

# **Fecal Source Tracking in the Wolf Run Watershed of Lexington, KY using molecular methods for *Bacteroides* bacteria**

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## **Introduction**

*Bacteroides* bacteria are obligate anaerobes found in the intestines and colon of humans and animals in large concentrations. Fortunately for those interested in fecal source tracking, they have been shown to have different 16S rRNA gene markers specific to the host animal. (Shanks, et. al. 2006) Identifying the presence of these specific markers in environmental water samples has been shown to correlate well with known source information and with other source tracking tools. (Shanks, et. al. 2006) (Gourmelon, et. al. 2007) (Dick, et. al. 2005) (Bernhard and Field, 2000) This study of the Wolf Run watershed in Lexington, KY attempts to determine the usefulness of these published PCR primers specific for cow or human markers in an urban environment of the southeastern United States and to compare the AC/TC (Brion, 2000) ratios and *E.coli* enumerations with the molecular signal.

## **Sampling Sites**

The creeks used in this study were selected from within the city limits of Lexington. The area is largely residential but also includes a horse track and some farming. The sites are part of the ongoing stream monitoring by Watershed Watch of Kentucky. Several years of historical data exists for these sites including chemical, physical, and microbial parameters. McConnell springs site is a karst spring. The sample ID's are as follows:

K54 McConnell Springs  
K184 Wolf Run at Gardenside Park  
K461 Cardinal Run at Davenport  
K465 Wolf Run below Cambridge bridge  
K470 Vaughn's Branch upstream of Wolf Run

## **Methods and Materials**

*E.coli* analysis was performed using Idexx Quantitray 2000 and Colilert media for most probable number calculation. AC/TC ratio was calculated from non-sheen and sheen colony counts by membrane filtrations grown on mF endo media. Samples were run at 3 filter volumes and each filter volume was run in duplicate.

The PCR primers used in this study were designed and published previously. (Bernhard and Field 2000) Bac32F is a general forward primer for all *Bacteroidales* bacteria and is not specific to any host animal. CF128F is a forward primer specific for bovine host. HF134F and HF183F are forward primers specific for human host.

Bac708R is a reverse primer general for all *Bacteroidales* and is used with each of the forward primers.

Each of the 5 sites was sampled on 5 dates from the end of July to mid August. Samples were collected in sterile 1L polypropylene containers and transported to the lab on ice. 500mL aliquots were filtered through 0.45 µm cellulose ester membrane filters. DNA was extracted from the filters using UltraClean Water DNA isolation kits (MoBio). Each 25 µL PCR reaction consisted of 12.5 µL GoTag Green master mix (Promega), 0.5 µL forward primer (Bac32F, CF128F, HF183F, or HF134F), 0.5 µL reverse primer (Bac708R), 9.5 µL sterile, nuclease free water, and 2 µL DNA template. PCR reaction mixtures were denatured at 94°C for 3 minutes and followed by 35 cycles of 94°C for 1 minute, 53°C for 1 minute and 72°C for 1.5 minutes and then held at 4°C until electrophoresis on 1.5% agarose gels.

Of the 25 samples collected, 2 were duplicated starting at filtration. A filtration blank was also carried through the entire method to ensure absence of contamination at each step. Waste water treatment plant influent was used as a positive control for human signal and a floor washings sample from a dairy barn was used as a positive control for bovine signal. To rule out the possibility of false negatives due to PCR inhibition by organic materials in the samples, a representative of each sample site was spiked with positive control and carried through the PCR protocol.

## Results

All of the samples and duplicates were positive for general *Bacteroidales* by the Bac32F primer with varying levels of intensity viewed on the gels. None of the samples from the Wolf Run study were positive for bovine signal by the CF128F primer. A sample was collected from West Hickman creek which is outside of the Wolf Run watershed and is impacted by agricultural activities. This sample gave a positive signal for bovine by the CF128F primer and was negative for human markers by both human specific primers. Of the 5 samples from McConnell Springs (K54), 2 were positive for human by HF183F and 4 were positive for human by HF134F. The majority of these were of average intensity and one was a very strong positive. Wolf Run at Gardenside (K184) was positive for human signal on 3 of the 5 sampling dates. Of the 5 samples from Vaughn's Branch (K470), 3 were positive for human by HF183F and 4 were positive for human by HF134F. The majority of these positives were of very weak intensity. None of the samples from Cardinal Run (K461) were positive for human markers by either primer. The one Wolf Run below Cambridge bridge sample (K465) that was positive for human on HF134F gave a very weak signal. All of the positive controls and the PCR inhibition control spikes were positive by their respective primers and the blank was negative on all primers.

Individual AC/TC ratios ranged from a low of 2.8 to a high of 614. The AC/TC ratios for individual samples were below 20 on 16 of the 25 samples. The *E. coli* enumerations ranged between <10 and almost 3000 per 100mL.

**Table 1. Wolf Run Watershed Data July-August 2007**

Sample ID	Date	AC/100mL	TC/100mL	AC/TC	EC/100mL	Bac 32F	CF 128F	HF 183F	HF 134F
West Hickman	7/25/2007					+	+		
K54	7/30/2007	29000	2950	9.83	546	+			+
K54	8/2/2007	13500	1300	10.38	148	+			+
K54	8/6/2007	34500	1800	19.17	161	+			
K54	8/8/2007	29000	4750	6.11	238	+		+	+
K54	8/13/2007	16000	1100	14.55	86	+		+	+
K184	7/30/2007	57500	3400	16.91	384	+		+	+
K184	8/2/2007	40500	2200	18.41	496	+			
K184	8/6/2007	80000	6000	13.33	2909	+			
K184	8/8/2007	67500	2950	22.88	1223	+			+
K184	8/13/2007	195000	27500	7.09	2755	+		+	+
K461	7/30/2007	164500	5800	28.36	650	+			
K461	8/2/2007	59500	21000	2.83	1467	+			
K461	8/6/2007	34000	3950	8.61	613	+			
K461	8/8/2007	35000	2900	12.07	393	+			
K461	8/13/2007	29500	1400	21.07	282	+			
K465	7/30/2007	89500	2950	30.34	481	+			
K465	8/2/2007	49500	4100	12.07	520	+			+
K465	8/6/2007	57000	6864	8.3	708	+			
K465	8/8/2007	475000	19000	25	295	+			
K465	8/13/2007	215000	350	614.29	<10	+			
K470	7/30/2007	90500	4850	18.66	959	+		+	+
K470	8/2/2007	62500	1800	34.72	691	+		+	+
K470 duplicate	8/2/2007	71500	2250	31.78	691	+			+
K470	8/6/2007	60500	3150	19.21	1106	+			
K470	8/8/2007	68000	1850	36.76	857	+			+
K470 duplicate	8/8/2007	88500	1700	52.06	663	+		+	+
K470	8/13/2007	91500	2050	44.63	204	+			+
Blank	8/6/2007	0	0	N/A	0				
cow + control						+	+		
human + control						+		+	N/A
K54 with + cow						N/A	+		
K54 with + human						N/A		+	N/A
K184 with + cow						N/A	+		
K184 with + human						N/A		+	N/A
K461 with + cow						N/A	+		
K461 with + human						N/A		+	N/A
K465 with + cow						N/A	+		
K465 with + human						N/A		+	N/A
K470 with + human						N/A		N/A	+

"+" very strong

"+" average

"+" very weak

**Table 2: Sites Ranked by Averaged AC/TC Values** (duplicates excluded from calculations)

Site ID	Average AC/TC	Geometric Mean <i>E.coli</i>	Frequency of Human Bacteriodes marker Presence	Signal Strength Comments
K54	12.0	193	4 of 5 samples	Mixed signal strengths, but average is medium
K184	15.7	1133	3 of 5	Strongest signal detected
K461	14.6	578	0 of 5	
K470	30.8	663	4 of 5	Faint signals with 1 medium signal strength
K465	138.0	478	1 of 5	Faint signal

### Table 3. Project Costs

<b>Microbial Methods</b>			
Supplies for E. coli			\$150
Supplies for AC/TC			\$440
Labor	50hrs	12.00/hr	\$600
<b>Molecular Methods</b>			
Supplies (not including method development)			\$892
Labor (not including method development)	50hrs	45.00/hr	\$2,250
Supplies for method development			\$1,520
Labor for method development	120hrs	45.00/hr	\$5,400
<b>Project Total</b>			<b>\$11,252</b>
Paid by Friends of Wolf Run	\$3,000		
ERTL contribution	\$8,252		

estimated price for analysis of samples by molecular methods using current protocol and assuming large batches of samples \$200/sample

estimated cost of method development for other existing primers (wild fowl, pig, etc) and development of greater sensitivity using nested PCR \$10,000

additional overhead may be required and will be negotiated with the University of Kentucky

### Discussion

Although the PCR inhibition spikes were positive and it can therefore be determined that the matrix is not causing false negatives, the sensitivity of the method has not been determined so it is not accurate at this time to assume the absence of human fecal material when there is no signal present. This data is convincing, however, that there is not a likely occurrence of false positives as the control samples repeatedly give a signal only with the appropriate primer.

There is an obvious trend in the positive signals for the human specific primers. K54 and K470 repeatedly give positive results showing that the determination is repeatable at the same sample location over time while K461 and K465 repeatedly show no positive signals.

Of the 16 samples with AC/TC ratios below 20, 8 were positive for human signal by one or both of the human specific primers. However, past research has shown that individual AC/TC values should not be used for ranking stream water quality or hot-spot

analysis, but averaged values from multiple days at the same site. (Brion, 2006) Average values for the AC/TC ratios were calculated and are shown in Table 2. The averaged AC/TC ratios on samples K54 and K184 (12.0 and 15.7 respectively) were lower than the average value of 19 found for Lexington's urban watersheds in a prior study calculated from 134 samples collected from flowing urban streams during dry weather over three years. (Table 4) (Brion, 2006) This can indicate that these sites are on average carrying fresher fecal material than expected, perhaps from human sources. The detection frequency for human specific *Bacteroides* was greater than 50% for both sites with 4 of 5 samples positive at K54 and 3 of 5 at K184. In addition to the high frequency of detecting human specific *Bacteroides* genomic material, the signal strength at these two sites was stronger than found in human specific *Bacteroides* detections at sites K465 and K470, both of which had average AC/TC ratios greater than 20. Samples from site K461 had an average AC/TC ratio of 14.6, a geometric mean for *E.coli* of 578, and showed no positive signals for human markers. These results may indicate the input of a fresh source of animal fecal material near to the sample site based on similar results in previous studies. (Brion, 2000) (Brion, 2006)

Table 4. Average bacterial ratios in surface water runoff sorted by land-use associated fecal sources

<i>Site Classification</i>	<i>Average AC/TC Ratio (n)</i>	<i>Average FC/FS Ratio (n)</i>
Impounded Suburban	102.6 (75)	3.7 (76)
Flowing Suburban	18.9 (134)	5.2 (124)
Flowing Agricultural	10.0 (59)	5.0 (45)
Raw Human Sewage	3.9 (11)	3.3 (11)

Reproduced from Brion 2006

The *E. coli* enumerations do not seem to correlate with the probability of finding a positive signal for human specific *Bacteroides* markers, or to the AC/TC value. The *E.coli* concentration in all of these streams is higher than one would like to see for recreational contact.

This molecular source tracking method proves to be very cost effective for determining the presence of human fecal pollution. At \$200 per sample, it is less expensive than some other source tracking methods including molecular library dependant methods and chemical methods

## Continuation

The efficacy of this molecular method for fecal source tracking in Kentucky can be further defined by the continuation of this study. The work should be expanded to include samples from rural, agricultural, and industrial environments as well as more primers and positive controls specific for other animal hosts. A close sanitary survey of the areas surrounding these sites is recommended to identify potential inputs of fresh human or animal fecal material.

The current method of presence or absence determinations could be altered with the use of Real-Time PCR to generate quantitative data with these markers. This would allow true quantitation of signal strength as opposed to the current estimation of strong, average, or weak from visual observation.

The sensitivity of the method should also be increased by using other molecular techniques including further concentration of the filter extract and nested PCR. Nested PCR may increase the sensitivity by using the general primer to amplify the signal initially and then performing a second PCR analysis with the specific primers while using the re-extracted product from the first run as the template. The sensitivity of the method should also be measured by running serial dilutions of a known positive control like the wastewater treatment plant influent until a visible band can no longer be seen on a gel. This will allow us to make a general determination of comparative concentrations in the stream.

As much of the Bluegrass region of Kentucky is inhabited by horse farms, more work should be done to develop a horse specific primer.

## References

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