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Long, Sharon C.; Stietz, Jamie R.
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FINAL REPORT

Project to Develop and Validate a Molecular Assay for *Rhodococcus coprophilus*

Prepared by:

Sharon C. Long and Jamie R. Stietz
Wisconsin State Laboratory of Hygiene

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Executive Summary

The need to distinguish between sources of microbial contaminants entering drinking water wells is a critical piece of information for public health, remediation and enforcement activities. A suite of microbial source tracking (MST) tests has been applied in Wisconsin. This suite included a culture-based method for detection of *Rhodococcus coprophilus* (*R. coprophilus)*, a grazing animal manure related organism that requires three to four weeks to complete. The research described here developed and validated molecular methods for detecting *R. coprophilus* in environmental samples. These methods shortened analysis time to as little as two to four days, although samples could be frozen and batched to provide greater cost-efficiency. One method involved traditional polymerase chain reaction (PCR) and provides presence/absence detection. A second method involved quantitative real-time PCR and can yield data in calibrated cell equivalents per 100 mL of sample. Overall, the molecular methods can be used to provide the same information as the culture method in a fraction of the time.

Introduction

The need to protect watersheds from fecal contamination has led to an investigation of better monitoring tools. A suite or toolbox of tests developed to supplement monitoring for indicator organisms is called "Microbial Source Tracking" (MST). *Rhodococcus coprophilus* is an emerging MST tool that has been demonstrated to identify the presence of grazing animal fecal matter or manure in the presence of fecal contamination (Rowbotham and Cross 1977, Mara and Oragui, 1981, Mara and Oragui 1983). *Rhodococcus coprophilus* is an aerobic Nocardioform actinomycete that is a natural inhabitant of the dung of grazing animals originating on the grass or hay eaten by herbivores. First discovered by Goodfellow in 1971 in a study of nocardioform bacteria, its value as an indicator organism was later explored by Rowbotham and Cross (1977a) and Oragui and Mara (1981).

The current method used to detect *Rhodococcus coprophilus* in water samples is both labor intensive and time consuming (Arango, 2000; Long *et al.*, 2002). Three to four weeks are needed to culture and confirm the presence of this organism in water samples. It is believed that molecular techniques could give reliable results in as little as 2-4 days using polymerase chain reaction (PCR) and gel electrophoresis (Savill *et. al.*, 2001). While some success with development of molecular detection methods for *R. coprophilus* in fecal samples have been reported in the literature (Savill *et al.*, 2001); further research is needed to translate and standardize these methods into practical methods that can be used in routine MST analysis. Development and validation of molecular detection method(s) for environmental water samples is the focus of this research.

Steps in developing and validating a PCR-based method include optimizing: (1) sample concentration, (2) extraction and purification of nucleic acid, (3) PCR amplification, (4) confirmation of presence of target amplicon, and (5) quantification of nucleic acid concentration against a standard curve. Using the U.S. EPA molecular method “Rapid, PCR-Based Method for Measuring *Enterococci* and *Bacteroides* in Water Samples” or US EPA Method 1606 as a launching point, sample concentration was accomplished using membrane filtration and nucleic acid extraction by bead beating. However, various nucleic acid purification and PCR approaches were explored to assess which approach best suited detection of *R. coprophilus*. To evaluate performance of the optimized PCR-based methods using environmental samples, results were compared against standardized plating methods for the enumeration of *R. coprophilus*.

Sample Concentration

The environmental densities of microbial targets can range from very low (single cells per 100 L) to very high (upwards to 10^6 cells per L) depending on the distance in time and space from the fecal contamination event. Therefore, samples are typically concentrated for microbial enumeration. Previous research has demonstrated that *R. coprophilus* is present in animal manure at densities of 10^1 to 10^6 per gram and in grazing animal contaminated surface waters at densities of 10^1 to 10^3 per 100 mL (Long *et al.*, 2002). Researchers report the ability to detect a single gene copy using PCR. If 5 µL of sample extract is used in a PCR reaction, then samples should be concentrated by a factor of 200 to detect one gene copy in 100 mL of sample.

Methodologies commonly used to concentrate bacteria in environmental samples include membrane filtration and centrifugation. Experiments to concentrate *R. coprophilus* spiked into drinking water and surface waters via centrifugation proved inefficient (Clark, 2007). Therefore, the membrane filtration approach described in EPA Method 1606 for enterococci and *Bacteroides* was evaluated. A target spike of 100 flow counted *R. coprophilus* cells was used. Duplicate volumes of 100, 500 and 1000 mLs were filtered, resuspended in AE buffer and bead beated and subject to PCR for quantification. Each duplicate volume was amplified using traditional PCR in duplicate. Visualization of PCR products on an agarose gel demonstrated that bands of increasing brightness were produced with increasing sample test volume. The membrane filtration concentration method proved quantitative for *R. coprophilus.*

Extraction of Nucleic Acid

Extracting *R. coprophilus* DNA from environmental samples is challenging because this organism produces a waxy protective layer in order to survive the environment of grazing animal gastrointestinal tracts and prevent desiccation once in the environment. Previously, both commercial extraction kits using lysozyme and protinase K and a traditional phenol/chloroform/alcohol extraction methods had met with limited success (Clark, 2007). The membrane filtration/bead-beat method described in EPA Method 1606 for molecular quantification of enterococci and *Bacteroides* was evaluated for its ability to lyse a known number of *R. coprophilus* cells. Phosphate buffer with magnesium chloride 99 mL dilution blanks were spiked in duplicate with 250, 2500, and 25000 flow counted *R. coprophilus* cultures, membrane filtered, resuspended in 600 µL of AE buffer and bead beated. Five microliters of the extract were then amplified via traditional PCR in duplicate and PCR products confirmed by size on an agarose gel. Visible bands of 443 base pairs confirmed that the filtration/beat-beat method was successful for all three spike levels. The bands were faint for the 250 cfu/100 mL spikes.

In order to lower detection limits, trials were conducted with resuspension in 300 µL of AE buffer and amplification with 5, 10 and 15 µL of extract in PCR reactions using the same spike levels. PCR products were visualized on an agarose gel. Bands of increasing brightness were observed and documented for increasing template volume used.

One hundred mLs of sample were filtered and resuspended with 300 µL of AE buffer. Fifteen (15) µL of the resuspension was used for PCR amplification with an expected theoretical detection limit of 20 calibrated cell equivalents. To test whether this was achievable, 300 mL of buffer was spiked with 60 flow counted *R. coprophilus* cells, filtered in triplicate 100 mL volumes, extracted into 300 µL of AE buffer and triplicate 15 µL volumes of each extract were amplified using PCR. The results are summarized in Table 1.

Table1. Results of 20 Flow Count Cell Challenge Study

Replicate Filtrate	PCR 1	PCR 2	PCR 3
1	+	+very weak	+weak
2	-	+	-
3	-	+weak	+weak

(+) visible band
(-) no visible band

Overall, the limits of the assay could be lowered by using smaller extraction volumes and larger PCR template volumes. The detection limit of the assay is approximately 20 cell equivalents per 100 mL for clean waters.

In order to challenge the assay with environmental waters, both a groundwater sample and surface water sample were collected. The groundwater sample was collected from a Madison municipal well known to contain significant amounts of manganese. Divalent metals are known to inhibit PCR reactions. The surface water was collected from Lake Mendota. Surface waters are known to contain natural organic matter that inhibit PCR reactions. Both the groundwater and surface samples were aliquoted into four 100 mL volumes and spiked with 25, 50, 100, and 500 flow counted *R. coprophilus* cells. Each aliquot was membrane filtered and extracted. Each extract was amplified in triplicate using PCR. For the groundwater spikes, each PCR reaction was positive, with increasing band brightness associated with increasing spike concentration. The results for surface water spikes were quite different. Significant inhibition was observed for all spiked concentrations. The samples containing 500 cells per 100 mL did produce very faint bands; however, this concentration is 25 times higher than the lower detection limit.

The results demonstrate that bead beating quantitatively releases the nucleic acid from intact *R. coprophilus* cells. Therefore, this method of nucleic acid extraction was carried forward through the remaining experiments. However, with environmental waters, the presence of inhibitors may be significant. Thus, evaluation of appropriate clean-up methodologies was required.

Purification of Nucleic Acid

As mentioned above, both ions and natural organic matter present in environmental samples can inhibit the PCR reaction. These substances may be dissolved or colloidal. The colloidal material will accumulate on the membrane and dissolved organic matter may absorb to the membrane. Thus, the inhibitors become concentrated with the nucleic acid and requires the purification of the nucleic acid extracted from environmental samples for PCR to be successful. One approach is to dilute the extracted material 1:10 and 1:100 before adding the target to the PCR reaction mixture. However, this increases the detection limit by the same order of magnitude. Another approach is separation methods.

Traditional methods for separating environmental compounds that inhibit the PCR reaction (organic matter and metals) include sephadex column clean-up and alcohol precipitation/resuspension and potential use of highly toxic compounds, guanidinium thiocyanate. These methods are labor intensive and are difficult to standardize for use in production laboratory settings. Therefore, commercial products were investigated. Based on commercial literature and discussions with company technical representatives, three products were chosen for evaluation:

- QIAgen DNeasy® Blood and Tissue kit

- MoBio PowerClean™ DNA Clean-Up Kit
- Zymo ZR Soil Microbe DNA Kit™

The results from each test are discussed below.

The QIAgen DNeasy® Blood and Tissue kit and the MoBio PowerClean™ DNA Clean-Up kit were evaluated for their ability to eliminate/reduce environmental PCR inhibitors, as well as how much target DNA was lost through kit processing. Groundwater and surface water 100 mL aliquots were each spiked in duplicate with 50 and 600 cells of flow sorted *R. coprophilus*. All samples were filtered and bead beated as described and evaluated above, then the second of each duplicate was processed according to the instructions on each kit, respectively. Each sample was analyzed via traditional PCR and qPCR. This experiment was repeated for each of the two kits. The results indicate that traditional PCR is more inhibited by lake water than qPCR. However, both kits were able to effectively eliminate PCR inhibitors at both spike concentrations. Both kits also showed some target DNA loss, which was expected.

The Zymo ZR Soil Microbe DNA Kit™ was evaluated for its ability to eliminate/reduce environmental PCR inhibitors, as well as recovery efficiency of target DNA. Groundwater and lake water 100 mL aliquots were each spiked in duplicate with 50 flow sorted cells (low) and 600 flow sorted cells (high) of *R. coprophilus*. All samples were filtered and bead beated as described in EPA method 1606, then the second of each duplicate pair was processed according to the instructions on the Zymo ZR Soil Microbe DNA Kit™. Each sample was analyzed via traditional PCR and qPCR. The results indicate that traditional PCR is more inhibited by lake water than qPCR. The Zymo kit was able to effectively eliminate PCR inhibitors at both spike concentrations, and it also showed some target DNA loss, which was expected. It was decided not to pursue this kit further because it is more labor intensive and demonstrated greater target DNA loss than both the QIAgen DNeasy® Blood and Tissue kit and the MoBio Power Clean™ kit.

Next, a number of targeted inhibitor tests were performed using standardized solutions containing humic acids and/or colloidal clay. Humic acids are a known PCR inhibitor and constitute a major fraction of the natural organic matter (NOM) in nearly all surface waters. NOM is a problem when attempting to concentrate, extract, and amplify DNA from environmental sources. The QIAgen DNeasy® Blood and Tissue kit and the MoBio PowerClean™ DNA Clean-Up kit were each evaluated, in separate experiments, for their ability to eliminate low to high concentrations of humic acids from samples.

Humic Acid addition/Clean-up kit evaluation

For each experiment, 2 liters of buffer solutions with specified concentrations of humic acids representing a range from low to high (4, 20 and 40 mg/L) were spiked with 50 and 600/100 mL of flow sorted *R. coprophilus* cells. The two spike levels from each concentration were membrane filtered, bead beated and resuspended in 300 µL of AE buffer. The second of each duplicate pair in each experiment was processed according to the instructions on each kit (QIAgen DNeasy® Blood and Tissue kit and MoBio PowerClean™ DNA Clean-Up kit), respectively. Each extract was analyzed via traditional PCR and qPCR.

Both kits were able to eliminate humic acid inhibition at the lowest (4 mg/L) test concentration. However, the MoBio PowerClean™ kit far outperformed the QIAgen DNeasy® kit at the mid (20 mg/L) and high (40 mg/L) humic acid concentrations based on inhibition removal and consistency of PCR results.

Turbidity addition/Clean-up kit evaluation

The QIAgen DNeasy® Blood and Tissue kit and the MoBio PowerClean™ DNA Clean-Up kit were each evaluated for their ability to eliminate mineral turbidity, another potential PCR inhibitor, from DNA extracts. Kaolin/kaolinite is a commercially available clay that does not dissolve into water, resulting in a cloudy (turbid) solution. Buffer solutions of approximately 2, 5, and 10 NTU to represent typical turbidities (from low to high) commonly found in Wisconsin waters. Two separate experiments were conducted, one using each kit.

For each experiment, buffer solutions with known amounts of kaolin representing a range from 2 NTU to 10 NTU were spiked with 50 or 600/100 mL flow sorted cells of *Rhodococcus coprophilus*. The two spike levels were processed as described above, and the second of each duplicate pair was processed according to the instructions on each kit, QIAgen DNeasy® Blood and Tissue kit and MoBio PowerClean™ DNA Clean-Up kit, respectively. Each sample extract was analyzed via traditional PCR and qPCR.

Both kits were able to eliminate turbidity inhibition at all levels. It is likely that the centrifugation step in the method (part of nucleic acid extraction) is sufficient to remove turbidity before the extract is processed via the kits. There was no significant loss of target DNA when kaolin was present, indicating that in the short contact times tested (approximately 1 hour) the DNA does not bind to the kaolin.

Humic acid with turbidity - MoBio PowerClean™ kit evaluation

Because the MoBio PowerClean™ kit outperformed the QIAgen DNeasy® Blood and Tissue kit in removing humic acid inhibition, further testing was conducted using this kit only. To test how well the MoBio PowerClean™ kit was able to eliminate a combination of humic acid and turbidity inhibition, 2 liters of buffer solution with 4 mg/L humic acid and 5 NTU turbidity were prepared and spiked with 50 or 600/100 mL flow sorted cells of *Rhodococcus coprophilus*. The two spike levels were processed as described earlier. The second of each duplicate was processed according to the instructions on the kit. Each sample was analyzed via traditional PCR and qPCR. The MoBio PowerClean™ kit was able to eliminate all PCR inhibition from the samples so that the DNA was effectively amplified in both traditional PCR and qPCR.

PCR Amplification and/or Quantification

There are two major formats for polymerase chain reaction (PCR) available to laboratories. There is the traditional PCR, where the target nucleic acid is amplified through repeated thermal program cycles, and the products are visualized using a gel. Amplification of a DNA sequence of the appropriate size (in base pairs) confirms a positive result. While the density of the amplicon (as evidenced by band width and brightness) is proportional to the starting number of targets, traditional PCR typically provides presence/absence information. The second format is quantitative real-time PCR or q PCR, which simultaneously amplifies and quantifies a targeted DNA . Quantitative real-time PCR in this experiment used TaqMan probes that utilize a fluorescent dye and quencher pair. As the target DNA is amplified, the dye begins to fluoresce as the quencher is cleaved during the extension cycle. The Ct or cycle threshold at which fluorescence is detected is related to the concentration of target DNA present in the sample. A standard curve of known cell or DNA sequence numbers is prepared and the Ct values of those standards are used to develop an equation for calculating sample concentrations. Key to designing an appropriate PCR assay is the selection of primer pairs (forward and reverse) and for

qPCR - the probe sequences. The sequences and concentration of primers and probe used in this research were determined by Savill *et al*. (2001).

A standard curve was created for qPCR to accurately determine the concentration of cells present in a qPCR reaction. The sample's Ct value is compared to a standard curve of known Ct values. To create a standard curve, precise amounts of cells must be filtered, extracted, and amplified on the thermocycler (a LightCycler in this research). The qPCR program as well as primers and TaqMan probe were synthesized based on Savill *et al*. (2001). It is especially challenging to achieve spikes of precise amounts of *R. coprophilus* because of its propensity to stick and clump. To disperse clumps, a 5-day culture of *R. coprophilus* was grown in Bennett's broth with 0.2% Tween 80, then poured through a 25 μm sieve to break up and exclude large clumps. A 2 mL aliquot was sonicated for 1 minute, then flow sorted into 60; 600; 6000; 60,000; and 600,000 cell aliquots. From this, a standard curve was created with an error of 0.0449, which is a very low and acceptable number.

To validate the qPCR method and the standard curve of *R. coprophilus*, flow sorted cells were spiked into 100 mL of groundwater (Madison municipal well) and processed as previously described. The extract was amplified via qPCR and compared to the standard curve. At the lower concentrations (25 and 50 cells), the software was only able to determine the concentration of one of the samples, and the rest were considered "positive" or "uncertain." For the 1000 cell spike, the average concentration was determined to be 687 cells. The internal control showed no inhibition factors for the test groundwater. At lower spike levels, qPCR could not determine the number but only a presence absence result.

As discussed above, the traditional PCR assay is capable of detecting approximately 20 to 25 cells per filter volume (typically 100 mL) if the concentration of inhibitors is low. The commercial clean-up kits did not change the detection of 20 to 25 cell per 100 mL. The qPCR lower level of detection was not determined in this research.

Other Methodological Considerations

Laboratory efficiency can be aided by batching samples. Therefore, it was important to determine the best step at which MST samples could be held in order to be batched without losing detection efficiency. To examine this, a factorial experimental designed was used to determine if holding times and temperatures had any effect on *R. coprophilus* DNA recovery and quantification.

A large volume of buffer was spiked with about 50 cells/100 mL of flow sorted *R. coprophilus*. Aliquots of this spike were filtered immediately for PCR analysis and plate counting. Another set of aliquots were filtered and the membranes frozen. A third set of spiked buffer samples aliquots were held at 4°C. On subsequent days, the "held" samples were analyzed for the quantity of target DNA/*R. coprophilus* cells present and were compared to "day 0" of the experiment. A summary of the results are presented in Table 2. Analysis consistency appeared to improve for both traditional and qPCR by freezing the membranes. Therefore, unless time is an issue in resolution of contamination situations, freezing and batching samples may benefit the accuracy of results.

Table 2. Comparison of 50 cell/100 mL Sample Holding Conditions

Sample		Plate (cfu/100 mL)	qPCR (cells/100 mL)	qPCR avg (cells/100 mL)	Traditional PCR
Day 0	1	<1	301		+
	2		74	188	+
	3		+, no conc.		weak +
	4		+, no conc.		+
Day 1	1		146		+
Frozen	2		Uncertain	71	+
	3		0		+
	4		67		+
Day 1	1	<1	0		weak +
Liquid	2		54	23	-
	3		16		+
	4		Uncertain		weak +
Day 3	1		65		weak +
Liquid	2		10	40	weak +
	3		Uncertain		weak +
	4		44		weak +
7 day	1		53		+
Frozen	2		82	50	+
	3		29		+
	4		38		+

Method Comparison

The filtration/resuspension/spread-plate culture method for *R. coprophilus* described by Arango has been the benchmark for MST studies in Wisconsin since 2006. Sixteen MST samples from around Wisconsin were submitted to WI State Lab of Hygiene through the duration of this project. These samples were analyzed in parallel by the culture, traditional PCR, and qPCR methods as the methods developed. Table 3 summarized the results. Overall, the results for traditional PCR and the culture method compare favorably. With the exception of samples with inconclusive results, seven of eight or 87.5% of samples enumerated by traditional PCR and the culture method matched. Four of the samples contained high background bacterial levels that resulted in interference and inconclusive results for the culture method, while traditional PCR provided clear positive or negative results. For qPCR versus the culture method, six of six samples with conclusive results matched. In two of the four cases where the culture method yielded inconclusive results, qPCR yielded a result. In two of the four cases where the culture method yielded inconclusive results, qPCR also yielded inconclusive results. Comparing the two molecular methods, eight of the ten or 80% yielded consistent results and two yielded conflicting results. The qPCR method was more susceptible to sample quality (*i.e.* presence of inhibitors and background DNA) than traditional PCR.

Table 3. Results of Method Comparisons from August 2008 through February 2009

Sample	Vol filtered	Traditional PCR	Kit	qPCR (100 mL)	Culture (CFU/100 mL)	Total coliforms (MPN/100 mL)	*E. coli* (MPN/100 mL)	Enterococci (MPN/100 mL)
1	250 mL	Neg, inhibition	No	ND	100	74.9	4.1	8.5
2	100 mL	Negative	No	ND	<10	5.2	2	<1
3	200 mL	Positive	No	ND	20	613	2	9
4	250 mL	Pos., QC failed	No	ND	<10	1783	19	19
5	250 mL	Pos., QC failed	No	ND	<10	<1	<1	<1
6	250 mL	Neg., QC failed	No	ND	<10	155	<1	1
7	150 mL	Negative	Q	Negative	<10	<1	<1	<1
8	250 mL	Negative	Q	Negative	<10	3.1	<1	<1
9	77 mL	Pos., inhibition	Q	50, inhibition	48	8.5	1	<1
10	250 mL	Positive	M	600	180	1310	435.2	344.8
11	20 mL	Positive	M	2668	Inc*	>240,000	9330	4960
12	250 mL	Positive	M	93	44	37.3	6.3	3.1
13	250 mL	Positive	M	1413	81	96.1	16.1	9.8
14	30 mL	Positive	M	Negative	Inc*	ND	ND	ND
15	40 mL	Positive	M	Negative[a]	Inc*	14,670	2	8.6
16	60 mL	Negative	M	Negative[a]	Inc*	36,540	1	3.1

ND - not done
Q - QIAgen DNeasy® Blood and Tissue kit
M - MoBio PowerClean™ DNA Clean-Up kit
Inc* - inconclusive, matrix spike was negative
Negative[a] - inconclusive, matrix spike was negative

These results demonstrate that no single method is superior to the others. A number of factors need to be considered when assessing the value of microbial testing. Those factors include cost (capital, supplies, and labor), time to results (days to weeks), and the type and reliability of the data obtained. Table 4 summarizes a number of these factors for the culture method, traditional PCR and qPCR.

Table 4. Comparison of *R. coprophilus* Detection Methods

	Culture	Quantitative PCR	Traditional PCR
Cost - Capital	$24,000	$53,000	$50,000
Cost - Consumables	$56	$258	$193
Labor	~11 hrs	~5-7 hrs	~5-7 hrs
Time to results	21-30 days	2+ days	2+ days
Detection limits	~12 CFU	~60 CEC	~20 CEC*
Provides time sensitive results	No	Yes	Yes
Common skills	+	-	0
Specialized training needed	Yes	0	0
Specialized instruments	No	Yes	Yes
Matrix interference/inhibition	0	-	-
Sample batching	-	+	+
Viability indicator	Yes	No	No

*Presence/absence
+ advantageous
0 neutral
- disadvantageous

The advantages of the culture method are that it is cheaper overall, does not require specialized equipment/instrumentation, is a viability indicator, is quantitative, and has the lowest detection limit provided background bacterial populations are not overly large. The drawbacks of this method are that it is time consuming, requires experience and specialized training needed to identify unique *R. coprophilus* colonies among background colonies, and it can only detect living cells.

For traditional PCR, to achieve desirable detection limits, 15 µL of template was added to 35 µL of master mix. This can create problems because the increased volume of extract can also result in increased amounts of PCR inhibitors. However, when the extract is cleaned using the MO BIO PowerClean™ clean-up kit, no inhibition problems were observed. Although traditional PCR is not able to determine the quantity of cell equivalents, there are programs available that can quantify the brightness of bands on an agarose gel, allowing for target concentrations to be estimated if a number of known cell extracts are also analyzed. Traditional PCR also has the advantage of being more reliable than qPCR, especially in the presence of high concentrations of non-target DNA. In the specific environmental samples tested (*i.e.* groundwater), because of the filtration, adsorption, and inactivation of organisms by soil, the mere presence of *R. coprophilus* cells signals manure contamination.

Although qPCR has the advantage of quantifying the amount of target DNA present, the assay is less reliable. Some issues may be a result of the type of thermocycler and master mix used. TaqMan probes are light sensitive and can degrade quickly. In this research, this phenomenon often resulted in the LightCycler software misreading negative samples as positives. This can create issues with QA/QC, as well as decreased confidence in calculated cell concentrations. Samples positive by traditional PCR were negative in two instances by qPCR and inclusive by the culture methods. These samples may appear "negative" in the qPCR assay because of high concentrations of non-target DNA present in the reaction mix. When compared to the *R. coprophilus* culture method and traditional PCR, qPCR also has the disadvantage of having higher capital costs, higher consumables cost, and a higher detection limit. However, qPCR allows for quantitative analysis, batching of samples, as well as a quick turnaround time from sample receipt to results.

Conclusion

The final method consists of membrane filtration of samples (up to 250 mL or until membrane refusal), resuspension in 300 mL AE with 150 mg of (212-300 µm, 50-70 US sieve) acid washed glass beads, bead beating on "homogenize" for one minute, clean-up using the MoBio PowerClean™ kit if needed, with traditional PCR of triplicate 15 µL volumes of target and with qPCR of triplicate 5 µL volumes of target.

This work supports moving to using molecular detection of *R. coprophilus* for MST samples in Wisconsin. Results can be obtained in as little as 2 to 4 days with detection limits similar to those of the culture-method.

Future work

The current method reports results as presence/absence for traditional PCR and calibrated cell equivalents for qPCR. In order to most accurately construct standard curves, it is important to know the number of copies of qPCR targets present in your typical target organism cell. Therefore, experiments determine the 16S gene copy number of *R. coprophilus* are necessary to calculate more accurate detection limits of molecular protocols. However, this work was beyond the scope and budget of this project. It is hoped that these experiments can be part of future MST initiatives within WI State Lab of Hygiene or Dr. Long's UW research program.

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