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Research article

Evaluation of Host Associated Genetic Markers for Rapid PCR Based Identification of Fecal Contamination Sources in Water

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Abstract

The water quality of many waterways in the state of Alabama, and in the nation as a whole, is deteriorating due to point and nonpoint source pollution from human and animal waste. Accurate identification of contamination sources is essential if we are to develop cost-effective pollution control strategies. The direct detection of host specific genetic markers by Polymerase Chain Reactions (PCR) has been widely used in identifying sources of fecal contamination in environmental waters. In this study, we conducted experiments to validate genetic markers associated with deer/elk, Canada goose, dog, and cattle for Microbial Source Tracking (MST) in Alabama. End point PCR was performed on 10 raw sewage samples and 133 fecal samples from nine animal species. Our results showed that CowM^3, GFD (goose), and deer/elk associated markers have acceptable specificity and sensitivity, making them suitable for MST studies. However, the dog marker and one of the cattle markers (CowM^2) exhibited cross reactions with other fecal samples. The performance of these host associated markers in environmental water was evaluated using both end point and quantitative PCR (qPCR). Human, goose, and dog markers were detected in several water samples by end point PCR; the human marker and CowM^2 marker were also detected by qPCR. Samples collected after a significant rainfall event showed the highest frequency of genetic marker detection. Both human and Canada geese contributed to fecal pollution in samples from Parkerson Mill Creek.

Abbreviations: PCR: Polymerase Chain Reactions; MST: Microbial Source Tracking; qPCR: quantitative PCR; ADEM: Alabama Department of Environmental Management's; FIB: Fecal Indicator Bacteria; NTC: No Template Controls; AE: Amplification Efficiencies

Introduction

The water quality of many of the waterways in our state, and in the nation generally, is deteriorating due to contamination by both point and nonpoint source pollution from human and animal wastes. Each year, millions of cases of infectious disease result from swimming and bathing in contaminated water or consumption of shellfish harvested from fecal polluted waters [1]. Parkerson Mill Creek, located in east Alabama, is rated "impaired" because it fails to meet the water quality criteria required to support its designated use as a fish and wildlife stream. In 2007, the creek was included on the Alabama Department of Environmental Management's (ADEM) 303(d) list of impaired waters for pathogens from point and non-point pollution sources. (http://adem.alabama.gov/programs/water/303d.cnt). Urban runoff, pet waste, wildlife, and leaky sewer lines have all been thought to contribute to the high level of *E. coli* concentration (Parkerson Mill Creek Watershed Management Plan, 2010). However, more information is needed to definitively identify the major sources of fecal pollution in order to develop better strategies to protect against the health risks posed by polluted water. Fecal Indicator Bacteria (FIB) such as *Escherichia coli* (*E. coli*) and *Entero cocci* have been used to indicate fecal pollution and potential human health risks in surface water for some time (USEPA, 1986). However, FIB methods are not specific to any fecal sources and require at least 24 h to obtain results [2,3]. It is also possible for *E. coli* and *Entero cocci* to survive and regrow in sediment after being released into the environment [4-6]. Given that the accurate identification of contamination sources is essential for developing cost effective pollution control strategies, increasing interest is now being directed towards the use of library and cultivation

independent Microbial Source Tracking (MST) methods based on the polymerase chain reaction (PCR) technique that target host specific molecular markers. Combining MST methods with end point PCR and qPCR should provide results that reflect the most recent fecal pollution and identify the sources, thus enabling us to develop better pollution control strategies [7-9].

Members of the order *Bacteroidales* are considered promising fecal indicators with which to monitor microbial water quality due to their host specificity, broad geographic stability, and high abundance in the gastrointestinal tract of warm-blooded animals [10-17]. However, *Acteroidales* are not present in the feces of every individual member of a species and the concentrations may also vary from one to another [18] suggested that *Bacteroides* in gulls are scarce and the horizontal transfer of Bacteroides from humans to gulls is common. As a result, the avian markers used in the current study targeted bacteria namely *Catellicoccus marimammalium.* The objective of this study was therefore to evaluate host specific genetic markers associated with dogs, cattle, geese and deer that are likely to affect the local watershed. Environmental water samples collected from Parkerson Mill Creek were used to determine the performance of these markers in the field.

Materials and Methods

Sample Collection

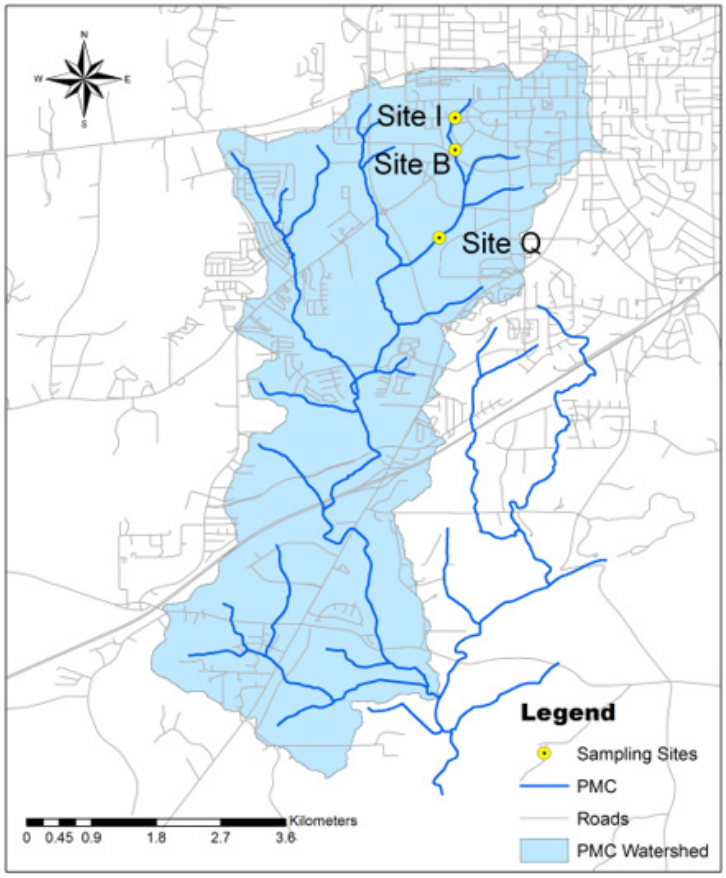

Figure 1: Sampling sites in the Parkerson Mill Creek watershed.

A total of 133 fecal samples and 10 wastewater samples were collected around three cities in east central Alabama, Auburn, Opelika, and Montgomery. The fecal specimens represented nine different animal species (cattle, Canada goose, cat, chicken, deer, dog, duck, goat, and horse) likely to affect the watersheds statewide and were collected with sterile wooden spatulas and placed in sterile polyethylene tubes. Ten raw human sewage samples were collected from nearby wastewater treatment plants in sterile 1-liter bottles. All samples were kept on ice and transported to the lab on the day of collection. Sewage samples were centrifuged at 5000 rpm at 4°C for 15 minutes to concentrate the solid materials 10-fold and fecal samples were stored at -80°C until use. On each of four days during the months of April and May in 2013 (April 12, 19, 26, and May 3rd), environmental water samples were collected from the surface of the water in three different sites in the Parkerson Mill Creek watershed (Figure1), a total of 12 samples were collected in sterile 1-liter plastic bottles. In order to extract the bacterial cells from the water, 500 ml of each sample was vacuum filtered through 0.45µm pore size, 47mm diameter nitrocellulose membrane filters (Thermo Fisher Scientific, Waltham, MA). The membrane filters were then stored at -20°C prior to DNA extraction.

DNA Extraction

All DNA extractions were performed using the Power Soil™ DNA Isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA). Following the manufacturer's instructions, 0.25 g of each fecal sample or 300µl of concentrated sewage were used for the DNA extraction. DNA from water samples were extracted from membrane filters that had been cut into small pieces prior to extraction. DNA concentrations were quantified using a NanoDrop ND-1000 UV spectrophotometer (Thermo Scientific, Wilmington, USA).

End-Point PCR and qPCR Assays

The primers used in this study are listed in Table 1. End-point PCRs were performed on a $TG_{RADIENT}$ thermal cycler (Whatman Biometra®, Germany). Each 25µl reaction mixture contained 5µl of 5x colorless GoTaq® Flexi buffer, 1.5mm of $MgCl_2$ solution, 0.2 mM of dNTPs, 0.5µm each of the forward and reverse primers for the genetic markers, 0.4 mg/ml of bovine serum albumin (BSA), 0.08 unit/µl GoTaq® DNA polymerase, 2.0µl template DNA, and an appropriate volume of PCR grade water. The thermal cycling parameters for each PCR assay were 94°C for 2 min, followed by 30 cycles of 94°C 60s, 60°C (for the different markers' annealing temperature please see (Table 1) 45 s, and 72°C 60 s, then 72°C for 7 min. The end-point PCR products were resolved using 1.5% agarose gel electrophorese and viewed under UV light to verify the absence or presence of the target gene. No Template Controls (NTC) containing PCR grade water only and positive controls were included in each instrument run for quality control. Real time PCR assays (All Bac, HF183, $CowM^3$, and $CowM^2$) were performed using the Step One real time PCR instrument (Applied Biosystems, NY). The reaction mixture (15µl) contained 1x SSO Advanced™ SYBR® Green Super mix (BIO-RAD, CA), 0.7µg/µl BSA, 0.2µm of each primer and 5µl of template DNA. All reactions were performed in duplicate and began with a hold at 95°C for 10 min, followed by 40 cycles of 95°C 15s, 60°C 30 s, and 72°C 30 s. For each set of experiments, a no template control with two replicates was included and a calibration curve with a concentration spanning the range from 10 to 10^6 gene copies per reaction with two replicates was constructed.

Table 1: Comparison of sensitivities and specificities of the host-associated genetic markers.

Fecal source	No	CowM3	CowM2	GFD	Elk	Dog
Cattle	11	11/11 (100%)	9/11 (81.8%)	0/11 (0%)	3/11 (27.3%)	0/11 (0%)
Canada Geese	26	0/26 (0%)	9/26 (34.6%)	22/26 (84.6%)	0/26 (0%)	0/26 (0%)
Cat	12	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)
Chicken	13	0/13 (0%)	7/13 (53.8%)	3/11 (27.3%)	0/13 (0%)	0/13 (0%)
Deer	26	2/26 (7.7%)	0/26 (0%)	0/26 (0%)	26/26 (100%)	0/26 (0%)
Dog	21	0/21 (0%)	15/21 (71.4%)	0/21 (0%)	0/21 (0%)	12/22 (54.5%)
Duck	9	0/9 (0%)	6/9 (66.7%)	0/8 (0%)	0/8 (0%)	0/8 (0%)
Goat	3	0/3 (0%)	0/3 (0%)	0/3 (0%)	3/3 (100%)	0/3 (0%)
Horse	12	0/12 (0%)	0/12 (0%)	0/14 (0%)	0/14 (0%)	14/14 (100%)
Sewage sample	10	2/10 (20%)	5/10 (50%)	0/10 (0%)	0/10 (0%)	10/10 (100%)

Data Analysis

The Amplification Efficiencies (AE) were calculated based on the following equation:

$$E = 10^{(-1/slope)} - 1$$

The related statistical analyses were performed using SAS® 9.3 software. ArcGIS 10.2 software for desk top was used to generate a sampling map for the Parkerson Mill Creek watershed.

Results

DNA extracts from a total of 133 fecal samples and 10 wastewater samples were analyzed and the results are shown in Table 1. The $CowM^3$ *Bacteroidales* marker exhibited 100% sensitivity and 97.1% specificity, so the assay met the 80% benchmark suggested by the USEPA (2005) for both specificity and sensitivity. However, false positive amplification was also observed in four non-target DNA samples (two sewage and two deer samples). The $CowM^2$ marker was present in 9 of 11 cattle fecal samples, resulting in 81.8% sensitivity. The CowM2 marker cross reacted with 42 non-target fecal DNA samples: 34.6% (n=9) Canada goose, 66.7% (n=6) duck, 50% (n=5) sewage, 71.4% (n=15) dog, and 53.8% (n=7) chicken, resulting in 68.2% specificity. Although the GFC marker for Canada goose was detected in 84.6% of the goose fecal samples it also exhibited a 100 % cross-reaction with human fecal samples, so in this case, we chose to use the GFD marker in our primer evaluation study instead. The GFD marker was positive in 84.6% and 27.3% of goose and chicken samples, respectively (Figure 2). The overall specificity and sensitivity of the GFD marker were 97.4% and 84.6%, respectively and although it had some cross reaction with chicken fecal samples (27.3%), it fully distinguished duck samples. Similarly, the elk marker was positive in 100% of the deer fecal samples, though it also cross reacted with the cattle (3/11) and goat (3/3) samples. The overall specificity and sensitivity of the elk marker were 94.9% and 100%, respectively. The dog marker was detected in 12 out of 22 dog fecal samples, 10 out of 10 sewage

samples, and 14 out of 14 horse fecal samples, giving the dog marker the lowest values for specificity and sensitivity, at 80.2% and 54.5%, respectively, of the species tested. The All Bac genetic marker targeting the general *Bacteroidales* was detected in all 12 environmental water samples (Table 2). The human marker was detected in 6 out of 12 and 7 out of 12 water samples in the end-point and real-time PCR assays, respectively. The Site B samples for all four sampling dates were positive for the human marker, as were the samples collected on April 12 and April 26 at site Q. Similarly, the dog marker was detected in one third (4 out of 12) of the water samples. The marker for Canada goose was detected in 58.3% (7 out of 12) of the water samples, with every sample from site B testing positive for this marker. Two samples from site Q, collected on April 12 and 26, showed positive results for the GFD marker. Neither CowM[3] nor elk markers were detected in any of the 12 water samples.

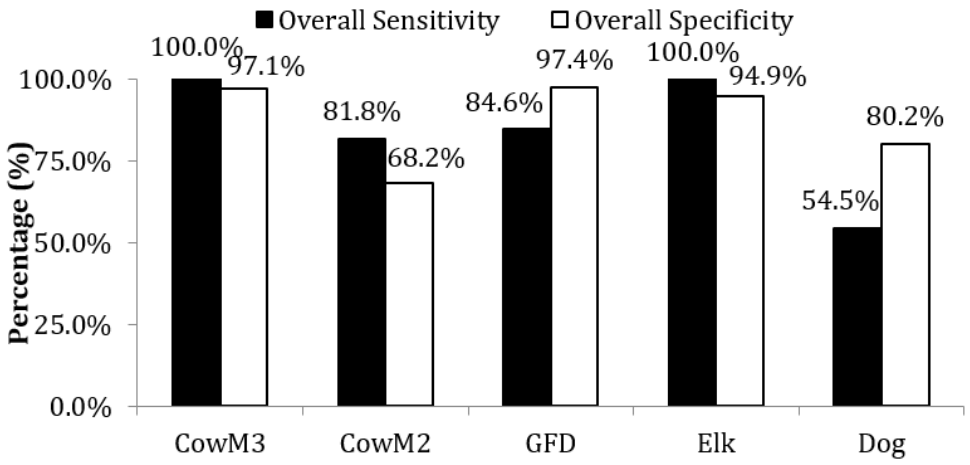

Figure 2: Overall sensitivity and specificity of the different markers tested.

Table 2: Detection of host associated genetic markers in water samples collected from Parkerson Mill Creek

Date	Site	AllBac		HF183		CowM3		CowM2		GFD	Elk	Dog
		PCR	qPCR	PCR	qPCR	PCR	qPCR	PCR	qPCR	PCR	PCR	PCR
12 Apr	I	+	4.49	-	UN	-	UN	-	3.13	+	-	+
	B	+	5.24	+	>6.00	-	UN	-	3.12	+	-	+
	Q	+	4.70	+	1.97	-	UN	-	2.95	+	-	+
19 Apr	I	+	4.22	-	UN	-	UN	-	2.70	-	-	-
	B	+	4.72	+	DNQ	-	UN	-	3.43	+	-	-
	Q	+	4.48	-	DNQ	-	UN	-	2.92	-	-	-
26 Apr	I	+	4.59	-	UN	-	UN	-	3.26	-	-	-
	B	+	5.78	+	4.39	-	UN	-	2.97	+	-	+
	Q	+	4.81	+	DNQ	-	UN	-	2.81	+	-	-
3 May	I	+	4.64	-	UN	-	UN	-	2.87	-	-	-
	B	+	5.05	+	DNQ	-	UN	-	3.01	+	-	-
	Q	+	4.59	-	UN	-	UN	-	2.77	-	-	-

(The unit for real-time PCR: log10 copies/100ml water).

All of the 12 environmental water samples collected from the three locations were positive for *E. coli* (Figure 3), with concentrations ranging from 225 CFU/100 ml on April 19 at site B to 5200 CFU/100 ml on April 26 at site I. Both Site B and Site Q had significant high concentrations on April 12, probably due to the rainfall on that day. Site I had the largest geometric mean for *E. coli* concentration and site B the lowest. All sites exceeded the USEPA's criterion for recreational water quality (USEPA 2012), which is a geometric mean of 126 CFU/100ml water. Eleven out of the 12 samples also exceeded the USEPA's single sample maximum for *E. coli* concentration, which is 410 CFU/100ml. The relationship between the *E. coli* concentration and the All Bac marker concentration was weak (results didn't show).

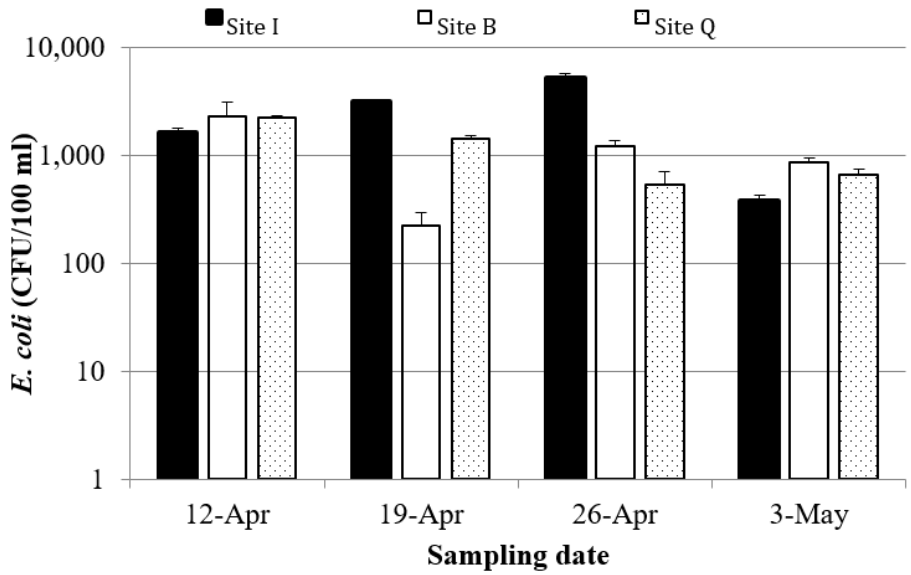

Figure 3: *E. coli* concentrations at all sampling locations for different dates (CFU/100ml).

Discussion

The Canada goose specific genetic markers (GFC and GFD) were chosen for evaluation in our study. The other genetic markers used in our study to identify the sources of fecal pollution were based on *Bacteroidales* and its relatives (Table 1), as the order of *Bacteroidales* is known to be both abundant and common in mammalian feces. However [19] characterized the fecal microbial community from Canada goose, suggesting that the majority of the genes sequenced were related to *Clostridia* or *Bacilli* or, to a lesser degree, *Bacteroidetes*. Canada goose *Bacteroidales* specific genetic markers have also been reported elsewhere [20], but these genetic markers were not chosen for the current study because although they are relatively temporally stable, they have low sensitivities. In Green [18] study, the GFC and GFD markers targeted were *Catellicoccus marimammalium* and *Helicobater spp*., respectively, with GFC occurring at a higher concentration as more ribosomal operons in *Catelli coccus*. However, the GFC marker failed to distinguish between waste pollution from human and goose samples in the present study, so we discontinued our evaluation of the GFC marker on other fecal samples. This result suggests that genetic markers need to be validated across a range of conditions, even when they appear to be highly specific when initially reported. Here, the GFD marker exhibited a 27.3% cross amplification with chicken samples, which is consistent with Green et al.'s research as GFD was originally developed to detect avian fecal samples. We found that it actually had a higher sensitivity (84.6%) on goose samples than the 68% reported in Green et al.'s original study. Therefore, our results for the GFD marker support the sensitivity and specificity of PCR assays for identifying Canada goose-associated fecal pollution in freshwater. CowM^2 and CowM^3 are both well-developed cattle associated *Bacteroidales* genetic markers that have been widely used in various MST research studies. Although CowM^2 was reported to perform better than CowM^3 by Raith et al. [21], a lower sensitivity for CowM^2 (50%) has also been reported elsewhere [22]. However, the applicability of those results to other regions is potentially limited due to factors such as host diet, climate and geographic location. In our study, we found a much lower specificity for CowM2 (68.2%) compared with previous studies, some of which have reported values of over 98% [21,23,24]. There are several possible reasons for this discrepancy. First, geographical differences could affect host associated *Bacteroidales* markers significantly due to differences in the diet and animal digestive tract physiology. Layton [17] found that *Bacteroidales* 16S rRNA gene sequences obtained from pig were more closely related to *Bacteroidales* 16S rRNA gene sequences obtained from humans than to cattle sources, even though pig and cattle are in the same order of Artiodactyla. Second, the evaluation of the same set of samples may produce different results when examined from a presence absence or quantitative perspective. When there are cross reactions with non-target feces in PCR assays, this is usually at a low level compared with the signal for the target feces and will thus tend to be classified as false positives in end point PCR evaluations but not in real time PCR evaluations [25]. Third, the decay rates for the host-associated markers in the environment may be different due to their size and function [26]. CowM^2 targets a 437 bp fragment as encoding an HDIG domain protein involved in energy metabolism and electron transport, while CowM3 targets a 569 bp fragment encoding a sialic acid-specific 9-O-acetylesterase secretory protein involving cell envelope biosynthesis and the degradation of surface polysaccharides and lipopolysaccharides [27-28], so the decay rate of these two proteins in environmental water after release from local animal tracts or the abundance variation of the proteins in fecal samples may explain the discrepancy.

The CowM^3 marker, on the other hand, had an overall specificity of 97.1 % and 100% sensitivity, which is consistent with previous studies that reported CowM^3 to have both a broader target host distribution and greater stability [21,27]. The relative abundance of the host associated genetic markers for CowM^3 was 32.6 times greater than the CowM^2 marker concentration in the same DNA sample, and this value compares favorably with the results previously reported by Shanks [28]. The amount of target gene in each cell may explain the different target copies detected in the same DNA samples by the different markers [29] pointed out that the CowM^2 marker targets a single copy gene involved in energy metabolism. Here we hypothesize that there may be two or more CowM^3 target genes involved in cell envelope biosynthesis and the degradation of surface polysaccharides and lipopolysaccharides. This result also indicates that not only is CowM^3 more specific, but it also has higher sensitivity and a lower detection limit than CowM^2 due to its greater abundance. Thus, it will be necessary to validate the specific genetic marker that will be used in each different geographic location because the performance characteristics may change and will thus affect the evaluation results. The dog associated marker DF475F was paired with *Bacteroidales* specific Bac708R and analyzed in our study against 143 target and non-target DNA samples. Dick [30] who developed this dog marker, found no cross amplifications with human, cat, cow, pig, chicken, or gull sources. However, they also pointed out that the horizontal transfer of fecal bacteria may occur among species in close contact, such as humans and their pets, which suggests the potential for cross reactions with the dog marker in human samples. This is probably why our results showed that this primer set amplified 100% of the sewage DNA representing human sources. Since this primer was the first and only dog specific primer that has yet been identified, the similar results for dog primer in Dick et al.'s research suggests the need for future work in this area to optimize the primer and reaction. Elk primer was found to amplify both the cattle and goat samples, which is consistent with previous studies that reported that deer/elk primer could not distinguish between *Bacteroidales* sequences from deer/elk and sheep. Our results suggest that combining the results from CowM^2, CowM^3, and elk markers should make it possible to distinguish between cattle and deer/elk fecal pollution.

The All Bac genetic marker was designed to target the 16S rRNA genes of *Bacteroides* spp. and provides a rapid direct measurement of fecal contamination in water due to feces from warm-blooded animal sources [16]. The positive results for the All Bac genetic marker in all the water samples in the present study provides an estimate of the total fecal contamination present in the water samples. The human marker was detected in 6/12 and 7/12 of the water samples using end point and real time PCR assays, respectively. All samples from site B has been detected with HF183 marker, which suggests a potential source of human fecal pollution close to this sampling site. However, there was no CowM^3 signal detected by either end-point PCR or real-time PCR. The discrepancy results were observed between end-point and real time PCR assays with CowM^2 marker probably due to the cross-reaction of CowM^2 marker with non-target feces since the low target concentration was detected. Given the lack of signal detected for the deer/elk marker, the positive signal for CowM^2 in the environmental water samples is probably due to the presence of human fecal pollution. The samples that were positive for human signals also amplified the dog marker; these sites were Site B and Site Q on April 12, and Site B on April 26. Since the dog marker was detected in 100% of the human samples, the positive signal for the dog marker in the water samples was probably due to the presence of human fecal pollution. Similar results for the GFD marker are likely to indicate the presence of fecal pollution from Canada goose; during the sampling season, Canada geese were observed around the sampling site, which is consistent with these results. Interestingly, there was no signal detected due to the CowM^3 marker in samples collected from site Q, even though this site is close to the beef teaching center at Auburn University and beef cattle were observed on site. That was probably because site Q was located at the upstream of beef teaching center. Future work may be needed to add more sampling sites locate downstream of beef teaching center. This result suggests the capacity of MST to identify major pollution sources from among many possible sources.

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