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Summary: The purpose of this work was to evaluate and analyze water samples collected from the Kings Creek watershed using a qPCR-based method to detect both total *Bacteroidale*s and *Bacteroidales* reported to be associated with human fecal contamination. Quantitative real-time PCR assays were used to significantly reduce processing times and at the same time yield estimates of target concentrations. Initial efforts focused on evaluation of various *Bacteroidales* primer sets reported in the literature tested against human and animal fecal samples collected from the Kings Creek watershed. Most samples, both animal and human, were positive with the universal (i.e. general or total) *Bacteroidales* assay. Strong positive signals were found with human sewage using the human-specific assay that was chosen for this study. Most animal scat samples were negative with respect to the human-specific *Bacteroidales* indicator. The few animal samples that were positive with the human-specific assay had very low signal intensity. Despite the generally pervasive drought conditions during this study, evidence of human contamination was detected at certain feeder stream locations, and was widespread after a significant rain event that occurred in late fall. Use of the human-specific *Bacteroidales* indicator holds promise as a tool to identify potential human, as opposed to animal, sources of contamination but will require a more comprehensive field monitoring and sample collection effort than could be managed in this preliminary study.

Introduction

In recent years bacteria belonging to the family *Bacteroidales* have been used or proposed as the basis for non-library based methods to detect fecal contamination. Library-based methods such as antibiotic resistance analysis (ARA) are cumbersome and criticisms include inadequate library size and source representation, a poor understanding of how stable library entries are over time, and diminished effectiveness when applied across different watersheds. Library-free methods targeting specific bacterial gene sequences avoid many of these problems and are now being evaluated for both risk assessment and detection of specific fecal sources. Bacteria belonging to the order *Bacteroidales* can be found at comparatively high densities in both human and animal

feces, as well as in sewage. Moreover, as obligate anaerobes inhabiting warm blooded animals, these bacteria would not be expected to grow outside of animal digestive tracks. Recent reports support the use of DNA gene sequences in the *Bacteroidales* group as human- or animal-specific indicators for fecal source tracking (Shanks et al. 2009; Haugland et al. 2010).

Because the bacteria targeted in this group are obligate anaerobes, enumeration by cultivation from field samples is difficult and time consuming. With the advent of PCR methods, which do not require cultivation and which can be designed to target specific regions of the bacterial 16S rRNA gene, a variety of universal or general PCR assays have been developed to detect total *Bacteroidales*. In addition, others have been designed to target either human-specific strains or only those strains that are animal-associated members of the group. However, none of these assays are 100% specific because many of the bacterial species have not been well characterized, especially with respect to their occurrence in animal sources. That being said Shanks et al. (2009) have recently described two sets of human-specific qPCR assays that showed relatively high specificity for human feces when they were tested against a variety of common animal fecal samples. Moreover, the human-specific qPCR assays did not amplify *Bacteroidales* present in cats or dogs in their samples. These assays show promise as fecal tracking tools to detect human-specific contamination.

The purpose of this study was to analyze water samples collected from both Kings Creek and its watershed using a qPCR-based method to detect both total *Bacteroidale*s and *Bacteroidales* reported to be associated with human fecal contamination. Quantitative real-time PCR assays can significantly reduce processing times and at the same time yield estimates of target concentrations. We proposed to assay a total of 50 samples using three primer sets coupled with TaqMan probes. During the preliminary portion of this study it became evident that considerably more work validating the different primer sets was necessary because of significant differences when the sensitivities of detection of primer sets against known fecal samples were compared. Thus, although a primer set might be highly specific for human sewage, several were found to be orders of magnitude less sensitive and therefore, less likely to detect contamination in field samples. This observation required a thorough comparative analysis before collected samples were processed.

During our initial site visit we met with local concerned stakeholders and discussed the kinds of wild and domestic animals that could affect water quality within the watershed. We used this information along with data from the literature to initially select which of the published assays would be the best match for the Northampton watershed. Some universal assays, for example, do not detect *Bacteroidales* from deer and our visit with the stakeholders indicated that

deer were likely to have a high presence in the watershed. Following these discussion we chose four human-specific assays and three universal assays (Table 1) for evaluation. Human sewage samples were obtained from the Cape Charles WWTP to validate and test the sensitivity of the human-specific assays. Unfortunately, stakeholders were unable to obtain septage samples for evaluation. The stakeholders collected animal fecal samples to confirm the specificities and sensitivities of the universal assay primer sets and to determine if they produced negative results with the human-specific assays. We also used a panel of available bacterial DNA from a variety of common bacteria (*E. coli*, *E. faecalis*, *S. flexneri*) as negative controls to confirm that the assays were specific to the *Bacteroidales* group. *B. thetaiotamicron* and *B. stercoris* are positive controls and used for qPCR calibration curves.

Table 1: Universal and human-specific assays used to detect *Bacteriodales* in this study.

Real-time Taqman Assay	**Results**	**Reference**
Human – BFD	cross-reacts: other bacteria; dog, goose, raccoon, deer	Converse et al. 2009
Human – HF183	no signal: *B. thetaiotamicron, B. stercoris;* influent +	Bernhard & Field, 2000
Human – BsteriF1	best human-specific assay, cross-reacts dog/raccoon	Haugland et al. 2010
Human – BacHum	no signal: *B. thetaiotamicron, B. stercoris;* influent -	Kildare et al. 2007
General – BthetaF2	low signal for influent samples; no signal for many animal samples	Haugland et al. 2010
General – GenBac3	best universal assay tested (also the assay used by the EPA)	Siefring et al. 2008
General – BacUni	no signal: *B. thetaiotamicron*	Kildare et al. 2007

Based on the results shown above the highlighted assays were determined best for this study. Sampling station locations were determined after an initial site visit and discussion with stakeholders (Fig. 1). We collected background samples at the station locations where water was present, as well as a Kings Creek sample (expected to be blank), and a sample from a pond regularly visited by geese (expected to have a high *Bacteroidales* signal with the universal assay). We provided stakeholders with collection bottles, disposable filters and other supplies needed for sampling four additional rain events and provided a vacuum pump for filtration of water samples. We demonstrated the sample filtration process and provided the stakeholders with a written protocol for sample processing.

No.	Sampling Site
1	US 13 N. (west)
2	Hardees (west)
3	Hardees (east)
4	Bus. 13 (west)
5	Bus. 13 (east)
6	Bayview Recycle
7	Bayview / Culls
8	KCL Spillway
9	Hogwood East

Fig. 1: Sampling sites for this study located within the King's Creek watershed. Inset lists the number and label assigned to each location

Methods

Sterile collection bottles were used to collect a 100 ml volume of water at each site. Two independent replicate samples were collected from each site on the last sampling date. The samples were stored in a cool, dark location for less than 12 hours before filtration. Water samples were filtered through 47 mm 0.22 micron pore membrane filters under vacuum to concentrate the microorganisms. Filters were placed in microcentrifuge tubes containing glass beads and frozen (-20°C) until collected for processing. DNA was extracted from the bacterial cells concentrated on the filters by the addition of 320 µl of elution buffer (AE, QIAGEN) followed by bead-beating (Haugland et al. 2005). DNA was recovered by centrifugation. Prior to analysis, 10x and 100x fold dilutions of the DNA were prepared to dilute out inhibitors that may be present. Environmental water samples commonly contain substances that interfere with and/or reduce the sensitivity of PCR-based assays and need to be removed. Accordingly, column purification was tested on some samples using a DNA-EZ DNA purification kit (GeneRite) a QIAGEN column purification protocol and a MO BIO column purification protocol. Each of these protocols involved adding a binding buffer to the recovered DNA, applying the DNA/binding buffer onto a purification column, washing the DNA on the column and then eluting the purified DNA off the column. All DNA extractions and purifications included a control blank.

Animal fecal samples were collected by stakeholders and frozen (-20°C) until collected for processing at VIMS. A small aliquot of each fecal sample was weighed and added to a microcentrifuge tube containing glass beads and 320 µl of QIAGEN elution (AE) buffer. DNA was extracted using the bead-beating method and dilutions of the recovered DNA were prepared as above. The DNA was purified using the DNA-EZ purification kit. A second aliquot of each fecal sample was weighed and added to a microcentrifuge tube. DNA from this aliquot was extracted using the QIAGEN QIAamp DNA Stool kit following the manufacturer's instructions. All DNA extractions and purifications included a control blank.

Both *Bacteroides thetaiotaomicron* (ATCC #29741) and *B. stercoris* (ATCC #43183) cultures were purchased for use as positive control standards. After resuscitating the cultures, DNA was isolated by the bead-beating method and then purified using the DNA-EZ purification kit. The DNA was quantified using a NanoDrop spectrophotometer and the concentration of DNA was used to calculate genome equivalents. Genome equivalents are based on the published weight of the bacterial chromosome, or its genome. It is generally assumed that one bacterial cell = one genome equivalent. Ten-fold serial dilutions were performed to prepare a five-point standard curve.

Each TaqMan qPCR assay was tested on the Applied Biosystems 7500Fast Real-Time PCR System using the TaqMan Universal PCR master mix from Applied Biosystems. The assays were tested using the *B. thetaiotaomicron* standard, DNA from influent sewage samples, and DNA from other bacteria. Each assay was initially tested using the Applied Biosystems kit protocol in 10 µl volumes with the published primer and probe concentrations. They were optimized for primer and probe concentration, the addition of bovine serum albumin (BSA), and the number of PCR cycles. The BsteriF1 (human-specific) and the GenBac3 universal assays were used to analyze all samples. An optimized reagent concentration for both of these assays was 0.4 µM of each primer, 0.08 µM of probe and 0.2 mg BSA. The optimized number of PCR cycles was 40 for the BsteriF1 assay and 45 for the GenBac3 assay. All samples were analyzed in duplicate with standard dilutions and the inclusion of a no-template control with each run.

Results and Discussion

Fecal samples

Based on a comparison of published assays we selected primer sets BsteriF1 (human-specific) and GenBac3 (universal) *Bacteroidales* assays for use in this study (Table 1). Almost all of the animal fecal samples tested for this study were positive using the universal GenBac3 assay. However, we did not detect *Bacteriodales* in the single sheep fecal sample and this may be due to sample degradation, rather than the true absence of these bacteria. All influent sewage samples gave strong positive signals with both the GenBac3 and human-specific BsteriF1 assays (Table 2). Most fecal samples from animal species in the watershed were negative or yielded comparatively low numbers (as genome equivalents) when tested using the human-specific BsteriF1 assay. Weak positive signals were seen with 3 dog and 2 raccoon samples. Both species have relatively close relationships with humans or their wastes, so it is possible that they share some *Bacteroidales* strains. These results were somewhat unexpected, however, since Haugland et al. (2010) reported the BsteriF1 assay was negative with dogs. On a genomic equivalent basis, the false-positive human-specific animal signal was only 0.11% of the average universal signal for animals in the watershed. By comparison, mean BsteriF1 *Bacteroidales* genomic equivalents were 425 times (v/wt) higher in Cape Charles sewage influent samples than in wet fecal samples from dogs and raccoons. It is unfortunate that we could not obtain septage samples to test. The universal assay (GenBac3) showed that most species of animals were a potential source of fecal contamination and *Bacteroidales* genome contributions were variable. The average *Bacteroidales* genome equivalent value for all animals using the universal assay was 639,909 genomic equivalents/g feces. The relatively weak signals present in a couple animal samples (i.e. one dog and the chicken) may be the result of diet, unique digestive process, or the presence of anaerobic *Bacteroidales* species that have not been characterized and are not detected with the assays used.

Weak positive signals from animal feces using the BsteriF1 assay are somewhat problematic if the assay is to be 100% human-specific. It may be possible to reduce this interference using other primer sets in conjunction with BsteriF2 as a "tuning" assay that would refine the initial result. Our results are coincident with the generally held belief that each watershed is unique and the animals present may harbor different bacterial populations based on diet, climate, location, etc.

Table 2. Results of testing animal and sewage samples with human-specific (BsteriF1) and universal (GenBac3) fecal *Bacteroidales* qPCR assays.

Animal fecal sample	Date received	Collector name	Human assay ge/g*	Universal assay ge/g *
Dog A	7/6/11	Boyd/Hogg	52	13,960
Dog B	7/6/11	Boyd/Hogg	0	630
Dog C	10/19/11	Boyd/Hogg	478	49,530
Dog D	10/19/11	Boyd/Hogg	526	50,670
Chicken	7/6/11	Boyd/Hogg	0	1,940
Goose/Duck	7/6/11	Boyd/Hogg	0	33,630
Goose	7/6/11	Boyd/Hogg		170,980
Sheep	7/6/11	Boyd/Hogg	0	0
Deer 1	7/6/11	Boyd/Hogg	0	31,860
Deer 2	10/19/11	Boyd/Hogg	0	122,570
Deer 4	10/19/11	Boyd/Hogg	0	176,120
Deer 5	10/19/11	Boyd/Hogg		13,051,470
Seagull 1	7/6/11	Boyd/Hogg	0	30,970
Seagull 2 (CBBT)	10/19/11	VIMS	0	517,390
Seagull 3 (CBBT)	10/19/11	VIMS	0	28,500
Seagull 4 (CBBT)	10/19/11	VIMS	0	149,750
Seagull 5 (CBBT)	10/19/11	VIMS	0	57,580
Seagull 6 (CBBT)	10/19/11	VIMS	0	86,450
Seagull 7 (CBBT)	10/19/11	VIMS	0	68,020
Raccoon 1	8/9/11	Boyd/Hogg	1,400	20,890
Raccoon 2	10/19/11	Boyd/Hogg	1,650	24,100
Raccoon 3	10/19/11	Boyd/Hogg	0	30,810
Muskrat	8/9/11	Boyd/Hogg	0	0

Sewage influent sample	Date collected	Human assay ge/ml*	Universal assay ge/ml*
Cape Charles AM1	8/9/1	106,000	819,675
Cape Charles AM2	8/9/11	301,000	429,150
Cape Charles PM1	8/9/1	607,500	643,057
Cape Charles PM2	8/9/11	148,000	547,002
Reedville #1	8/8/11	8,025	162,586
Reedville #2	8/8/11	1,248	13,794
Reedville #3	8/8/11	6,183	96,106
Reedville #4	8/8/11	8,566	100,173
James River #1	7/28/08	22,395	467,765
James River #2	7/29/08	11,660	2,449,105

* *B. stercoris* genomic equivalents per g of fecal sample or milliliter of sewage influent sample.

Watershed samples

Results of analyses of feeder stream and standing water samples collected by stakeholders and VIMS personnel are shown in Table 3. These data revealed two patterns. First, based on the universal assay (GenBac3) there is a background of animal fecal contamination at all the stations sampled under dry and wet conditions. Genomic equivalent values on a per ml basis are approximately 1-2 orders of magnitude lower than values found in animal feces. Although environmental dilution factors are unknown, they are presumably substantial during large precipitation events and the concentrations seen at various watershed locations following the October rainfall (i.e samples collected Oct. 19, 2011) suggest widespread animal contamination. Values of the human-specific indicator, BsteriF1 (highlighted in gray), were either zero or generally several orders of magnitude less than those *Bacteroidales* equivalent densities determined using the universal assay and tended to be largest following the October precipitation event. Overall, the data suggest that a relatively low level of human contamination may be present in the watershed. However, given the fact that some dog and raccoon samples were weakly positive with the BsteriF1 assay, it is not possible to rule out that dogs and raccoons contributed to these signals. Locations that stand out with regard to repeat BsteriF1 positives are Bayview Recycle, Bayview Culls, Business 13 East and West, and Hardees locations. Several small older homes abut against the feeder stream (the Business 13 West location) that leads to Hardees East and are apparently on septic systems. Therefore these sites may warrant further evaluation. Bayview Recycle and Culls should also be evaluated for potential human sources or as animal attractants. It is difficult to make additional recommendations since this study was designed to evaluate new molecular methods and neither the scope or coverage and sampling design of the watershed were sufficient to provide data on which to base more definitive conclusions concerning human contamination.

Results of this study suggest that management of domestic dog waste by stakeholders and reducing activities that attract raccoons could lead to reductions in fecal landscape waste, as well as provide more confidence in the use of the BsteriF1 assay to specifically detect human contamination in the system. Other animal species in the watershed, while presumably responsible for elevated fecal coliform counts, were negative with respect to the BsteriF1 indicator.

Sample Site	Sample Date	Weather	Collector	Human Assay ge/ml*	Universal Assay ge/ml*
Creek "Blank"	7/6/11	Dry	Boyd/Hogg /VIMS	0	62
Goose Pond	7/6/11	Dry	Boyd/Hogg /VIMS	0	248
US 13 N	7/6/11	Dry	Boyd/Hogg /VIMS	0	134
	7/9/11	0.5" rainfall quickly absorbed	Boyd/Hogg	0	140
	9/17/11	after 0.5" rainfall	Boyd/Hogg	0	418
	9/21/11	after 0.5" rainfall	Boyd/Hogg	0	172
	10/19/11	during heavy rainfall	VIMS	24	2559
	10/19/11	during heavy rainfall	VIMS	53	1453
Hardees West	7/6/11	Dry	Boyd/Hogg /VIMS	14	14
	7/9/11	0.5" rainfall quickly absorbed	Boyd/Hogg	0	302
	9/17/11	after 0.5" rainfall	Boyd/Hogg	0	1020
	9/21/11	after 0.5" rainfall	Boyd/Hogg	0	0
	10/19/11	during heavy rainfall	VIMS	7	2007
	10/19/11	during heavy rainfall	VIMS	11	2007
Hardees East	7/6/11	Dry	Boyd/Hogg /VIMS	0	8
	10/19/11	during heavy rainfall	VIMS	18	3702
	10/19/11	during heavy rainfall	VIMS	76	5118
Bus. 13 West	7/9/11	0.5" rainfall quickly absorbed	Boyd/Hogg	28	576
	9/17/11	after 0.5" rainfall	Boyd/Hogg	10	278
	9/21/11	after 0.5" rainfall	Boyd/Hogg	0	256

	10/19/11	during heavy rainfall	VIMS	76	5118
	10/19/11	during heavy rainfall	VIMS	0	9774
Bus. 13 East	7/6/11	Dry	Boyd/Hogg /VIMS	0	1100
	7/9/11	0.5” rainfall quickly absorbed	Boyd/Hogg	0	124
	9/17/11	after 0.5” rainfall	Boyd/Hogg	66	624
	9/21/11	after 0.5” rainfall	Boyd/Hogg	0	678
	10/19/11	during heavy rainfall	VIMS	40	3416
	10/19/11	during heavy rainfall	VIMS	0	3150
Bayview Recycle	7/6/11	Dry	Boyd/Hogg /VIMS	0	6
	7/9/11	0.5” rainfall quickly absorbed	Boyd/Hogg	2	1060
	9/17/11	after 0.5” rainfall	Boyd/Hogg	0	302
	9/21/11	after 0.5” rainfall	Boyd/Hogg	136	186
	10/19/11	during heavy rainfall	VIMS	0	2102
	10/19/11	during heavy rainfall	VIMS	38	3150
Bayview/Culls	9/17/11	after 0.5” rainfall	Boyd/Hogg	0	576
	10/19/11	during heavy rainfall	VIMS	38	4014
	10/19/11	during heavy rainfall	VIMS	34	4720
KCL Spillway	7/6/11	Dry	Boyd/Hogg /VIMS	0	4
	7/9/11	0.5” rainfall quickly absorbed	Boyd/Hogg	0	214
	9/17/11	after 0.5” rainfall	Boyd/Hogg	0	172
	9/21/11	after 0.5” rainfall	Boyd/Hogg	0	0

	10/19/11	during heavy rainfall	VIMS	0	1788
	10/19/11	during heavy rainfall	VIMS	0	9014
Hogwood East	7/6/11	Dry	Boyd/Hogg /VIMS	0	152
	7/9/11	0.5” rainfall quickly absorbed	Boyd/Hogg	0	302
	9/17/11	after 0.5” rainfall	Boyd/Hogg	0	1100
	9/21/11	after 0.5” rainfall	Boyd/Hogg	0	452
	10/19/11	during heavy rainfall	VIMS	0	51
	10/19/11	during heavy rainfall	VIMS	3	55

Conclusions

- Use of recently published *Bacteroidales* primer sets to discriminate human from animal contamination were tested against a fecal samples from a variety of animal species and sewage (i.e WWTP influent) in the Kings Creek watershed.

- With the exception of some raccoon and dog samples, animals feces were negative with the BsteriF1 human-specific assay. Dogs and raccoons tested weakly positive (compared with the signal from human sewage).

- Watershed samples collected under both dry and varied rainfall conditions reveal widespread animal contamination throughout the watershed.

- Samples positive using the BsteriF1 assay were found throughout the watershed and genomic equivalents were much lower than corresponding universal assay values. The highest equivalent values with the human-specific assay tended to occur following the heavy rain event in October.

- Because of the apparent false-positive reactions of the BsteriF1 assay with the dog and raccoon fecal samples provided, we recommend additional testing of other primer sets and/or gene targets with fecal samples from these species as a means to improve the specificity of the assay.