# Testing the applicability of microbial source tracking method using qPCR for determining the source of high levels of fecal contamination in Western Slope waterways

**Final Report**

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**Executive Summary:**

High levels of *E. coli* above the regulatory standards were measured in Adobe and Leach Creeks in Mesa County, indicating fecal contamination, with a need to implement total maximum daily loading (TMDL) on these creeks. However, the traditional *E. coli* method only determines the number of bacteria present and not the source of fecal contamination. Mesa County must be able to determine the fecal contamination sources to properly mitigate the issue.

*Bacteriodales* are bacteria found in the excrement of animals and because of evolution, have become species specific. One microbial source tracking (MST) method employs the extraction of *Bacteriodales* DNA and quantifying them using specific primers of *Bacteriodales* found in different organisms and quantitative polymerase chain reaction (qPCR), allowing the fecal contamination source to be determined. Currently, no MST method has been tested in the Western Slope region to determine if MST is applicable in the area. Our project tested qPCR for its specificity in determining fecal contamination from different animals (cows, chicken, and horse) and humans in the area.

Animal feces samples, from horses, cows, chicken were collected along with waste water obtained from a local treatment plant for the human samples. DNA was extracted and used in the qPCR analysis. *Bacteriodales* primers specific to different host animals were used for qPCR amplification.

Among the studied animals, it was determined that the HF183/BacR287 primers could reliably identify human *Bacteriodales* DNA without amplifying any of the other organisms' *Bacteroidales*. This was also true for the CowM2FR that only amplified cow *Bacteriodale* DNA. Using a combination of primer tests, it is possible to rule out cow and human fecal contaminants; however, a distinction between chicken and horse *Bacteriodale* DNA could not be differentiated.

**Introduction**:

High levels of *E. coli* and fecal coliform in the waterways are dangerous for local wildlife and humans. A study conducted in Michigan tested if *E. coli* could accumulate in fish mucus and tissues (Hamilton 2020). There was an overwhelming amount of data that both wild fish and fish born in hatcheries that were exposed to *E. coli* had accumulated the bacteria in their system. In addition, local anglers were tested for contamination as well. After simply dipping their hands in the river, 78% of the anglers showed contamination on their hands (Hamilton 2020). According to the Mayo clinic, ingestion of *E. coli* could cause diarrhea, stomach cramping, and vomiting, while the exposure to E. coli O157:H7 could lead to kidney failure (Mayo 2020). Even if the species of *E. coli* found in the local waterways is not the toxic strain, *E. coli* is an indicator for other potentially harmful microorganisms often found in feces.

High *E. coli* loading is detected in some of the region's streams and is an indication of fecal contamination. However, the source of the fecal contamination is often unknown, creating obstacles for developing sound mitigation plans. For instance, previous measurements of *E. coli* concentrations in Adobe and Leach Creeks have shown values that exceeded regulatory limits, with total maximum daily loading (TMDL) limits to be imposed on these creeks. However, because traditional *E. coli* enumeration method can only quantify the number of bacteria present in the water, it does not provide information about the animal origin of the fecal waste –

i.e. whether the *E. coli* originate from human sources (e.g. leaking septic tanks), domesticated animals (e.g. cattle and dogs), or wild animals. To determine the appropriate TMDL limits, it is important to determine the origin of the fecal contamination.

**Objectives of the project:**

Our study aims to test the effectiveness of using microbial source tracking (MST) to determine the origin of fecal contamination in the Western Slope region. Microbial source tracking is a technique that could be used to determine the sources of microbial contamination. One type of bacteria that has been used is *Bacteroidales* which are commonly found in the intestinal tracts and excrements of animals and have evolved alongside their host species. Many of these bacteria have thereby become host specific. Using this knowledge, scientists have detected and quantified the genetic material, specifically the 16srRNA genes from *Bacteroidales,* to determine the sources of fecal contaminants (Ahmed et al., 2016). This method could be used to complement traditional *E. coli* enumeration method, which does not provide information about the origin of the fecal waste, allowing counties and cities to develop better fecal contamination mitigation plans.

Currently, the USEPA has approved a method (Method 1696) using *Bacteriodales* to determine if the source of *E. coli* is human or non-human (EPA 2019), but this method does not distinguish between the different animal sources. For instance, this method is unable to differentiate *Bacteroidales* that come from cows and horses.

There is value in using the non-approved method in the Western Slope region to provide data for the specific sources of fecal contamination – data that Mesa County is interested in. Kildare et al. (2007) and Odagiri et al. (2015), for instance, quantified the total or universal *Bacteriodales* community from different sources and found that the method could effectively identify *Bacteriodales* from all the animals tested (humans, cats, dogs, seagulls, cows, and horses). However, when Kildare et al. (2007) used a method specific for cows, they found cross-reactivity between the bovine and horse samples, but not with humans, dogs, swine, horse, seagull, or cat stools, indicating that this method can provide information if fecal contamination comes from farm animals versus humans or wild animals.

The possibility of cross reactivity makes it necessary to test the effectiveness of any MST method within the local region. Our goal was thus to test the applicability of the MST method in determining the sources of fecal contamination in the Western Slope region. Additionally, we also attempted to use digital PCR (dPCR) technology to help quantify the data. Previous studies have mostly used qPCR or real-time PCR to examine the *Bacteriodales*. The downfall of qPCR is that it must be normalized to a standard curve and variations in amplification can alter the results (Bio 2021). The dPCR improves upon previous methods as it does not need a standard curve and could detect extremely rare events in the nucleotide sequence, increasing its sensitivity. While dPCR has been used to test for fecal contamination along several coastlines (Cao et al. 2015; Steele et al. 2018), the number of studies that have used dPCR for MST purposes is limited. Furthermore, the effectiveness of using dPCR for determining fecal contamination origin has not been conducted in the Western Slope region.

If the newer MST method using dPCR is successful, it would provide a novel and more efficient way to determine sources of fecal contamination in the Grand Valley. Successful results from this testing phase would allow Mesa County to use this method to preliminarily locate the source(s) of fecal contamination in its impaired streams and determine sound methods to reduce fecal contamination in the region's streams.

**Methods:**

*Fecal Sample collection*

Fresh animal fecal samples were collected between May-September of 2021 with sterile wooden sticks and placed in 50mL sterile conical tubes in the field. We did our best to obtain samples from the top of manure, but this was not always possible, especially with smaller chicken manure samples, which could thus contain some soil particles. Human fecal samples were obtained at Persigo Wastewater treatment plant. All samples were transported on ice to the laboratory. Animal fecal samples were stored at -80°C freezer until DNA extraction. Human samples were processed right away.

*DNA extraction*

DNA was extracted using the QIAamp® PowerFecal Pro DNA Stool Kit (Qiagen, Hilden, Germany). Human samples were first centrifuged to obtain the pellet of solid material. The liquid phase was decanted into a glass beaker and discarded after bleach treatment. The manufacturer's instructions were followed for DNA extraction. DNA elution was done with 50 µL of Solution 6, aliquots were stored at -20°C. Chicken manure DNA samples were diluted after amplification failure likely due to PCR inhibitors found in bird manure. Following 1:10 dilution amplifcations were successful.

*TaqMan assays*

*Bacteriodales* DNA was amplified using a TaqMan system with several primer/probe sets (Odagiri et al 2015 and EPA 2019). Reactions were 20µL and contained 2x BIORAD iTaq PCR Probe mix, Primer/probe mix at the previously published concentrations (Table 1) and 1µL of target DNA. The amplification conditions were set at 10 minutes at 95°C for initial denaturation followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Fluorescence emission was measured after each annealing/extension phase.

For each biological sample, three technical replicates were performed. Each 96 well plate was run using one primer pair/probe mix for all the samples and replicates available.

*Amplification analysis*

The RFU threshold was set at 25. Cq values below 32 were considered as positive amplification. In cases where one of the triplicates was not amplified, if the average Cq value of the other two was below 32, it was considered as amplified.

**Results and Discussion:**

The use of HF183/BacR287 primers allowed for the detection of human *Bacteroidales* DNA. These primers did not amplify chicken, cow, or horse samples. CowM2FR primers allowed for the detection of cow *Bacteroidales* DNA. They did not amplify chicken, cow, human, or horse samples. BacCow primers reliably identified the presence of cow *Bacteroidales* DNA. However, they also amplified horse and chicken *Bacteroidales* DNA although not 100% of the time (Table 1). Since BacCow primers did not amplify the human *Bacteroidales* DNA, these could be used to confirm the absence of human *Bacteroidales* DNA. BacUni primers amplified human and cow *Bacteroidales* DNA 100% of the time but were not reliable for horse and chicken *Bacteroidales* DNA.

Table 1: Summary of the primers used in this study, their target animals, and the percentage of manure samples from each animal group amplified with the particular primer pair. The numbers next to the animal group titles indicate the number of individual animals that were tested for that group.

| | | % of animal samples amplified (# of samples) | | | |
|---|---|---|---|---|---|
| **Primers used** | **Target animal** | **Humans (1)** | **Cows (10)** | **Horses (9)** | **Chickens (10)** |
| **BacUni** | Universal (all animals) | 100% | 100% | 56% | 80% |
| **HF183/BacR287** | Humans | 100% | 0% | 0% | 0% |
| **CowM2FR** | Cows | 0% | 100% | 0% | 0% |
| **BacCow** | Ruminants, livestock/domestic | 0% | 100% | 89% | 50% |

BacUni and BacCow runs should be completed as a general control. If there is no amplification in any of the tests, this could identify a problem with the primers and/or the sample, or the absence of fecal contamination, especially with respect to the animals tested in this study.

HF183/BacR287 and CowM2FR primer runs should allow human and cow *Bacteroidales* DNA to be identified. It should be noted that If no amplification occurs, it is likely neither cow nor human.

If the HF183/BacR287 and CowM2FR runs do not yield amplification, but the BacUni and BacCow yield results, the sample could be from animals other than cows. However, distinguishing between chicken, horse, or another animal definitively is not possible based on the primers tested.

Figure 1 outlines the recommended order of operations for analyzing an unknown sample using the primer pairs listed above. If the user arrives at "Error" on the flow chart, it suggests one of the following:

1. That the sample was neither cow nor human with a high probability that it also did not originate from a horse (or chicken if BacUni was also not amplified);
2. The sample was not viable;
3. There was an error in the amplification process and the test should be repeated; or
4. A qPCR using universal 16S rRNA primers could be used as a positive control for proper DNA extraction and qPCR reaction mix as almost all biological samples are expected to have a detectable bacterial load.

It should be noted that the results above are only applicable amongst the animals and human waste samples selected for this study. Odagiri et al. (2015), for instance, showed differences in primer reactivity between individual human stools and mixed sewage samples. In addition, they found that the human *Bacteriodales* primer cross reacted with *Bacteriodales* from dog manure, which was not tested in this study. Nevertheless, the method tested in this study could provide a preliminary glance towards sources of fecal contamination in water bodies.

An attempt was made to run the samples using digital PCR (dPCR). However, despite amplification with qPCR, the same reactions would not amplify when we used dPCR. More research needs to be performed to determine why this was the case.

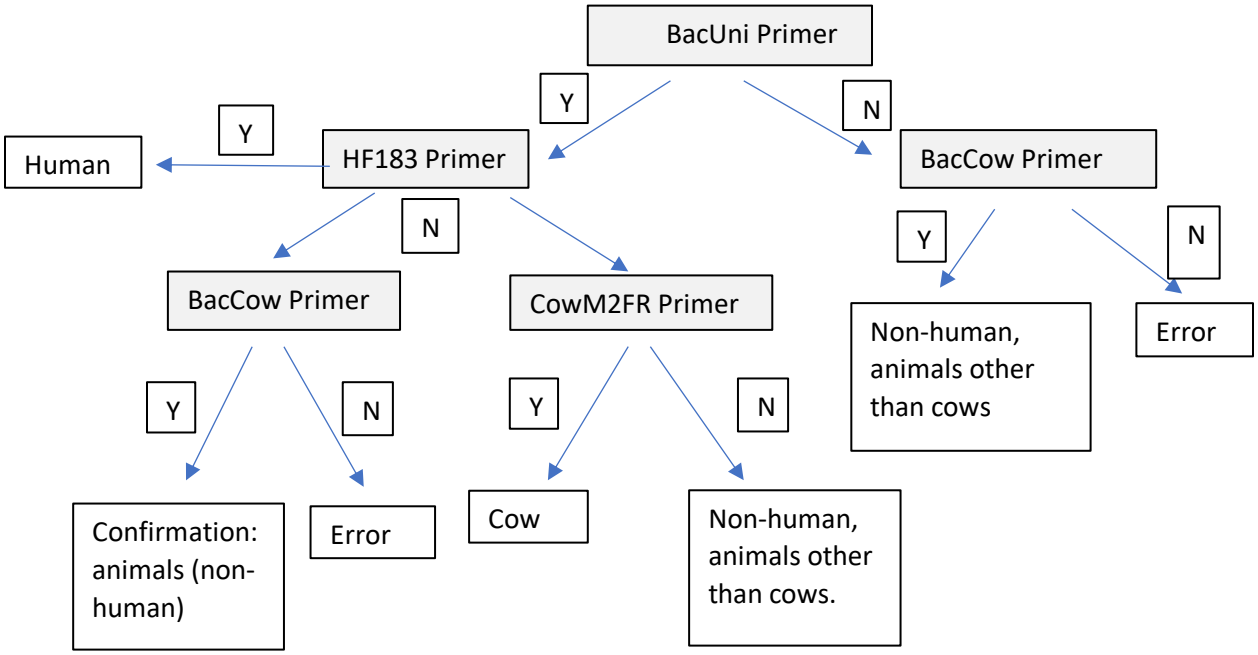


Figure 1: Flowchart of suggested workflow. Y indicates amplification, N indicates lack of amplification. Shaded boxes are the primer sets, clear boxes indicate the suggested source of *Bacteriodales.*

**Acknowledgements:**

We would like to thank the Ruth Powell Hutchins Water Center (org #2634) and Mesa County for providing funding for this project, and Dr. Denise McKenney for her guidance in this project.

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