCTACAATGGCATATACAATGAGACGCAATACCGCGAGGAT CCGCAATCTATAAAATATGTCCAGTTCGAGATTGTCTCTGCAACTCG AGAGCATGAAGCCATCG	This study

^a The CowM2 and CowM3 qPCR assays are patented by the U.S. Environmental Protection Agency (EPA) (33). Any use of these methods is prohibited without a license granted by the EPA.

could graze on wheat, Bermuda, and natural grasses while nursing. In month six, diets were supplemented with grain on a daily basis (grain ingredients: 25% rolled corn, 28% soyhull pellets, 33% corn gluten, 1.77% liquid molasses, 0.1% trace minerals, 0.05% vitamins [A, D, and E], 0.05% salt, 0.019% Bovatec 91Gr [ionophore], 1.101% calcium, and 10.12% distiller's grain). Fecal samples were initially placed on ice in the field and then immediately frozen to -20°C and express shipped overnight to the U.S. Environmental Protection Agency research laboratory in Cincinnati, OH. Upon receipt (≤ 24 h since sample collection), samples were stored at -80°C until time of analysis (≤ 12 months).

DNA extractions. All DNA extractions were performed with the FastDNA kit for soils (Q-Biogene, Carlsbad, CA) as previously described (13). Prior to DNA extraction of fecal samples, approximately 1 g (wet weight) of fecal material was mixed with GITC buffer (5 M guanidine isothiocyanate, 100 mM EDTA [pH 8.0], 0.5% Sarkosyl) to create a fecal slurry. Eight hundred microliters of each fecal slurry was bead homogenized at 4.0 m/s for 30 s using an MP FastPrep-24 instrument (MP Bio-medicals, LLC, Solon, OH). Extraction controls were performed with each extraction preparation to monitor for potential extraneous DNA contamination. Each DNA extract was diluted to a fixed concentration of total DNA (1 ng/ μl) to standardize measurements. Total DNA extract yields were measured with a NanoDrop ND-1000 UV spectrophotometer (NanoDrop Technologies, Wilmington, DE). All DNA extracts were stored at 4°C in GeneMate Slick low-adhesion microtubes (ISC BioExpress) until time of amplification (< 48 h).

Oligonucleotides and plasmid DNA standards. A total of 9 PCR-based assays were used in this study (Table 1), including four endpoint PCR assays (CF128 and CF193) and seven qPCR assays (CowM2,

CowM3, Entero1, EC23S857, GenBac3, CampF2, and ttr-6) (14–21). The plasmid DNA construct used as an absolute DNA standard for the CowM2 and CowM3 qPCR assays is described elsewhere (18). Two additional plasmid constructs (Integrated DNA Technologies) were generated for the remaining qPCR assays (Table 1). Plasmids were linearized by NotI restriction digestion (New England BioLabs, Beverly, MA), quantified with a NanoDrop ND-1000 UV spectrophotometer, and diluted in 10 mM Tris and 0.1 mM EDTA (pH 8.0) to generate 10-fold dilutions ranging from 10 to 1×10^6 copies/ $2 \mu\text{l}$ for use to generate calibration curve models. All dilutions were stored in low-retention microtubes at -20°C until the time of analysis.

Endpoint PCR amplification. Twenty-five-microliter endpoint PCR amplifications were performed on a DNA Engine 2 Tetrad thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA) for 35 cycles. Reaction conditions and thermal cycling parameters are described elsewhere (21) with the exception that TaKaRa Ex Taq Hot Start (TaKaRa Bio, Inc., Japan) DNA polymerase was used for each assay. All reactions were performed in duplicate in 96-well polypropylene plates. Results were visualized by loading 5 μl of PCR product onto a 2% agarose gel containing a $1 \times$ final concentration of GelStar nucleic acid stand (Cambrex BioScience, Rockland, ME). Agarose gels were photographed with a Gel Logic 100 imaging system (Carestream Health, Inc.) and a UV transilluminator (model 614A; Fisher Scientific) at the maximum setting and with an exposure time of 2.5 s.

qPCR amplification. All amplifications were performed in a 7900 HT Fast real-time sequence detector (Applied Biosystems). Reaction conditions and thermal cycling parameters for CowM2, CowM3, GenBac3, EC23S857, and Entero1 are reported elsewhere (14, 17, 18). For CampF2

TABLE 2 Calibration model performance characteristics for each qPCR assay

Assay	Calibration equation	r^2 ^a	E ^b	Nondetection threshold ^c	LLOQ ^d
CowM2	$y = 42.3 - 3.53x$	0.989	0.92	0.659	38.4
CowM3	$y = 41.3 - 3.50x$	0.992	0.93	0.375	37.7
GenBac3	$y = 38.3 - 3.38x$	0.981	0.98	0.086	34.5
Enterol	$y = 38.1 - 3.34x$	0.977	0.99	0.183	34.3
EC23S857	$y = 39.1 - 3.31x$	0.983	1.01	0.483	35.3
CampF2	$y = 38.1 - 3.50x$	0.969	0.98	-0.534	34.6
ttr-6	$y = 37.7 - 3.38x$	0.982	0.93	-0.698	34.2

^a Coefficient of determination representing the proportion of variability in the data set accounted for by a linear model.

^b Amplification efficiency, where $E = [10^{(1/\text{slope})} - 1]$.

^c Log₁₀ threshold where the corresponding C_q value cannot be distinguished from false-positive background.

^d Mean C_q based on repeated ($n = 18$) plasmid DNA standard at 10 copies per reaction.

and ttr-6 qPCR assays, reaction mixtures contained 1× TaqMan universal master mix, 0.2 mg/ml bovine serum albumin (Sigma-Aldrich, St. Louis, MO), 1 μM each primer, 80 nM 6-carboxyfluorescein (FAM)-labeled TaqMan probe (Life Technologies), and fecal DNA extracts containing 1 ng total DNA or 10 to 1 × 10⁶ target copies (plasmid DNA standards) in a total reaction mixture volume of 25 μl. The thermal cycling conditions were 2 min at 95°C, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. All reactions were performed in triplicate. Data were initially analyzed with Sequence Detector software (version 2.3.2; Life Technologies) at a threshold determination of either 0.08 (CowM2 and CowM3) or 0.03 (all other qPCR assays). Quantification cycle (C_q) values were exported to Microsoft Excel in preparation for further statistical analysis.

Experiment controls. An internal amplification control (IAC) designed to evaluate the suitability of isolated DNA for PCR-based amplifications was performed on each DNA extract using the CowM2 IAC multiplex assay as described previously (18). The criterion for concluding the presence of amplification inhibition in fecal DNA preparations was established as a C_q value of 35.9 ± 1.5, based on repeated experiments used to calculate a mean C_q value from control reaction mixtures containing 50 copies of the CowM2 IAC in buffer alone. To monitor for false positives, no-template and extraction blank controls were included, where laboratory-grade water was substituted for fecal samples for each instrument run and sample batch, respectively.

Data analysis. Master calibration curves, unknown DNA concentration estimates, and credible intervals were determined using a Markov chain Monte Carlo (MCMC) approach (22, 23). The linearity of C_q measurements determined from DNA standards was expressed as a coefficient of determination (r^2). Amplification efficiency (E) was determined, where $E = [10^{(1/\text{slope})} - 1]$. The lower limit of quantification (LLOQ) was reported as the mean C_q based on repeated measurements ($n = 18$) of the 10-copy plasmid DNA standard. To report frequency of detection and abundance of genetic markers over time, fecal samples were divided into seven groups based on calf age (group 1, 25 to 55 days; group 2, 56 to 85 days; group 3, 86 to 115 days; group 4, 116 to 145 days; group 5, 146 to 175 days; group 6, 176 to 205 days; and group 7, ≥260 days). For a given assay and time series group, all mean C_q measurements from each individual sample were used to estimate mean log₁₀ copies per nanogram of total DNA. Individual mean C_q values were either (i) in the range of quantification (above 10 copies), (ii) between the nondetection threshold (the log₁₀ value where the measurement cannot be distinguished from background based on no-template control data) and 10 copies, or (iii) below the nondetection threshold. A normal distribution was assumed for the first group. A truncated normal distribution was used with bounds (LLOQ, 1) or (-1, LLOQ) for the second and third groups, respectively. A chi-square for binomial proportions was used to compare CF128- and CF193-positive detection data between cow and calf measurements at each time series group and to identify any significant differences between these two endpoint PCR assays. A t test with unequal variance was used to compare mean estimates between cow and calf samples at each time series group number for qPCR assays.

RESULTS

Calibration curve parameters and experiment controls. Calibration curve performance parameters are listed in Table 2. qPCR assays indicated excellent linearity, with r^2 values ranging between 0.969 (CampF2) and 0.992 (CowM2). All E values were ≥0.92. IAC detection levels from the CowM2 IAC multiplex assay (expected mean C_q of 35.9 ± 1.5) indicated the absence of amplification inhibitors in 97.9% of DNA preparations (8 failures of 381 DNA extracts). DNA extracts that failed this criterion were discarded from the study. No-template controls and extraction blanks indicated the absence of extraneous DNA molecules in 99.3% (4 false positives of 613 reactions) of PCR and qPCR experiments in this study.

Abundance of host-associated bacterial genes in cow and calf populations. Figure 1 illustrates the frequencies of positive detections for CF128 and CF193 endpoint PCR assays (Fig. 1A) and estimated mean log₁₀ copies/ng of total DNA for CowM2 and CowM3 qPCR assays (Fig. 1B). For endpoint PCR assays, genetic markers are absent in group 1 and are detected at frequencies below 15% in groups 2 and 3 in calf samples. A chi-square binomial proportions analysis indicated a significant difference ($P \leq 0.0034$) between cow and calf samples for all time series groups except group 7 ($P = 0.947$ for CF128) and group 6 ($P = 0.1043$ for CF193). For cows, no significant difference was observed between CF128 and CF193 across all time series groups ($P = 0.99$) except groups 5 and 6 because all reactions were positive. No significant differences ($P \geq 0.0973$) were observed between CF128 and CF193 measurements in calf samples at time series groups 2, 3, and 6. However, a significant difference ($P \leq 0.0136$) was shown at time series groups 4, 5, and 7. A comparison at time series group 1 was not suitable because all reactions were negative for both assays. The abundances of CowM2 and CowM3 markers exhibit a similar pattern in calves, remaining virtually undetected in groups 1 to 3 and then increasing in abundance by at least one order of magnitude in group 5. A comparison of cow and calf mean estimates by time series group indicated a significant difference ($P \leq 0.0072$) across all time series groups except for CowM3 at time series group 5 and 6 ($P \geq 0.0576$).

Abundance of general fecal indicator bacterial genes in cow and calf populations. The abundances of genetic markers for general fecal indicators, including *Bacteroidales* (GenBac3), *E. coli* (EC23S857), and *Enterococcus* spp. (Enterol), were also determined for all cow and calf fecal samples. Estimated mean log₁₀ copies/ng of total DNA exhibited different distribution patterns over the sampling period (Fig. 2). *E. coli* abundance exhibited the

FIG 1 Simple straight-line and scatter plots indicating the frequency of positive detections for CF128 and CF193 (A) as well as the mean log₁₀ copies/ng of total DNA for CowM2 and CowM3 (B) over the sampling period for cows and calves. Fecal samples were divided into seven groups based on calf age: group 1, 25 to 55 days; group 2, 56 to 85 days; group 3, 86 to 115 days; group 4, 116 to 145 days; group 5, 146 to 175 days; group 6, 176 to 205 days; and group 7, ≥ 260 days. Calves typically gain the ability to ruminate by 115 days (group 3). Calves were weaned and introduced to grain diets during time periods 5 and 6. Error bars in panel B represent the standard deviation for each mean estimate. The time series group number denotes the age range in days for calves and the respective maternal parent.

largest variability between calves and adult cows, with adult levels ranging from 0.44 to 1.54 mean log₁₀ copies/ng of total DNA, while those for calves ranged from 2.16 to 3.97. The abundances of *E. coli* and *Enterococcus* spp. in calves were always greater than or equal to those in paired adult cow samples. *E. coli* genetic marker measurements in calves were almost 3 log₁₀ higher than those in mothering adults at time period 1 and then rapidly declined until time period 3 (Fig. 2, EC23S857). A comparison of cow and calf estimates for *E. coli* and *Enterococcus* spp. indicated a significant difference ($P \leq 0.0072$) across all time series group numbers except *Enterococcus* spp. at time series group 7 ($P = 0.6983$). *Bacteroidales* levels exhibited a different pattern, where there was no significant difference between cow and calf estimates at time series groups 1, 2, 5, and 6 ($P \geq 0.0072$).

FIG 2 Simple straight-line and scatter plots indicating the mean log₁₀ copies/ng of total DNA for *Bacteroidales* (GenBac3), *Enterococcus* spp. (Enterol), and *E. coli* (EC23S857) over the sampling period for cows and calves. Fecal samples were divided into seven groups based on calf age: group 1, 25 to 55 days; group 2, 56 to 85 days; group 3, 86 to 115 days; group 4, 116 to 145 days; group 5, 146 to 175 days; group 6, 176 to 205 days; and group 7, ≥ 260 days. Calves typically gain the ability to ruminate by 115 days (group 3). Calves were weaned and introduced to grain diets during time periods 5 and 6. Error bars represent the standard deviation for each mean estimate. The time series group number denotes the age range in days for calves and the respective maternal parent.

Abundances of pathogen genes in cow and calf populations. *Salmonella* spp., estimated by the *trr-6* genetic marker, were absent in all calf and adult cow samples in this study across the sampling time period (Fig. 3). *CampF2* genetic marker abundance ranged from 0.23 to 2.20 mean log₁₀ copies/ng in calves and from -0.59 to 1.7×10^{-4} in adult cows (Fig. 3). The abundance of *Campylobacter* spp. peaked at time series group 5 (146 to 175 days) in calves and then decreased to adult levels by group 7 (≥ 260 days). A comparison of cow and calf estimates for *Campylobacter* spp. indicated no significant difference ($P \leq 0.0209$) across all time series groups except group 7 ($P = 0.0981$).

DISCUSSION

Trends in host-associated genetic indicator shedding. An ideal host-associated genetic indicator of cattle fecal pollution should be uniformly distributed across populations regardless of animal age. Analysis of paired fecal samples from calves and mothering cows revealed that host-associated genetic markers are virtually absent in calves for up to 115 days (time period 3) from birth,

FIG 3 Simple straight-line and scatter plots indicating the mean log₁₀ copies/ng of total DNA for *Campylobacter* spp. (CampF2) and *Salmonella* spp. (ttr-6) over the sampling period for cows and calves. Fecal samples were divided into seven groups based on calf age: group 1, 25 to 55 days; group 2, 56 to 85 days; group 3, 86 to 115 days; group 4, 116 to 145 days; group 5, 146 to 175 days; group 6, 176 to 205 days; and group 7, ≥260 days. Calves typically gain the ability to ruminate by 115 days (group 3). Calves were weaned and introduced to grain diets during time periods 5 and 6. Error bars represent the standard deviation for each mean estimate. The time series group number denotes the age range in days for calves and the respective maternal parent.

suggesting that physiological changes in calf rumen function play an important role in fecal source identification genetic marker shedding. The primary difference between ruminant and non-ruminant animals is that ruminants have a four-compartment stomach which enables the animal to consume and subsist on grasses and other plants predominately composed of cellulose. However, for the first couple of months or so of life, juvenile ruminants such as calves are functionally monogastric, potentially explaining the large differences in host-associated indicator shedding observed in this study. Interestingly, host-associated genetic marker shedding does not appear to be altered due to calf weaning or introduction of a grain supplement (Fig. 1, time periods 5 and 6). A shift from milk to grass/grain as the primary source of subsistence represents a large change in diet. In adult cattle, more subtle changes in diet, such as shifts from one type of grain to another, are reported to severely alter host-associated genetic marker shedding (2). A minimal change in genetic marker shedding may be due to the fact that grasses were available through the study period and supplemental grains were introduced to calves at month six of sampling before animals were fully weaned. Perhaps the overlapping of diet regimens helped minimize shifts in host-associated genetic marker shedding as animals transitioned from one diet to another. While it is evident that animal age plays an important role in host-associated genetic marker shedding in calves, additional studies are needed to further characterize the influence that dietary shifts can have during calf development.

Trends in general fecal indicator genetic marker shedding. Measurements of general fecal indicator markers for *Bacteroidales*, *E. coli*, and *Enterococcus* spp. exhibited three separate density and distribution trends across time, indicating that these bacteria respond differently to age-related physiological and/or dietary changes during calf development. On average, the highest indica-

tor levels were observed with GenBac3, supporting previous reports that *Bacteroidales* make up a large portion of the bovine fecal community (3, 24). Findings indicate that *Bacteroidales* genetic marker concentrations are relatively similar in calves and mothering adults over the sampling period (Fig. 2, GenBac3), suggesting that *Bacteroidales* shedding remains consistent regardless of animal age, onset of ruminant function, or dietary shifts.

In contrast, levels of *Enterococcus* spp. were consistently higher in calves than in mothering adults for the first six time periods and then converged by the seventh time period (Fig. 2, Enter1) ($P = 0.693$), suggesting that *Enterococcus* genetic marker shedding is governed by factors different from those for host-associated or *Bacteroidales* indicators. Two factors may explain this trend. First, supplement grains were introduced during the sixth time period, which included not only corn and soyhull but also an ionophore. Ionophores are inhibitory to Gram-positive bacteria (25, 26) and are commonly fed to cattle to improve feed efficiency (27). The introduction of ionophores into the bovine diet has been shown to alter the bacterial community composition in rumen (25, 28, 29), in particular for Gram-positive microorganisms (25, 26). Thus, the introduction of an antimicrobial agent into the calf diet may play a role in the reduction of enterococcus shedding. Second, weaning occurred around this time, representing not only a change in diet but also the type and amount of contact between mother and calf. These changes may also influence changes in enterococcus shedding. It remains unclear which factor or combination of factors is responsible for the trends observed in this study. Regardless of which factors are accountable, there is clear evidence that factors involved in calf rearing can influence the shedding of the *Enterococcus* genetic markers by at least one order of magnitude compared to that in mothering adults.

It is interesting to note that *E. coli* and host-associated indicators exhibit remarkably different shedding patterns during calf development. For example, *E. coli* levels rapidly declined as the calf shifted from a monogastric to a ruminant digestion physiology, while host-associated indicators were essentially absent. After the onset of rumination, host-associated indicator levels rapidly increased, while *E. coli* concentrations remained consistent. It remains unclear why *E. coli* levels were initially so high in newborn calves, and this will require additional research to describe. Finally, *E. coli* levels began to increase during the introduction of supplemental grains, but host-associated indicators remained relatively stable. It has been documented that Gram-negative bacteria are generally resistant to ionophores (25, 26) and that feeding cattle grains containing ionophore additives can lead to increased *E. coli* shedding (30). Trends in this study support these assertions.

Trends in shedding of *Campylobacter* spp. Some *Campylobacter* species are known to cause spontaneous abortions in cattle (31), and there are at least a dozen species that have been implicated in human disease (32). While none of the animals sampled during this study were diagnosed with any *Campylobacter* infections (data not shown), the CampF2 genetic marker was consistently detected in both calves and mothering adults (Fig. 3). Previous studies report that the levels of many *Campylobacter* species are significantly higher during the first 3 months of life in calves than at the time the time of weaning (11). However, findings in this study suggest a different trend, where levels of *Campylobacter* spp. are very low in newborn calves and then steadily increase until the grain supplement/weaning stage (time periods 5 and 6). Unlike for the Gram-negative *E. coli*, levels of *Campylobacter* spp.

then rapidly decrease, suggesting that these two groups of microorganisms respond differently during this stage of calf development.

Implications for future research. Currently available host-associated PCR-based water quality indicator technologies used for assessing bovine fecal pollution can under- or overestimate fecal pollution originating from calves. Because calves make up a considerable proportion of the bovine population and are reported to be supershedders of several human pathogens, the development of novel calf-associated fecal source identification methods designed to target calves between the ages of 1 and 115 days is needed. Combined with the observation that many bovine-associated genetic markers are impacted by shifts in grain diets in adult cattle (2), it is clear that the successful characterization of fecal pollution from bovine sources will require more than one method, as well as some *a priori* knowledge of the age and diet of animals in the watershed of interest.

The study findings also raise important questions about general fecal indicator selection in watersheds where cattle may be a significant contributor to the fecal load. Measurements of enterococci, *E. coli*, and *Bacteroidales* indicate different trends in shedding for calves and, in some instances, the mothering adults. None of the general indicators exhibit the same trend as host-associated indicators, further complicating efforts to link fecal source identification and general fecal indicator measurements, especially when there are animals of various ages under different feeding regimens present in the study area. The most promising general fecal indicator may be the GenBac3 method targeting the *Bacteroidales* group, because data suggest that this genetic marker is shed with the most consistency in both calves and mothering cows over the sampling period.

Characterization of *Campylobacter* spp. also led to some interesting observations. First, none of the host-associated or general fecal indicator bacteria exhibited the same shedding trends in calves, suggesting that none of these methods is adequate for estimating the risk of exposure to *Campylobacter* spp. for this animal group. Measurements of *Enterococcus* spp. appear to share the most similar trend, where levels rapidly decline after grain supplementation/weaning (time periods 5 and 6); however, Entero1 may underestimate levels of *Campylobacter* spp. in calves <115 days of age. Finally, it is worth noting that the trends observed in this study are likely to occur in other relevant agricultural sources, suggesting that a “tool box” fecal source identification approach for each animal group may be needed to accurately estimate pollution levels.

In summary, we have described the density and distribution of genetic markers from 11 PCR- and real-time quantitative PCR-based assays targeting bovine-associated, general fecal indicator, and pathogen genetic markers measured from individual fecal samples collected from 31 mother and calf pairings sampled over a 10-month time period. The results indicate that indicators can exhibit dramatic differences in shedding between calves and mothering cows. These age-related trends not only can be triggered by physiological developmental changes in calves but also may be influenced by changes in calf diet.

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Agency's peer and administrative review and has been approved for external publication. Any opinions expressed in this paper are those of the authors and do not necessarily reflect the official positions and policies of the U.S. EPA. Any mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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