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Evaluation of Bovine Feces-Associated Microbial Source Tracking Markers and Their Correlations with Fecal Indicators and Zoonotic Pathogens in a Brisbane, Australia, Reservoir

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This study was aimed at evaluating the host specificity and host sensitivity of two bovine feces-associated bacterial (BacCow-UCD and cowM3) and one viral [bovine adenovirus (B-AVs)] microbial source tracking (MST) markers by screening 130 fecal and wastewater samples from 10 target and nontarget host groups in southeast Queensland, Australia. In addition, 36 water samples were collected from a reservoir and tested for the occurrence of all three bovine feces-associated markers along with fecal indicator bacteria (FIB), *Campylobacter* spp., *Escherichia coli* O157, and *Salmonella* spp. The overall host specificity values of the BacCow-UCD, cowM3, and B-AVs markers to differentiate between bovine and other nontarget host groups were 0.66, 0.88, and 1.00, respectively (maximum value of 1.00). The overall host sensitivity values of these markers, however, in composite bovine wastewater and individual bovine fecal DNA samples were 0.93, 0.90, and 0.60, respectively (maximum value of 1.00). Among the 36 water samples tested, 56%, 22%, and 6% samples were PCR positive for the BacCow-UCD, cowM3, and B-AVs markers, respectively. Among the 36 samples tested, 50% and 14% samples were PCR positive for the *Campylobacter* 16S rRNA and *E. coli* O157 *rfbE* genes, respectively. Based on the results, we recommend that multiple bovine feces-associated markers be used if possible for bovine fecal pollution tracking. Nonetheless, the presence of the multiple bovine feces-associated markers along with the presence of potential zoonotic pathogens indicates bovine fecal pollution in the reservoir water samples. Further research is required to understand the decay rates of these markers in relation to FIB and zoonotic pathogens.

Identification of the source of fecal pollution in recreational, fish harvesting, and drinking waters is vital in order to minimize public health risks from exposure to various enteric bacteria, protozoa, and viruses (1, 2, 3). Fecal indicator bacteria (FIB) such as fecal coliforms, *Escherichia coli*, and *Enterococcus* spp. have been commonly used as indicators of the microbiological quality of source waters. These bacteria are found in the gastrointestinal tracts of all warm-blooded animals, including humans. An important shortcoming of the FIB monitoring approach, however, is that it does not provide information on whether these bacteria originated from animals or humans.

Library-independent microbial source tracking (MST) methods have been developed to detect animal and human feces-associated markers in environmental waters using PCR assays (4, 5, 6). The commonly used PCR-based MST markers include anaerobic bacterial gene markers (7), bacterial toxin gene markers (8, 9), and viral markers (5, 10). Ideally, these markers should have certain characteristics: (i) they should be associated with the feces of a target host group (also known as host specificity) that is suspected as a source of fecal pollution; (ii) they should be present in all members of the target host group (also known as host sensitivity); (iii) they should be distributed evenly in all members of a target host group; (iv) they should exhibit temporal and geographical stability; (v) they should correlate with the presence of FIB or pathogens; and (vi) their decay rates should be similar to those of FIB or pathogens (11, 12). Among these characteristics, host specificity and host sensitivity are considered important because they can influence the false-positive and -negative detection of fecal pollution in environmental waters. The host specificity and host sensitivity of a particular marker can be determined by analyzing fecal samples from the target and nontarget host groups with the aid of mathematical formulas (12, 13).

Several studies have reported the development of PCR- and quantitative PCR (qPCR)-based assays for the detection and quantification of bovine feces-associated bacterial or viral markers in environmental waters (7, 14, 15, 16, 17, 18, 19). Some of these markers showed absolute host specificity when tested against fecal samples from nontarget host groups. For example, the bacterial marker cowM3 could not be detected in 144 fecal samples from 16 nontarget host groups in the United States (18). A follow-up study also reported the absolute host specificity of cowM3 in Canada (20). Among the 320 fecal samples tested from 15 nontarget host groups, none was positive for cowM3. Similarly, Ahmed et al. (21) also reported the absolute host specificity of the bovine adenoviruses (B-AVs) in Australia. Among the 154 fecal samples tested from 10 nontarget host groups, none was positive for the B-AVs marker. In contrast, bacterial markers such as BacCow-UCD (22) and BoBac (16) have been reported to be detected in a small number of samples from nontarget host groups in the United States. Because of variable host specificity results, validation of MST markers against a panel of reference fecal samples from target and nontarget host groups has been recommended (11, 12).

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TABLE 1 Sequences of primers and probes used for real-time PCR assays

Marker or organism in real-time PCR assay	Target	Primer or probe sequence (5′–3′)[a]	Amplicon size (bp)	Reference(s)
BacCow-UCD	16S rRNA	F: CCA ACY TTC CCG WTA CTC R: GGA CCG TGT CTC AGT TCC AGTG P: FAM-TAG GGG TTC TGA GAG GAA GGT CCC CC-TAMRA	177	7, 22
cowM3	Sialic acid-specific 9-*O*-acetylesterase secretory protein homolog	F: CCT CTA ATG GAA AAT GGA TGG TAT CT R: CCA TAC TTC GCC TGC TAA TAC CTT P: FAM-TTA TGC ATT GAG CAT CGA GGCC-TAMRA	122	18
Bovine adenoviruses	Hexon	F: GRT GGT CIY TRG ATR TRA TGGA R: AAG YCT RTC ATC YCC DGG CCA F: ATT CAR GTW CCW CAR AAR TTT TTT GC R: CCW GAA TAH RIA AAR TTK GG ATC	641 430	14
Bacteroides HF183	16S rRNA	F: ATC ATG AGT TCA CAT GTC CCG R: TAC CCC GCC TAC TAT CTA ATG	82	7, 27
Campylobacter spp.	16S rRNA	F: CAC GTG CTA CAA TGG CAT AT R: GGC TTC ATG CTC TCG AGTT P: FAM-CAG AGAA CAA TCC GAA CTG GGA CA-BHQ1	108	37
E. coli O157	*rfbE*	F: GCAGATAAACTCATCGAAACAAGG R: CGATAGGCTGGGGAAACTAGG P: TET-TCCACGCCAACCAAGATCCTCAGC-TAMRA	141	38
Salmonella spp.	*invA*	F: ACA GTG CTC GTT TAC GAC CTG AAT R: AGA CGA CTG GTA CTG ATC GAT AAT	244	39

[a] F, forward primer; R, reverse primer; P, probe; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; BHQ, black hole quencher.

The primary objective of this study was to evaluate the host specificity and host sensitivity of the bovine feces-associated bacterial (BacCow-UCD and cowM3) and viral (B-AVs) markers in fecal samples collected from various target and nontarget host groups in Brisbane, Australia. Environmental water samples were also collected from a reservoir in Brisbane that was potentially polluted with bovine feces. The reservoir water samples were also tested for the presence of bovine feces-associated markers using real-time PCR assays. In addition, water samples were also tested for the FIB (*E. coli* and *Enterococcus* spp.) using culture-based methods and for potential zoonotic bacterial pathogens (*Campylobacter* spp., *E. coli* O157, and *Salmonella* spp.) using real-time PCR assays. The occurrence of these markers was used to provide evidence of bovine fecal pollution in the reservoir. Finally, agreement on the occurrence and nonoccurrence among the bovine feces-associated markers, FIB, and zoonotic bacterial pathogens is discussed.

MATERIALS AND METHODS

Primers, probes, and positive controls for real-time PCR assays. For the real-time PCR detection of the bovine feces-associated markers and zoonotic bacterial pathogens, previously published primers and probes were used (Table 1). For BacCow-UCD, cowM3, and B-AVs real-time PCR assays, positive controls were derived from bovine wastewater collected from an abattoir. In summary, the PCR-amplified products were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA), cloned into the pGEM-T Easy vector system (Promega, Madison, WI), transferred into *E. coli* JM109 competent cells, and plated on Luria-Bertani (LB) agar plates containing ampicillin, IPTG (isopropyl-β-D-thiogalactopyranoside), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as recommended by the manufacturer. Plasmid DNA was isolated using the plasmid minikit (Qiagen). DNA sequencing was carried out at the Australian Genome Research Facility (St. Lucia, Queensland, Australia). For real-time PCR assays of *Campylobacter* 16S rRNA, *E. coli* O157 *rfbE*, and *Salmonella invA* genes, genomic DNA was isolated from *Campylobacter jejuni* NCTC 11168, *E. coli* O157:H7 ATCC 35150, and *Salmonella enterica* serovar Typhimurium ATCC 14028, respectively.

Target and nontarget host group sampling. To determine the host specificity and host sensitivity of the bovine feces-associated markers, fecal and wastewater samples were collected from 10 target and nontarget host groups (Table 2). Individual bovine fecal samples were collected from 10 beef cattle farms, whereas composite bovine wastewater samples were collected from an abattoir located on the outskirts of Brisbane. Five of the 10 cattle farms were located around the reservoir. Individual bovine fecal samples were not included in composite bovine wastewater samples. Bird fecal samples were collected from the City Botanical Garden and Currumbin Wildlife Sanctuary Hospital at Currumbin on the Gold Coast. The bird species include plover, crow, ibis, seagull, wood duckling, noisy miner, fantail cuckoo, rainbow lorikeet, crested tern, and topknot pigeon. Chicken fecal samples were collected from the backyard of a household and a chicken-processing farm in Brisbane. Dog fecal samples were collected from a dog park on the Sunshine Coast. Duck fecal samples were collected from areas adjacent to ponds and lakes in the Sunshine Coast region. Kangaroo fecal samples were collected from the University of the Sunshine Coast located on the Sunshine Coast. Pig fecal samples were collected from two pig farms in Brisbane. Possum fecal samples were collected from the rooftops of various households within Brisbane. Horse fecal samples were collected from a horse racecourse in the Sunshine Coast. Human wastewater samples were collected from the primary influent of two sewage treatment plants (STPs) in Brisbane. For the individual animal fecal samples, a fresh sample was collected from the fresh defeca-

TABLE 2 Evaluation of PCR inhibitors in DNA isolated from target and nontarget host groups and reservoir water samples

DNA sample source	No. of samples	Sample vol or wt[a]	C_T value (range) of real-time PCR	
			Undiluted DNA	10-fold dilution[b]
Cattle[c]	20	180–220 mg	27.1–27.9	—
Bovine wastewater[d]	20	10 ml	27.3–27.6	—
Birds	10	50–150 mg	27.5–38.1	27.3–28.1
Chickens	10	150–200 mg	26.8–27.1	—
Dogs	10	180–220 mg	27.1–27.6	—
Ducks	10	100–200 mg	27.2–28.0	—
Kangaroos	10	180–220 mg	26.9–27.4	—
Pigs	10	180–220 mg	27.3–27.9	—
Possums	10	180–220 mg	27.3–27.6	—
Horses	10	180–220 mg	27.6–31.9	27.1–27.8
Human wastewater[d]	10	10 ml	24.6–25.8	—
R1	4	9 liters	26.3–28.7	25.5–27.1
R2	4	9 liters	26.0–28.1	25.8–27.3
R3	4	9 liters	26.4–28.1	26.1–27.2
R4	4	9 liters	26.3–27.6	25.9–27.4
R5	4	9 liters	26.6–28.0	26.2–27.2
R6	4	9 liters	25.9–27.1	—
R7	4	9 liters	26.1–28.3	25.7–27.2
R8	4	9 liters	26.5–27.5	—
R9	4	9 liters	26.5–27.0	—

[a] The amount used for DNA isolation.
[b] —, 10-fold dilution was not performed.
[c] Individual fecal samples.
[d] Composite samples.

tion of an individual animal. All samples were transported on ice to the laboratory, stored at 4°C, and processed within 6 h.

Water sampling sites in the reservoir. Water samples were collected between November 2011 and April 2012 (during summer) from nine different sites (designated R1 to R9) (Fig. 1). Water samples were collected from each site (midpoint of the reservoir) using a boat. Four samples were collected from each site, one each on four separate occasions, giving a total number of 36 samples. The reservoir area is used for camping and other outdoor recreational activities, such as swimming, boating, and fishing. The suspected sources of fecal pollution are (i) waste from intensive grazing of cattle with direct access to the reservoir, (ii) native and feral wildlife, (iii) human recreational activities (to a lesser extent), and (iv) treated wastewater from STPs located upstream of the reservoir. A 10-liter water sample was collected from each site in 10-liter sterile carboy containers (Nalgene Labware, Rochester, NY) at 30 cm below the water surface. The water samples were transported on ice to the laboratory and processed within 6 to 8 h.

Enumeration of fecal indicator bacteria (FIB). The membrane filtration method was used for the isolation and enumeration of FIB. Serial dilutions of water samples were made in sterile MilliQ water, and filtered through 0.45-μm pore size (47-mm diameter) nitrocellulose membranes (Millipore, Tokyo, Japan), and placed on modified membrane-thermotolerant *Escherichia coli* agar (modified mTEC agar) (Difco, Detroit, MI) and membrane-*Enterococcus* indoxyl-D-glucoside (mEI) agar (Difco) for the isolation of *E. coli* and *Enterococcus* spp., respectively. Modified mTEC agar plates were incubated at 35°C for 2 h to recover stressed cells, followed by incubation at 44°C for 22 h (23), while the mEI agar plates were incubated at 41°C for 48 h (24).

Sample concentration and DNA isolation. The composite human and bovine wastewater samples were concentrated and desalted with Amicon Ultra centrifugal filters (Ultracel 50K; Millipore, Billerica, MA) as previously described (21). DNA was isolated from the concentrated human and bovine wastewater samples using a DNeasy blood and tissue kit (Qiagen) (Table 2). A QIAamp stool DNA kit (Qiagen) was used to isolate DNA from 50 to 120 mg of fresh feces from each individual animal.

The water samples were concentrated by passing 9 liters of water sample through a hollow-fiber ultrafiltration system (HFUS), using Hemoflow HF80S dialysis filters (Fresenius Medical Care, Lexington, MA) as previously described (25). Briefly, each water sample was pumped with a peristaltic pump in a closed loop with high-performance, platinum-cured L/S 36 silicone tubing (Masterflex; Cole-Parmer Instrument Co.). Tubing was sterilized by soaking overnight in 10% bleach, washed with sterile distilled water, and autoclaved at 121°C for 15 min. At the end of the sample concentration process, pressurized air was passed through the filter cartridge from the top to recover as much samples as possible. A new filter cartridge was used for each sample. The samples were concentrated to approximately 100 to 150 ml, depending on the turbidity of the water. Each sample was further centrifuged at 3,000 × *g* for 30 min at 4°C to obtain a pellet. The supernatant was discarded, and the pellet was resuspended in 5 ml of sterile distilled water. DNA was isolated from the 1.5 ml of the 5-ml concentrated water samples using a Power Soil DNA isolation kit (Mo Bio Laboratories). All DNA samples were quantified using a NanoDrop spectrophotometer (ND-1000; NanoDrop Technology, Wilmington, DE). Each DNA sample was amplified using a universal bacterial PCR assay as described elsewhere (26) to confirm successful DNA extraction process.

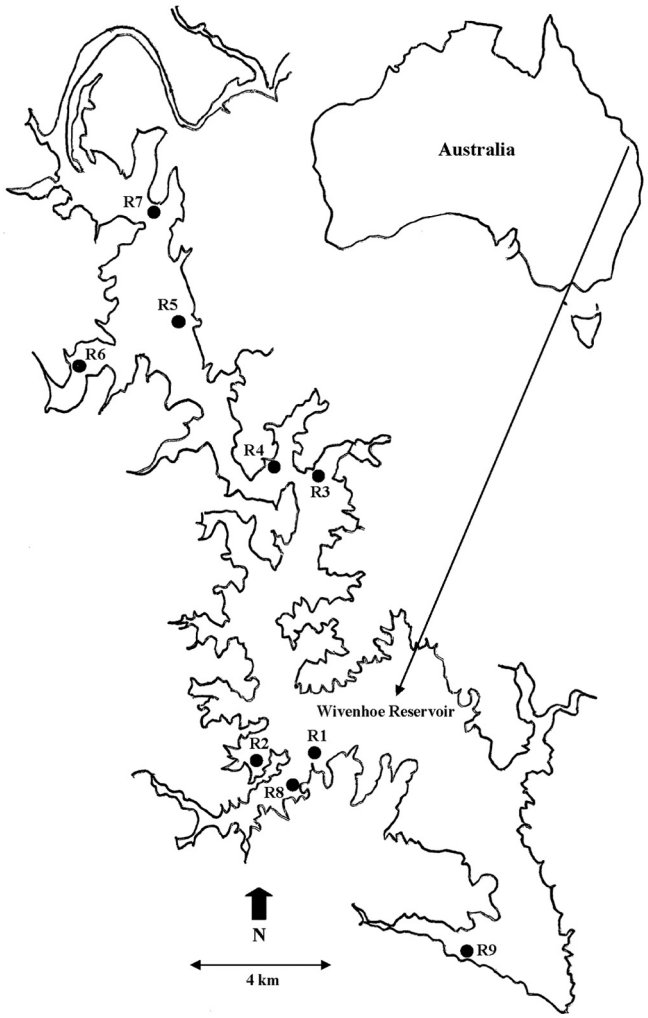

FIG 1 Map of the reservoir showing sampling sites R1 to R9.

Evaluation of PCR inhibition. An experiment was conducted to determine the potential presence of PCR inhibitors in composite wastewater and individual fecal DNA samples isolated from target and nontarget host groups. All DNA samples were diluted 10-fold with sterile water. Undiluted and 10-fold-diluted animal composite wastewater and fecal DNA samples were spiked with 10^3 gene copies of the sewage-associated *Bacteroides* HF183 marker (27). Human wastewater DNA samples were spiked with 10^3 gene copies of the cowM3 marker (18). An experiment was also conducted to determine the potential presence of PCR inhibitors in DNA isolated from the reservoir water samples. Water samples were spiked with 10^3 gene copies of the HF183 marker. The threshold cycle (C_T) values of the spiked undiluted and 10-fold-diluted DNA samples were compared to those of the distilled water spiked with the same number of gene copies of the HF183 and cowM3 markers to obtain the information on the PCR inhibition level (21).

Real-time PCR assays. The BacCow-UCD 16S rRNA, cowM3, *Campylobacter* 16S rRNA, and *E. coli* O157 *rfbE* real-time PCR assays were performed in 25-μl reaction mixtures using iQ Supermix (Bio-Rad Laboratories, Hercules, CA). The PCR mixture contained 12.5 μl of Supermix, a 400 nM concentration of each primer and 80 nM probe (for the BacCow-UCD 16S rRNA gene assay), an 800 nM concentration of each primer and 80 nM probe (for the cowM3 assay), a 500 nM concentration of each primer and 400 nM probe (for the *Campylobacter* 16S rRNA gene assay), or a 300 nM concentration of each primer and probe (for *E. coli* O157 *rfbE* gene assay), and 5 μl of template DNA. The real-time PCR conditions were as follows: 10 min at 95°C followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C (for BacCow-UCD and cowM3 assays); 10 min at 95°C followed by 45 cycles of 15 s at 95°C, 30 s at 58°C, and 30 s at 72°C (for the *Campylobacter* 16S rRNA assay); or 2 min at 95°C followed by 35 cycles of 15 s at 95°C, 45 s at 57°C (for the *E. coli* O157 *rfbE* assay).

The HF183 16S rRNA (for the PCR inhibition assay), *Salmonella invA*, and B-AVs hexon gene amplifications were performed in 20-μl reaction mixtures using Sso Fast EvaGreen Supermix (Bio-Rad Laboratories). The PCR mixture for all three assays contained 10 μl of Supermix, a 300 nM concentration of each primer, and 5 μl of template DNA. The HF183 real-time PCR consisted of 10 min at 95°C followed by 45 cycles of 30 s at 95°C, 1 min at 53°C, and 1 min at 60°C. The *Salmonella invA* gene real-time PCR consisted of 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 35 s at 59°C, and 2 min at 72°C. For the detection of the B-AVs marker, two rounds of the real-time PCR protocol were used. Both rounds of B-AVs PCR consisted of 4 min at 94°C followed by 30 cycles of 60 s at 92°C, 30 s at 52°C, and 75 s at 72°C. The second round of real-time PCR was performed using the same conditions as in the first round of PCR, except that 1 μl of the PCR products from the first round was added to a new 10-μl PCR mixture and 30 amplification cycles were performed. For each real-time PCR assay, a positive control (corresponding to plasmid DNA or genomic DNA) and a negative control (sterile water) were included. The real-time PCR assays were performed using the Bio-Rad iQ5 real-time PCR detection system (Bio-Rad Laboratories).

Real-time PCR limit of detection (LOD). To determine the real-time PCR limit of detection (LOD), plasmid DNA (BacCow-UCD, cowM3, and B-AVs markers) and genomic DNA (*C. jejuni* NCTC 11168, *E. coli* O157:H7 ATCC 35150, and *S.* Typhimurium ATCC 14028) were quantified using a spectrophotometer. Ten-fold serial dilutions (ranging from 500 to 5 copies) were made and tested with the real-time PCR assays.

Quality control. To minimize PCR contamination, DNA isolation and PCR setup were performed in separate laboratories. To prevent cross contamination, a method blank was included for each batch of water samples. A reagent blank was included to prevent cross contamination of samples during DNA isolation. To separate the specific product from nonspecific products, DNA melting curve analysis was performed for the HF183, *Salmonella invA*, and B-AVs real-time PCR assays. During melting curve analysis, the temperature was increased from 57°C to 95°C at approximately 2°C per min. Samples were considered positive when the sample had the same melting temperature as the positive control for each PCR assay.

Data analysis. The host specificity and host sensitivity of the markers were determined as follows: sensitivity = $a/(a + c)$ and specificity = $d/(b + d)$, where a is true positive (samples were positive for the marker of its own species), b is false positive (samples positive for the marker of another species), c is false negative (samples were negative for the marker of its own species), and d is true negative (samples were negative for the marker of another species) (13). Pearson's correlation (r_p) was used to test the relationship between *E. coli* and *Enterococcus* sp. concentrations in the reservoir water samples.

Bayes' theorem was used to calculate the conditional probability that the detection of bovine feces-associated markers in the reservoir water samples originated from bovine feces rather than feces from the nontarget host groups. The following formula was used to calculate the conditional probability (22, 28): $P(H \diagdown T) = [P(T \diagdown H)P(H)] \diagdown [P(T \diagdown H)P(H) + P(T \diagdown H')P(H')]$, where $P(H \diagdown T)$ is the probability (P) of bovine fecal pollution (H) in a water sample given a positive test result (T) for the sample, $P(T \diagdown H)$ is the true positive, $P(H)$ is the background probability of detecting a marker in a water sample, $P(T \diagdown H')$ is the false positive, and $P(H')$ is the background probability that a marker was not detected in a water sample. The value of $P(H')$ is $1 - P(H)$.

A binary logistic regression analysis was also performed to obtain correlations between the presence of FIB concentrations with bovine feces-associated markers and zoonotic bacterial pathogens (Minitab version 16; Minitab Inc., State College, PA). Binary logistic regression is a technique commonly used to model the binary (presence/absence) results from water samples. The presence/absence of bovine feces-associated markers and zoonotic bacterial pathogens was treated as the dependent variable (a binary variable). When a marker or pathogen was present, it was assigned the value 1, and when a marker or pathogen was absent, it was assigned the value 0. Relationships were considered significant when the P value for the model chi square was <0.05 and the confidence interval for the odds ratio did not include 1.0. Greater odds ratios indicate a higher probability of change in the dependent variable with a change in the independent variable.

RESULTS

PCR inhibition. All composite wastewater and individual fecal DNA samples ($n = 130$) isolated from target and nontarget host groups were checked for the presence of PCR inhibitors. For the HF183-spiked distilled water, the C_T values ranged from 26.6 to 27.1. For the HF183-spiked undiluted bovine wastewater and individual cow, chicken, dog, duck, kangaroo, pig, and possum fecal DNA samples, the C_T values ranged from 26.8 to 28.0 (Table 2). According to Student's paired t test, no significant difference was observed between the mean C_T values for spiked distilled water and undiluted DNA, which indicated that the DNA extracted from bovine wastewater and individual cow, chicken, dog, duck, kangaroo, and possum fecal DNA samples was free of PCR inhibitors. Based on the results, undiluted DNA samples were used for the PCR assays for the above host groups.

For the HF183-spiked undiluted individual bird and horse fecal DNA samples, the C_T values ranged from 27.5 to 38.1 and 27.6 to 31.9, respectively. Bird ($n = 4$) and horse ($n = 3$) fecal DNA samples had higher C_T values than the HF183-spiked distilled water, which suggested the presence of PCR inhibitors in undiluted fecal DNA samples. For the HF183-spiked 10-fold-diluted bird and horse fecal DNA samples, the C_T values, however, ranged from 27.3 to 28.1 and 27.1 to 27.8, respectively. No significant difference was observed between the mean C_T values for spiked distilled water and 10-fold-diluted DNA; therefore, inhibition was

TABLE 3 Host specificity and host sensitivity of bovine-feces-associated markers

DNA sample source	No. of samples	No. with positive PCR results for:		
		BacCow-UCD	cowM3	B-AVs
Cattle[a]	20	17	16	6
Bovine wastewater[b]	20	20	20	18
Birds	10	0	0	0
Chickens	10	4	0	0
Dogs	10	9	5	0
Ducks	10	3	4	0
Kangaroos	10	5	0	0
Pigs	10	1	0	0
Possums	10	2	2	0
Horses	10	4	0	0
Human wastewater[b]	10	3	0	0
Host specificity		0.66	0.88	1.00
Host sensitivity		0.93	0.90	0.60

[a] Individual fecal samples.
[b] Composite samples.

not observed. Based on the results, 10-fold-diluted bird and horse DNA samples were used for the PCR assay.

For the cowM3-spiked distilled water, the C_T values ranged from 24.2 to 25.1. For the cowM3-spiked undiluted human wastewater DNA samples, the C_T values ranged from 24.6 to 25.8. No significant difference was observed between the mean C_T values for spiked distilled water and undiluted DNA, indicating that human wastewater DNA samples were potentially free of PCR inhibitors. Based on the results, undiluted human wastewater DNA samples were used for the PCR assays.

DNA isolated from all reservoir water samples were also checked for the potential presence of PCR inhibitors. For the HF183-spiked distilled water, the C_T values ranged from 25.1 to 26.4. For the HF183-spiked undiluted DNA isolated from water samples, the C_T values ranged from 26.0 to 28.7. No significant difference was observed between the mean C_T values for spiked distilled water and undiluted DNA, demonstrating that the reservoir water samples were potentially free of PCR inhibitors. Based on the results, undiluted DNA samples were used for the PCR assays.

Host specificity and host sensitivity of the bovine feces-associated markers. Among the 90 fecal DNA samples isolated from the nontarget host groups, 34% and 12% samples were PCR positive for the BacCow-UCD and cowM3 markers, respectively. The BacCow-UCD marker was detected in chicken, dog, duck, kangaroo, pig, possum, horse, and human wastewater DNA fecal samples (Table 3). The cowM3 marker, however, was detected in dog, duck, and possum fecal DNA samples. The B-AVs could not be detected in DNA fecal samples of any nontarget host groups. The overall host specificity values of the BacCow-UCD, cowM3, and B-AVs markers for differentiating between bovine and other nontarget host groups were 0.66, 0.88 and 1.00, respectively (maximum value of 1.00).

Among the 40 DNA samples isolated from the target host groups, 93%, 90%, and 60% samples were PCR positive for the BacCow-UCD, cowM3, and B-AVs markers, respectively. The prevalence of B-AVs in individual bovine fecal DNA samples was low compared to that of BacCow-UCD and cowM3. The prevalence of all three bovine feces-associated markers in composite bovine wastewater DNA samples was high. The overall host sensitivity values of the BacCow-UCD, cowM3, and B-AVs markers, however, in composite bovine wastewater and individual bovine fecal DNA samples were 0.93, 0.90, and 0.60, respectively (maximum value of 1.00).

PCR limit of detection (LOD). PCR LOD assays were performed using plasmid DNA and genomic DNA. To determine the reproducibility of the assays, several replicates ($n = 9$) of serially diluted plasmid DNA and genomic DNA were tested. The LODs were as low as 5 gene copies for the all target genes.

Fecal indicator bacteria (FIB) and prevalence of bovine feces-associated markers and zoonotic bacterial pathogens in the reservoir water. Among the 36 samples from the reservoir, 44% and 75% samples yielded culturable *E. coli* and *Enterococcus* spp., respectively. The concentrations of *E. coli* in the water samples ranged from 0.30 to 2.31 $\log_{10}$ CFU per 100 ml of water. The concentrations of *Enterococcus* spp. ranged from 0.70 to 3.40 $\log_{10}$ CFU per 100 ml of water. Pearson's correlation was used to test the relationship between *E. coli* and *Enterococcus* sp. concentrations. The concentrations of *E. coli* were found not to correlate with the concentrations of *Enterococcus* spp. ($r_p = 0.009$; $P > 0.956$).

Among the three bovine feces-associated markers tested, BacCow-UCD was more prevalent than the cowM3 and B-AVs markers (Table 4). Of the 36 samples tested, 56%, 22%, and 6% samples were PCR positive for the BacCow-UCD, cowM3, and B-AVs markers, respectively. Similarly, among the 36 samples tested, 50% and 14% were PCR positive for the *Campylobacter* 16S rRNA and *E. coli* O157 *rfbE* genes, respectively. The *Salmonella invA* gene was not detected in any of the samples tested.

Application of Bayes' theorem to estimate the conditional probability of accurately detecting the presence of bovine fecal pollution in the reservoir water samples. Bayes' theorem was used to estimate the conditional probability of accurately detecting bovine fecal pollution in the reservoir water samples for the BacCow-UCD and cowM3 markers, since these markers were detected in fecal samples from nontarget host groups. The background probabilities, $P(H)$, of detecting the BacCow-UCD and cowM3 markers in the reservoir water samples were determined to be 0.56 and 0.22, respectively. The background probability that these markers were not detected in the reservoir water samples were $1 - P(H)$, or 0.44 (for the BacCow-UCD marker) and 0.78 (for the cow M3 marker). $P(T \diagdown H)$ is the true-positive rate of the assays, and the values were calculated from the host sensitivity assays as reported in this study (0.93 for BacCow-UCD and 0.90 for cowM3). $P(T \diagdown H')$ is the false-positive rate of the assays, and the values were calculated from the host specificity assays in this study (0.34 for BacCow-UCD and 0.12 for cowM3). Based on the occurrence and nonoccurrence results of the BacCow-UCD marker in the reservoir water samples and fecal samples from target and nontarget host groups, there was a 78% probability that the detection of the BacCow-UCD marker in a reservoir water sample was due to true bovine fecal pollution and not to nontarget hosts such as chickens, dogs, ducks, kangaroos, pigs, possums, horses, and humans. Similarly, there was a 68% probability that the detection of the cowM3 marker in a reservoir sample was due to the true bovine fecal pollution and not from nontarget hosts such as dogs, ducks, and possums.

Agreement, disagreement, and correlations between fecal indicator bacteria (FIB) and bovine feces-associated markers and zoonotic bacterial pathogens. The occurrences of FIB, bovine

TABLE 4 Concentrations of *E. coli* and *Enterococcus* spp. and real-time PCR positive/negative results of bovine feces-associated markers and zoonotic bacterial pathogens in the reservoir water samples

Sampling site[a]	Fecal indicator range (avg), $\log_{10}$ CFU per 100 ml		No. of samples with positive PCR results for:					
	E. coli	*Enterococcus* spp.	BacCow-UCD	cowM3	B-AVs	*Campylobacter* 16S rRNA	*E. coli* O157 *rfbE* gene	*Salmonella invA* gene
R1 (4)	0.00–1.38 (0.78)	0.70–2.41 (2.01)	0	0	0	0	0	0
R2	0.00–1.63 (1.11)	0.00–2.28 (1.98)	0	0	0	0	0	0
R3	0.00–2.01 (1.41)	1.36–3.04 (2.53)	2	0	0	1	0	0
R4	0.00–1.92 (1.32)	0.00–2.65 (2.26)	2	1	1	2	0	0
R5	0.00–2.31 (1.71)	0.00–3.20 (2.69)	2	1	0	2	2	0
R6	0.00–2.13 (1.59)	0.00–3.18 (2.71)	3	3	1	2	2	0
R7	0.00–2.05 (1.52)	0.00–3.40 (2.93)	3	0	0	3	0	0
R8	0.00–1.49 (0.90)	0.00–2.48 (2.03)	4	0	0	4	1	0
R9	0.00–2.11 (1.80)	0.00–2.23 (1.96)	4	3	0	4	0	0
Total ($n = 36$)			20	8	2	18	5	0

[a] Four samples were obtained from each site.

feces-associated markers, and zoonotic bacterial pathogens were compared pairwise for all the pooled reservoir water samples (Table 5). The percentage of total agreement was calculated by adding the percentage of co-occurrence and non-co-occurrence for each pairwise comparison. The BacCow-UCD marker and the *Campylobacter* 16S rRNA gene had the highest percentage (47%) of co-occurrence agreement. In contrast, the *Salmonella invA* gene had no co-occurrence agreement with the FIB, bovine feces-associated markers, and other zoonotic bacterial pathogens. *E. coli* and *Enterococcus* spp. had 39% co-occurrence agreement. Among the markers, BacCow-UCD and cowM3 had the highest percentage (22%) of co-occurrence agreement, whereas B-AVs marker had the lowest percentage (6%) of co-occurrence agreement with both BacCow-UCD and cowM3. Among the zoonotic bacterial pathogens, the *Campylobacter* 16S rRNA gene had 14% co-occurrence agreement with the *E. coli* O157 *rfbE* gene. No co-occurrence

TABLE 5 Agreement on the co-occurrence and non-co-occurrence among fecal indicator bacteria (FIB), bovine feces-associated markers, and zoonotic bacterial pathogens in water samples from the reservoir

Pairwise comparison	Co-occurrence agreement (%)	Non-co-occurrence agreement (%)	Total agreement (%)	Total disagreement (%)
E. coli vs. *Enterococcus* spp.	39	19	58	42
E. coli vs. BacCow-UCD	22	22	44	56
E. coli vs. cowM3	8	42	50	50
E. coli vs. B-AVs	3	53	56	44
E. coli vs. *Campylobacter* 16S rRNA	22	28	50	50
E. coli vs. *E. coli* O157 *rfbE*	3	44	47	53
E. coli vs. *Salmonella invA*	0	56	56	44
Enterococcus spp. vs. BacCow-UCD	42	14	56	44
Enterococcus spp. vs. cowM3	17	19	36	64
Enterococcus spp. vs. B-AVs	6	25	31	69
Enterococcus spp. vs. *Campylobacter* 16S rRNA	39	14	53	47
Enterococcus spp. vs. *E. coli* O157 *rfbE*	11	22	33	67
Enterococcus spp. vs. *Salmonella invA*	0	25	25	75
BacCow-UCD vs. cowM3	22	44	66	34
BacCow-UCD vs. B-AVs	6	44	50	50
BacCow-UCD vs. *Campylobacter* 16S rRNA	47	42	89	11
BacCow-UCD vs. *E. coli* O157 *rfbE*	11	42	53	47
BacCow-UCD vs. *Salmonella invA*	0	44	44	56
cowM3 vs. B-AVs	6	78	84	16
cowM3 vs. *Campylobacter* 16S rRNA	17	44	61	39
cowM3 vs. *E. coli* O157 *rfbE*	6	72	78	22
cowM3 vs. *Salmonella invA*	0	78	78	22
B-AVs vs. *Campylobacter* spp.	3	47	50	50
B-AVs vs. *E. coli* O157 *rfbE*	0	81	81	19
B-AVs vs. *Salmonella invA*	0	94	94	6
Campylobacter 16S rRNA vs. *E. coli* O157 *rfbE*	14	50	64	36
Campylobacter 16S rRNA vs. *Salmonella* spp. *invA*	0	50	50	50
E. coli O157 *rfbE* vs. *Salmonella invA*	0	86	86	14

TABLE 6 Correlations among fecal indicator bacteria (FIB) with bovine-feces-associated markers and zoonotic bacterial pathogens using binary logistic regression analysis

Comparison	Concordance (%)	Odds ratio	*P* value[a]
E. coli vs. BacCow-UCD	55.8	0.99	0.140
E. coli vs. cowM3	44.3	0.99	0.459
E. coli vs. B-AVs	41.7	0.98	0.378
E. coli vs. *Campylobacter* 16S rRNA	38.9	1.00	0.752
E. coli vs. *E. coli* O157 *rfbE*	45.5	0.99	0.614
Enterococcus spp. vs. BacCow-UCD	64.6	1.00	0.131
Enterococcus spp. vs. cowM3	46.4	1.00	0.883
Enterococcus spp. vs. B-AVs	88.3	1.00	0.163
Enterococcus spp. vs. *Campylobacter* 16S rRNA	43.7	1.00	0.709
Enterococcus spp. vs. *E. coli* O157 *rfbE*	45.5	1.00	0.835

[a] The *P* value for the model chi-square was <0.05, and the confidence interval for the odds ratio did not include 1.0.

agreements were observed between the *Salmonella invA* gene and either the *Campylobacter* 16S rRNA gene or the *E. coli* O157 *rfbE* gene.

For most pairwise comparisons, the percentages of non-co-occurrence agreement were higher than co-occurrence agreement. The B-AVs marker and the *Salmonella invA* gene had the highest (94%) non-co-occurrence agreement. The lowest (19%) non-co-occurrence agreement was found between *Enterococcus* spp. and both *E. coli* and the cowM3 marker. Among the markers, cowM3 and B-AVs had the highest (78%) non-co-occurrence agreement, whereas B-AVs and cowM3 had lower (44%) non-co-occurrence agreement with the BacCow-UCD marker. Among the zoonotic bacterial pathogens, the *E. coli* O157 *rfbE* gene had 86% non-co-occurrence agreement with the *Salmonella invA* gene, whereas the *Campylobacter* 16S rRNA gene had lower (50%) non-co-occurrence agreement with the *E. coli* O157 *rfbE* and *Salmonella invA* genes.

BacCow-UCD and the *Campylobacter* 16S rRNA gene had the highest (89%) total agreement and *Enterococcus* spp. and the *Salmonella invA* gene had the lowest (25%) total agreement when co-occurrence and non-co-occurrence percentages were added for each pairwise comparison. The overall mean pairwise codetection agreement value (12%) was lower than the non-codetection agreement value (46%). Variable percentages of disagreement were observed for each pairwise comparison. The percentages of disagreement were as low as 6% (for B-AVs versus *Salmonella invA*) and as high as 75% (for *Enterococcus* spp. versus *Salmonella invA*).

Binary logistic regressions were used to determine whether any correlation existed between the concentrations of FIB and the presence/absence results for bovine feces-associated markers and zoonotic bacterial pathogens (Table 6). The PCR results for the *Salmonella invA* gene were not included in the analysis, as *Salmonella invA* could not be detected in any water samples tested. The presence/absence of the bovine feces-associated markers and zoonotic bacterial pathogens did not correlate with the concentrations of either of the FIB.

DISCUSSION

In all, 3% and 58% of the reservoir water samples exceeded Australian and New Zealand Environment Conservation Council (ANZECC) water quality guideline values of 150 fecal coliforms and 35 *Enterococcus* organisms per 100 ml of water, respectively, for primary contact (29). The FIB concentrations were pooled for each site and compared with the guideline values. All sites complied with the *E. coli* guideline value. In contrast, all sites did not comply with the *Enterococcus* guideline value. One important drawback of using FIB, however, is that their presence does not necessarily indicate the presence of pathogens or their source(s). The concentrations of *E. coli* in water samples collected during the first sampling event were approximately half a log higher than those of *Enterococcus* spp. *Enterococcus* spp. could not be detected in any water samples collected during the second sampling event. Only two water samples from sites R5 and R7 yielded *E. coli*; however, the concentrations were below the guideline value. The reservoir received 12 to 15 mm of rainfall during the second sampling event. It is possible that increased water flow may have diluted the concentrations of FIB in the reservoir, which has a total storage capacity of 2.61 km^3 and a surface area of 109.4 km^2. The prevalence and concentrations of *Enterococcus* organisms were generally higher in samples collected during sampling events 3 and 4, when the reservoir did not receive any rainfall. The mean concentrations of *Enterococcus* organisms in the reservoir water samples were approximately one order of magnitude higher than that of *E. coli*. It is possible that *E. coli* persisted for a shorter period in the reservoir than *Enterococcus* spp.

The markers BacCow-UCD and cowM3 were detected in 34% and 12% of fecal samples from nontarget host groups. It is desirable that a marker should be highly host specific, preferably with a value close to 1.00. The U.S. Environmental Protection Agency (EPA) has suggested that a marker with a specificity of <0.80 may not be useful for MST field studies (30). It has also been recommended that the host specificity and host sensitivity of any MST marker need to be tested prior to its field application (11). In this study, the host specificity and host sensitivity of the bovine feces-associated markers were evaluated by screening 130 fecal samples from 10 target and nontarget host groups. For each target and nontarget host group, at least 10 fecal samples were included as recommended (30). The overall host specificity values of the BacCow-UCD and cowM3 markers for differentiating between bovine and other nontarget host groups were 0.66 and 0.88, respectively, suggesting that the host specificity of cowM3 is higher than that of BacCow-UCD in southeast Queensland.

The host specificity of a marker can be influenced by false-positive results of the assay, and therefore, care was taken to prevent false-positive results. To prevent cross-contamination, fecal samples from nontarget host groups were collected and processed before the target host groups. Reagent blanks were included to minimize DNA cross-contamination for each batch of reservoir water samples. The PCR cycling parameters were kept the same as in the studies that reported the development of these markers (14, 18, 22). Despite that, the BacCow-UCD marker was detected in 32 fecal samples from chickens, dogs, ducks, kangaroos, pigs, possums, horses, and human wastewater. Similarly, the cowM3 marker was detected in 11 fecal samples from dogs, ducks, and possums. The presence of the BacCow-UCD marker in horse fecal samples has been reported in California (22). The cowM3 marker, however, has been reported to have absolute host specificity among the nontarget host groups (18). The discrepancies between previous studies and the present study could be due to the fact that the previous studies normalized (used 1 ng per μl of DNA for the

host specificity assay) the DNA concentration prior to testing with PCR (18, 31). In contrast, in the present study, the DNA concentrations were not normalized. The concentrations of DNA from target and nontarget host groups ranged from 10 to 30 ng per μl, and therefore, the detection sensitivity of the assays was higher than that of previous studies. PCR-positive amplicons were further confirmed by visualization on agarose gels in addition to melt curve analysis. Finally, up to two amplicons for each target host group were sequenced, and we verified that they were >97% identical to the published sequences (data not shown). The B-AVs marker also showed an absolute host specificity value of 1.00. The high specificity of the B-AVs marker has been reported in two previous studies (14, 21), suggesting its suitability for tracking bovine fecal pollution.

The overall host sensitivity values of the BacCow-UCD, cowM3, and B-AVs markers in composite bovine wastewater and individual bovine fecal DNA samples were 0.93, 0.90, and 0.60, respectively. For composite bovine wastewater samples, the BacCow-UCD and cowM3 markers exhibited absolute host sensitivity, whereas B-AVs exhibited a host sensitivity value of 0.90. For individual bovine fecal samples, BacCow-UCD had the highest host sensitivity value, 0.85, followed by the cowM3 (0.80) markers. The host sensitivity values obtained in this study for BacCow-UCD and cowM3 markers were similar to those in previous studies (18, 22). The host sensitivity of the B-AVs marker (0.30), however, in individual bovine fecal samples was low. These results are also consistent with a previous study, which was undertaken in Spain (14). It is possible that the concentrations of B-AVs could be low in bovine feces compared to bovine feces-associated bacterial markers. More research is needed to determine the prevalence and concentration of the B-AVs marker in bovine feces, preferably in a quantitative fashion. The host sensitivity value of a marker can be influenced by false-negative PCR results, and therefore, care was taken to prevent false-negative results. All fecal DNA samples were amplified with the universal bacterial PCR assay to confirm the presence of DNA after the isolation procedure. All fecal DNA samples from target and nontarget host groups were checked for the presence of PCR inhibitors. The DNA samples were serially diluted and retested with the PCR when PCR inhibitors were detected. We conclude that the host specificity and host sensitivity results obtained in this study in fact were true positive and negative and were not due to any artifacts of the analysis.

Among the markers, BacCow-UCD was the most prevalent in the reservoir water samples followed by the cowM3 and B-AVs markers. All the PCR-negative water samples were checked for the presence of PCR inhibitors to confirm that PCR inhibition did not mask the amplification. The high prevalence of BacCow-UCD in water samples could be due to the fact that this marker was detected not only in fecal samples from the target bovine sources but also in fecal samples from a range of nontarget host groups (Table 3). Similarly, the cowM3 marker was also detected in several water samples but to a lesser extent than the BacCow-UCD marker. Only two of 36 samples were positive for the B-AVs marker despite the fact that a nested PCR was performed for the B-AVs marker to increase detection sensitivity. The presence of B-AVs in water samples, nonetheless, indicates that true bovine fecal pollution of the reservoir water occurs, since this marker showed absolute host specificity. The presence of the bovine feces-associated bacterial markers in water samples, especially BacCow-UCD, should be interpreted with care, as these markers were detected in fecal samples from nontarget host groups. Bayes' theorem has been used by several researchers to overcome this issue as it indicates the conditional probability of true results and can be valuable to water quality managers for mitigating the contaminating sources (22, 28, 32). Based on Bayes' theorem, there was a 78% probability that the detection of the BacCow-UCD marker in a reservoir water sample was due to true bovine fecal pollution and not due to fecal pollution from nontarget hosts such as chickens, dogs, ducks, kangaroos, pigs, possums, horses, and humans. Similarly, there was a 68% probability that the detection of the cowM3 marker in a reservoir sample was due to true bovine fecal pollution and not due to fecal pollution from nontarget hosts such as dogs, ducks, and possums.

A single marker may not be sufficient to identify the source of fecal pollution unless the marker exhibits absolute host specificity and host sensitivity. BacCow-UCD and cowM3 had 66% agreement and 34% disagreement, whereas B-AVs had 50% agreement with BacCow-UCD. cowM3 had 84% agreement with B-AVs. All three markers tested in this study had disagreement among each other and could generate misleading information if used incorrectly. The consequences of inaccurate source tracking may lead to expensive treatment plans that may not improve the water quality or to overly restricted access to water for recreational purposes. It has been recommended that a "toolbox" approach should be used for the accurate identification of contaminating sources using MST tools (33, 34, 35, 36). The results from the present study also concur that a combination of MST markers would be required to obtain confirmatory results. An obvious disadvantage of using multiple methods is increased expense. Despite any increased costs resulting from the use of a toolbox of MST markers, however, this method would still be more accurate than merely relying on FIB or attempting to directly detect microbial pathogens and would be preferable to dealing with an outbreak of disease caused by an uncontrolled contamination source.

Among the zoonotic bacterial pathogens tested, the *Campylobacter* 16S rRNA gene was the most prevalent. It has to be noted that all *Campylobacter* spp. are not pathogenic. The *E. coli* O157 *rfbE* gene was also detected in 14% of samples, while *Salmonella invA* was not detected in any of the samples. Agreement ranging from 42% to 94% was observed among the markers and the co-occurrence and non-co-occurrence of zoonotic bacterial pathogens. A binary logistic regression was performed to identify the correlation between FIB with the bovine feces-associated markers and zoonotic pathogens. None of the markers or pathogens correlated with the concentrations of FIB. It is possible that inactivation of FIB occurs more rapidly than that of bovine feces-associated markers and pathogens which were detected with PCR. We acknowledge that the PCR results are expressed as the presence/absence for the bovine feces-associated markers and pathogens and do not provide information regarding the degree of fecal pollution. Another limitation of current PCR assays is that they do not provide information regarding the pathogenicity of the target organisms. The inability of PCR assays to distinguish between viable and nonviable pathogenic microorganisms is another issue. Therefore, in this study, the possibility that in some cases, the PCR assays may have detected DNA from nonviable pathogenic microorganisms cannot be ruled out.

In conclusion, the BacCow-UCD and cowM3 markers were detected in fecal samples from both target and nontarget host groups in southeast Queensland, Australia. On the other hand, the

B-AVs marker showed absolute host specificity but low host sensitivity, as its prevalence was low in bovine feces and wastewater. The application of a single marker may not be sensitive enough to provide the evidence of bovine fecal pollution, and therefore, it is recommended that multiple bovine feces-associated markers be used if possible. Nonetheless, the prevalence of these markers in water samples collected from the reservoir suggests that the quality of water may be affected by bovine fecal pollution. The presence of multiple bovine feces-associated markers also suggests the presence of potential zoonotic pathogens in the water. This is further supported by the detection of potential bacterial zoonotic pathogen genes such as *Campylobacter* 16S rRNA and *E. coli* O157 *rfbE* genes in the reservoir water samples. No correlations were observed between the concentrations of FIB and the bovine feces-associated markers or bacterial zoonotic pathogens, thus indicating that FIB could not be relied upon alone to obtain information on the microbiological quality of the reservoir water. An important area for further research is to understand the decay rates of these bovine markers in environmental water samples in relation to FIB and zoonotic pathogens. Additionally, quantitative PCR data would be required to assess the magnitude of fecal pollution and associated public health risks.

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