EVALUATION OF A METHOD TO IDENTIFY SOURCES OF FECAL CONTAMINATION FOR URBAN WATERSHED MANAGEMENT IN WESTERN FINLAND USING 16S rDNA GENE FRAGMENTS AS A GENETIC MARKER

L. A. McLaughlin[1], O. Kaarela[2], M. M. Keinänen-Toivola[3], U. Munster[4], T. Tuhkanen[5], J. Puhakka[6] and J. S. Meschke[7]

(1) Tampere University of Technology, Environmental Engineering and Biotechnology, P.O. Box 541, 33101 Tampere, Finland, University of Washington, email: laurita6@gmail.com
(2) Tampere University of Technology, Environmental Engineering and Biotechnology, P.O. Box 541, 33101 Tampere, Finland email: outi.kaarela@tut.fi
(3) Vesi-Instituutti, Water Institute, Kalliokatu 10 B, FI- 26100 Rauma, FINLAND, email: minna.keinanen-toivola@vesi-instituutti.fi
(4) Tampere University of Technology, Environmental Engineering and Biotechnology, P.O. Box 541, 33101 Tampere, Finland email: uwe.munster@tut.fi
(5) Tampere University of Technology, Environmental Engineering and Biotechnology, P.O. Box 541, 33101 Tampere, Finland email: tuula.tuhkanen@tut.fi
(6) Tampere University of Technology, Environmental Engineering and Biotechnology, P.O. Box 541, 33101 Tampere, Finland email: jaakko.puhakka@tut.fi
(7) University of Washington, Department of Environmental and Occupational Health Sciences, 4225 Roosevelt Way NE, Suite 100, Seattle, Washington, 98195, U. S., email: jmeschke@u.washington.edu

ABSTRACT

Fecal contamination of drinking, recreational, and industrial waters is a worldwide issue that causes economic and public health problems. This paper evaluates a previously developed microbial source tracking method to identify the source of fecal contamination in Finland, using human and bovine-specific protocols. The specificity and precision of these protocols were evaluated using fecal, wastewater, and environmental samples. The bovine-specific protocol showed 16 out of 18 (89%) cow samples to be positive. The human-specific protocol showed four out of four (100%) of wastewater samples, but only two out of five (40%) individual human samples to be positive. The results from this evaluation study showed presence of multiple amplicons and non-target amplicons in the PCR product of some samples. This emphasizes the importance of a confirmation step that could be incorporated into the protocol in the future. This method has the potential to distinguish between human and bovine water contamination, thereby improving watershed management practices and preventing economic losses and health problems in Finland.

Keywords: Source-Tracking, Bacteroides, Finland, Microbial

1. INTRODUCTION

Fecal contamination of drinking water is a worldwide problem in both rural and urban areas. Consumption of fecally contaminated water is a major cause of health and economic problems. Beach closures and drinking water contamination cause public inconveniences as well as waterborne illness. Industries that depend on clean water for production, such as the shellfish industry, suffer substantial economic losses when water is contaminated (Bernhard & Field, 2000a). Many of these problems could be prevented by water quality monitoring techniques that aim to improve watershed management and prevent contamination.

Traditional methods for monitoring drinking water for fecal contamination use indicator organisms such at E. coli, Enterococci, coliphage, or coliforms (Long, Shafer, Arango, & Siraco, 2003; Simpson, Santo Domingo, & Reasoner, 2002). These traditional methods monitor for the presence of fecal material that originated from warm blooded animals (Lamendella, Domingo, Oerther, Vogel, & Stoeckel, 2007). Theoretically, the potential presence of enteric pathogens will correlate to the level of fecal indicator organisms present in a sample (Dufour, 1984). However traditional methods do not identify the source of the contamination or give information on how to remediate the problem.

In recent years, various microbiological and molecular methods have been developed to identify the source of fecal contamination (Field et al., 2003; Myoda et al., 2003; Simpson et al., 2002; Stewart et al., 2003). Certain methods that require either a labor intensive library of organisms or culturing of the target organism have been proposed (Stewart et al., 2003). These methods are time and labor intensive and may not be reliable due to lab conditions required to culture. A rapid, non-library dependent source tracking method could improve watershed management and reduce contamination.

Organisms in the Bacteroidetes phylum are obligate anaerobes, that constitute approximately half of the fecal flora in warm blooded animals (Holdeman, Good, & Moore, 1976). They can only survive for short periods in the environment and cannot reproduce. Additionally they adapt to the gut conditions of their host, leading to genetic differences between organisms that originated different hosts. Molecular techniques allow these genetic differences to be detected without culturing. These characteristics of organisms in the Bacteroidetes phylum make them good candidates for source tracking. In addition, specific markers for humans and cows in Baceroidetes DNA have been identified (Bernhard & Field, 2000b).

This study evaluated and tested the geographic stability of a previously described microbial source tracking method that distinguishes between human and bovine fecal contamination (Bernhard & Field, 2000b) for application in Finland. The method has been widely applied in the United States (Lamendella et al., 2007; Shanks et al., 2006).

2. METHOD

2.1. Sample Collection

We collected fecal samples from cow, human, and other potential sources of contamination in the southwestern Finland region. Samples were collected from healthy adult human volunteers using sterile collection containers. Researchers collected fresh animal fecal samples also using sterile containers and spatulas that were sterilized with alcohol. Mixed human samples were collected from Tampere municipal waterworks on five separate days in sterile 50 ml conicals. Fecal and mixed wastewater samples were transferred to one milliliter eppendorf tubes and stored at -80ºC.

Water samples were collected in sterile, one-liter glass bottles from sewage outfall impacted surface water, wells, and irrigation ditches near a farm. All sites were in the greater Tampere area in southwest Finland. All samples were tested for Enterococci, one of the regulated indicators in Finland, according to the method described by Kinzelman et al. (Kinzelman *et al.*, 2003). Fifty to 500 ml of each sample was concentrated on 0.2 micron, 47 mm filters and stored at -80ºC until extraction.

All samples were collected between December, 2006 and April, 2007. They were all stored on ice and processed within 24 hours of collection. A total of ten high-risk water samples, five human fecal samples, 17 bovine fecal samples, four sewage samples, and 12 non target animal fecal samples were collected.

2.2. DNA Extraction:

DNA was extracted from all samples using the Powersoil DNA Isolation kit according to the manufacturer’s protocol (Mo Bio Laboratories, Inc., Carlsbad, CA). For filter concentrated samples, the clean outside area of each filter was trimmed off with a sterile scalpel for ease of placement of the filters into the Powersoil extraction tubes.

2.3. PCR Amplification:

All water samples were initially run with a general Bacteroidetes primer set to screen for the presence of Bacteroidetes before running the specific PCR reactions. If Bacteroidetes was not present, the sample was considered to be negative for both human and bovine contamination. The specific CF128 and HF183 primer sets were not run on negative BAC32 samples.

Specific primer sets CF128/BAC708 and HF183/BAC708, developed by Bernhard and Field (Bernhard & Field, 2000b), were optimized and tested on all fecal, wastewater, and water samples.

Table 1: Sequences of specific human and bovine primers (Bernhard & Field, 2000b)

Name	Target	Direction	Sequence
BAC32F	general	forward	AAC GCT AGC TAC AGG CTT
CF128F	bovine	forward	CCA ACY TTC CCG WTA CTC
HF183	human	forward	ATC ATG AGT TCA CAT GTC CG
BAC708R	general, bovine, and human	reverse	CAA TCG GAG TTC TTC GTG

JumpStart™ REDTaq® ReadyMix™ Reaction Mix kits (St. Louis, MO) were used for all PCR reactions in this study. Each 25 µl reaction included the 12.5 µl of the ReadyMix reaction mix, 9.5 µl of ultrapure water, and 1 µl of the forward primer (10 µM), the reverse primer (10 µl), and of the DNA template.

After optimizing the cycle number and the annealing temperatures for each primer set, the final PCR programs were obtained. The PCR cycle for the HF183 primer set had an initial denaturation step of three minutes at 94ºC. Forty cycles of denaturation (30 seconds, 94ºC), annealing (30 seconds, 56ºC), and extension (one minute, 72ºC) were followed by a final extension step of 72ºC for seven minutes. The CF128 primer set had the same cycle with the following changes: the annealing temperature was 52ºC, 35 cycles, and a final extension of six minutes.

For initial optimization of the PCR reactions, fecal DNAs were amplified in 10-fold serial dilutions with ultrapure water. However, the zero dilution always produced the most clearly visible band, thus relieving the need to dilute in subsequent reactions.

Environmental samples were initially processed using the general Bacteroidetes primer set. If the general reaction was negative, the specific primer protocols were not attempted and the samples were assumed to be negative for fecal contamination. If the general reaction was positive, the sample was assayed by both the human and bovine specific protocols. Positive bovine fecal DNA was used as the positive control for the CF128 primer set, and a wastewater DNA extract was used as a positive control for the HF183 primer set. At least one negative control was used per gel.

2.4. Agarose Gel Electrophoresis:

PCR amplicons were visualized by electrophoresis on a 10% agarose gel stained with ethidium bromide.

3. RESULTS

This study evaluated the host specificity and precision of this method in Finland. The host specificity is represented in table 2 and was determined by amplifying DNA from each host with each specific primer set.

Table 2: Number of positive results on each sample type (human feces, cow feces, or sewage) from each specific primer set

Marker	PCR results					
	Human		Cow		Sewage	
	# tested	# positive	# tested	# positive	# tested	# positive
Human (HF183)	5	2	18	1	4	4
Cow (CF128)	5	1	18	16	4	0

3.1. Human Specific Protocol

The precision of the human-specific protocol was examined by testing individual human fecal samples and population wastewater samples. Two out of the five human fecal samples tested positive with the human protocol. All four of the mixed wastewater samples were positive.

To validate the specificity of the human protocol, cow and a selection of non-target fecal samples were tested. Ideally, none of the non-human fecal samples would test positive with the human-specific protocol. However, one of the cow DNA samples (out of 18 samples) tested positive (Table 2). In addition, one of the four horse samples and two of the three goat samples were positive. All dog, pig, and rabbit fecal samples were negative using the human protocol (Table 3).

Table 3: Human specific protocol results on potential non-target sources of contamination

Animal feces	Number of samples tested	Number of positive PCR results	Number of other sized bands
Horse	4	1	4
Dog	3	0	0
Pig	1	0	1
Rabbit	1	0	1
Goat	3	2	0

3.2. Bovine Specific Protocol

Sixteen of the 18 individual cow fecal samples were amplified by the CF128 primer set (Table 2), 89% of the samples tested. Since a large portion of individual fecal samples tested positive with this primer set, there was no need to test mixed samples.

The specificity of the bovine-specific protocol was further examined by testing human and the other non-target animal DNA. One of the five individual human samples and none of the four mixed human samples were positive. None of the other (non-human, non-cow) animal fecal samples, except the goat samples-- also bovine animals, were positive (Table 4).

Table 4: Bovine specific protocol results on potential non-target sources of contamination

Animal	Number of samples tested	Number of positive PCR results	Number of other sized bands
Horse	4	0	0
Dog	3	0	0
Pig	1	0	0
Rabbit	1	0	0
Goat	3	3	0

To verify that the method is sensitive enough to detect human contamination at environmental concentrations, we also selected ten sites that were likely to have fecal contamination. Environmental samples were collected from four wells, four sites that were downstream of sewage effluent and two sites that were downstream of cattle farms. One well sample was negative for Bacteroidetes. The remaining three were positive for Bacteroidetes but negative for human or cow- specific contamination. Both surface sites that were at risk for cow contamination tested positive using the bovine-specific protocol and negative using the human-specific protocol. Three out of the four high-risk human sites tested positive with the human-specific protocol (Table 5). None of the environmental samples tested positive for both human and bovine contamination.

Table 5: Bovine and human specific protocol results on high-risk water samples

	High-Risk water PCR results			
	Well water		Surface water	
Marker	# tested	# positive	# tested	# positive
Human (HF183)	4	0	6	3
Cow (CF128)	4	0	6	2

4. DISCUSSION

While the specificity and precision were not ideal in this validation study, the results compare to the specificity and sensitivity in other published studies that used these primer sets. For example, Lamendella et al. used the same human and bovine-specific primer sets in their study that assessed the fecal pollution sources in the Plum Creek Watershed in Nebraska. Ninety-six percent (97/101) of their cattle fecal samples were positive with the bovine-specific primer set. However, all six of their horse fecal samples were positive, and two out of 66 (3%) wildlife samples also tested positive. One of their 101 cattle fecal samples tested positive with the human-specific primer set and only four out of ten septic samples were positive. They did not test individual human fecal samples (Lamendella et al., 2007). Bernhard and Field, who developed this method, found results that were closer to the ideal situation (Tables 6 and 7).

Table 6: Comparison of this study's specific bovine primer set specificity and precision with other published studies and the ideal scenario

	Percent positive with CF128					
Study	human fecal	cow fecal	wastewater	septic	wildlife	other non-target fecal
Ideal	*0%*	*100%*	*0%*	*0%*	*0%*	*0%*
Lamendella et al., 2007	-	96%	-	0%	3%	100% (horse)
Bernhard and Field, 2000	0%	100%	0%	-	-	-
this study	20%	89%	0%	-	-	25%

Table 7: Comparison of this study's specific human primer set specificity and precision with other published studies and the ideal scenario

	Percent positive with HF183					
Study	human fecal	cow fecal	wastewater	septic	wildlife	other non-target fecal
Ideal	*100%*	*0%*	*100%*	*100%*	*0%*	*0%*
Lamendella et al., 2007	-	0.01%	-	40%	0%	0% (horse)
Bernhard and Field, 2000	85%	0%	100%	-	-	-
this study	40%	6%	100%	-	-	25%

Ideally, all human individuals would carry this marker and therefore test positive. However, since contamination is typically present in the environment from a *population* of individuals, it is most important for population samples (wastewater or septic) samples to test positive. Previous studies have found this marker to be more present in individual human fecal samples than the current study (Bernhard & Field, 2000b).

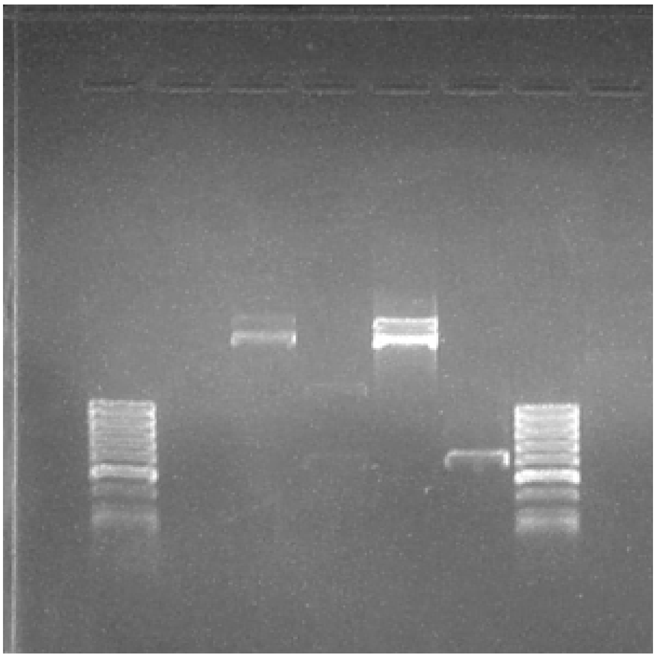

Figure 1: This method occasionally amplifies non-target regions of DNA. This is a gel with non specific amplicons in the second and fourth lanes from the left. Both of these samples show multiple non-specific amplicons.

This study emphasized the need for a validation step, such as sequencing or Southern blotting. There were non-specific amplicons that were visible in some samples (Figure 1), in addition to the band that was consistently present in the positive controls (Tables 3 and 4). While additional bands appeared to be visually distinguishable on the gels, they still present the need for a further verification step. The effects of these non-specific amplicons were not evaluated in this study; however they may affect the specificity and efficiency of the PCR reactions. The non-specific amplicons could deplete the reaction resources (primers, dNPTs, enzymes, etc.) before the target sequences are amplified. This could lead to false negatives. Furthermore, if non-specific amplicons are not distinguishable from the target amplicons, false positive would result. For immediate use, it is recommended to sequence a portion of the samples on which this method is used. However there is need for a rapid confirmatory test that can be easily carried out on each sample.

5. CONCLUSION

While traditional indicator assays identify the presence of fecal contamination, microbial source tracking techniques additionally identify the *source* of fecal contamination (bovine or human). This is advantageous over traditional indicator methods because it could reduce watershed management and industrial costs that result from beach closures or contaminated drinking water. Furthermore, this microbial source tracking method does not rely on culturing organisms and therefore has the potential to obtain results faster than with traditional indicators that must be cultured for 24 or 48 hours.

Neither protocol evaluated in this study was 100% specific or 100% precise, however our results were similar to those achieved in other (Bernhard & Field, 2000a, 2000b; Lamendella et al., 2007) and show potential as a source tracking method for use in Finland.

While this method correctly identifies bovine and human fecal contamination, a rapid validation step is needed to verify the size and sequence of the PCR product. This is particularly important in this method because of the multiple bands and bands of different sizes than those of the positive controls that were produced in some samples.

ACKNOWLEDGEMENTS

We would like to acknowledge Clarita Lefthand at the University of Washington for her advising and cooperation on this project.

REFERENCES

Bernhard, A. E., and Field, K. G. (2000a): Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes. *Applied Environmental Microbiology,* 66(4): 1587-1594.

Bernhard, A. E., and Field, K. G. (2000b): A PCR assay To discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA. *Applied Environmental Microbiology,* 66(10): 4571-4574.

Dufour, A. P. (1984): Bacterial indicators of recreational water quality. *Can J Public Health, 75*(1): 49-56.

Field, K. G., Chern, E. C., Dick, L. K., Fuhrman, J., Griffith, J., and Holden, P. A. (2003): A Comparative Study of Culture-independent, Library-independent Genotypic Methods of Fecal Source Tracking. *Journal of Water and Health,* 1(4): 181-193.

Holdeman, L. V., Good, I. J., and Moore, W. E. (1976): Human fecal flora: variation in bacterial composition within individuals and a possible effect of emotional stress. *Applied Environmental Microbiology,* 31(3): 359-375.

Kinzelman, J., Ng, C., Jackson, E., Gradus, S., and Bagley, R. (2003): Enterococci as indicators of Lake Michigan recreational water quality: comparison of two methodologies and their impacts on public health regulatory events. *Applied Environmental Microbiology,* 69(1): 92-96.

Lamendella, R., Domingo, J. W., Oerther, D. B., Vogel, J. R., and Stoeckel, D. M. (2007): Assessment of fecal pollution sources in a small northern-plains watershed using PCR and phylogenetic analyses of Bacteroidetes 16S rRNA gene. *FEMS Microbiology Ecology,* 59(3): 651-660.

Long, S. C., Shafer, E., Arango, C., and Siraco, D. (2003): Evaluation of three source tracking indicator organisms for watershed management. *Journal of Water Supply: Research and Technology - AQUA,* 52(8): 565-575.

Myoda, S. P., Carlson, A., Fuhrmann, J. J., Hahm, B.-K., Hartel, P. G., and Yampara-lquise, H. (2003): Comparison of Genetypic-based Microbial Source Tracking Methods Requiring a Host Origin Database. *Journal of Water and Health,* 1(4): 167-180.

Shanks, O. C., Nietch, C., Simonich, M., Younger, M., Reynolds, D., and Field, K. G. (2006): Basin-wide analysis of the dynamics of fecal contamination and fecal source identification in Tillamook Bay, Oregon. *Applied Environmental Microbiology,* 72(8): 5537-5546.

Simpson, J. M., Santo Domingo, J. W., and Reasoner, D. J. (2002): Microbial source tracking: State of the science. *Environmental Science and Technology,* 36(24): 5279-5288.

Stewart, J. R., Ellender, R. D., Gooch, J. A., Jiang, S., Myoda, S. P., and Weisberg, S. B. (2003): Recommendations for Microbial Source Tracking: Lessons from a Methods Comparison Study. *Journal of Water and Health,* 1(4): 225-231.