Comparison of PCR and quantitative real-time PCR methods for the characterization of ruminant and cattle fecal pollution sources

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ABSTRACT

The State of California has mandated the preparation of a guidance document on the application of fecal source identification methods for recreational water quality management. California contains the fifth highest population of cattle in the United States, making the inclusion of cow-associated methods a logical choice. Because the performance of these methods has been shown to change based on geography and/or local animal feeding practices, laboratory comparisons are needed to determine which assays are best suited for implementation. We describe the performance characterization of two end-point PCR assays (CF128 and CF193) and five real-time quantitative PCR (qPCR) assays (Rum2Bac, BacR, BacCow, CowM2, and CowM3) reported to be associated with either ruminant or cattle feces. Each assay was tested against a blinded set of 38 reference challenge filters (19 duplicate samples) containing fecal pollution from 12 different sources suspected to

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impact water quality. The abundance of each host-associated genetic marker was measured for qPCR-based assays in both target and non-target animals and compared to quantities of total DNA mass, wet mass of fecal material, as well as Bacteroidales, and enterococci determined by 16S rRNA qPCR and culture-based approaches (enterococci only). Ruminant- and cow-associated genetic markers were detected in all filters containing a cattle fecal source. However, some assays cross reacted with non-target pollution sources. A large amount of variability was evident across laboratories when protocols were not fixed suggesting that protocol standardization will be necessary for widespread implementation. Finally, performance metrics indicate that the cattle-associated CowM2q PCR method combined with either the BacR or Rum2Bac ruminant-associated methods are most suitable for implementation.

INTRODUCTION

The presence of fecal contamination in recreational waters from ruminant animals, in particular cattle can pose a threat to public health (Soller *et al.* 2010). For example, cattle feces are commonly associated with the spread of Salmonella, *Escherichia coli* O157:H7, Giardia, and Cryptosporidium. Human populations may be exposed to cattle-derived fecal pathogens via a number of routes (MacKenzie *et al.* 1994, Fayer and Lewis 1999) including swimming or bathing in recreational waters (Cabelli *et al.* 1982, Keene *et al.* 1994). Waterborne disease outbreaks due to suspected cattle fecal contamination are documented worldwide (i.e., Cryptosporidium in Milwaukee, Wisconsin, USA in 1993). Currently, health authorities rely on the enumeration of fecal indicators (i.e., enterococci or *E. coli*) to identify the presence of fecal contamination. However, a variety of warm-blooded, and even some cold-blooded (Harwood *et al.* 1999, McLain *et al.* 2009), animals contain these same fecal indicators making these approaches insufficient for the determination of cattle fecal pollution. As a result, many methods have been developed to detect and/or quantify ruminant fecal pollution sources (Bernhard and Field 2000, Reischer *et al.* 2006, Kildare *et al.* 2007, Shanks *et al.* 2008, Mieszkin *et al.* 2010). A recent study designed to assess the performance of several of these methods with a collection of cattle fecal samples collected from different geographic locations across the United States found that the shedding of ruminant-associated fecal indicators dramatically changed based on

local animal feeding practices (Shanks *et al.* 2010). The notion that performance can vary from one geographic location to another due to local animal diets or other uncharacterized factors suggests that these methods must be tested before implementation in a particular region.

In California, it is estimated that there are over six million ruminant animals including cattle (5.35 million), sheep (570,000), goat (3500), deer (445,000), as well as alpaca and llama (1800); USDA 2012). Because of the prevalence of ruminant animals in this geographic region, cattle- and ruminant-host associated fecal identification approaches were included in a large multiple laboratory fecal source identification method evaluation study to identify top performing technologies for the State of California (Boehm *et al.* 2013). The overall report of this study provides an excellent overview of the findings submitted by 27 different laboratories using a total of 42 different fecal source identification technologies designed to identify fecal animal sources ranging from cattle to pigeons. However, the overall report leaves several important factors that may influence the performance of ruminant/cattle-associated methods unaddressed warranting further study in the present work. This report describes the performance of two end-point PCR assays (CF128 and CF193) and five qPCR assays (Rum2Bac, BacR, BacCow, CowM2, and CowM3) previously reported to be associated with either ruminant and/ or cattle feces (Bernhard and Field 2000, Reischer *et al.* 2006, Kildare *et al.* 2007, Shanks *et al.* 2008, Mieszkin *et al.* 2010) using reference fecal samples collected from the state of California. Issues such as lack of standardization of protocols, use of extremely high concentrations of fecal material, influence of selected performance benchmark definition (unit of measure and test concentration), and the high degree of similarity in primer design between most ruminant methods are explored.

METHODS

Sample Collection and Preparation

Fecal material was collected from more than 100 individual animals representing 10 different species (human, horse, cow, deer, pig, goose, chicken, pigeon, gull, and dog), 9 primary effluent wastewater samples, and 6 septage samples collected from Northern, Central and Southern California (Ervin *et al.* 2013). Fecal slurries were prepared for each

pollution source by mixing equal wet weight masses or volumes of respective individual samples to generate composites. Blinded, composite samples, of both single sources and mixed sources (two pollution types), were prepared for each slurry at two concentrations (undiluted and 1:10) using 47 mm diameter, 0.4 μm polycarbonate membranes and distributed to participating laboratories in duplicate sets (n = 38 filters/laboratory). More detailed information about fecal sample collection and creation of blinded reference samples is reported elsewhere (Boehm *et al.* 2013).

Participating Laboratories and Method Selection

Eleven laboratories from the United States (n = 7) and the European Union (n = 4) contributed data from seven host associated methods (Table 1). Methods originally reported to be ruminant associated included two end-point PCR approaches, CF128 andCF193 (Bernhard and Field 2000), as well as three qPCR technologies, Rum2Bac (Mieszkin *et al.* 2010), BacR (Reischer *et al.* 2006), and BacCow (Kildare *et al.* 2007). Note that the BacCow qPCR method was originally reported to be

cattle associated, but is considered to be ruminant-associated in this study. The rational for this change is based on findings reported elsewhere (Wang *et al.* 2010, Boehm *et al.* 2013) and similarities in primer design to other ruminant-associated methods (data not shown). Two qPCR cattle-associated methods were also submitted including CowM2 and CowM3 (Shanks *et al.* 2008).

Molecular Protocols

Method protocols, reagents, instrumentation, and data analysis procedures were not standardized across laboratories in many instances. A complete description of each individual laboratory nucleic acid isolation kit, PCR or qPCR reagents, thermal cycling instrumentation, and key data analysis information are reported elsewhere (Boehm *et al.* 2013). Briefly, for end-point PCR methods, two nucleic acid isolation kits, two DNA polymerase reagent types, and three different thermal cycling instruments were used. For qPCR methods, three nucleic acid isolation kits, five DNA polymerase reagent types, and three thermal cycling instruments were used. In addition, no two participating laboratories used the same values for the lower limit of quantification (LLOQ)

Table 1. Originally reported performance information for ruminant- and cow-associated PCR and qPCR methods.

Method	Platform[a]	Presumptive Animal Target(s)	Fecal Reference Sources		Performance Benchmark (per reaction)	LLOQ (target copies)	Initial Report (%)	
			Target	Non-Target			Sen	Spec
Cow M2	qPCR	Cow	Cow	Alpaca, Canada Goose, Cat, Chicken, Deer, Dog, Duck, Goat, Horse, Human, Pelican, Pig, Gull, Sheep, Turkey, Wastewater	1 ng total DNA	10	NR	100
Cow M3	qPCR	Cow			1 ng total DNA	10	NR	98
BacCow	qPCR	Cow	Cow	Horse, Dog, Cat, Gull, Wastewater, Human	5000 copies Bacteroidales	31	100	62
Rum2Bac	qPCR	Ruminant	Bovine, Sheep	Human, Pig, Horse, Wild Bird	NR	1.6	97	100
BacR	qPCR	Ruminant	Cattle, Deer, Chamois	Human, Pig, Fox, Cat, Dog, Chicken, Turkey, Swan, Horse, Duck, Black Goose	0.1 mg wet mass	6	100	100
CF193	PCR	Ruminant	Cow, Deer, Elk	Cat, Dog, Duck, Pig, Gull	NR	NR	NR	100
CF128	PCR	Ruminant	Goat, Sheep, (Llama)					100

[a] "Platform" denotes quantitative real-time PCR (qPCR) or end-point PCR (PCR).

"Performance Benchmark" refers to unit of measure definition and concentration of fecal material used per reaction to assess sensitivity and specificity.

"LLOQ" indicates reported lower limit of quantification.

"Sen" represents sensitivity.

"Spec" denotes specificity.

"NR" indicates not reported.

() indicates pseudo-ruminant animal source.

for a given qPCR method. Each laboratory then submitted results in binary (presence/absence) and quantitative (estimated $\log_{10}$ copies/sample) formats for performance comparisons.

Performance Metrics

Four metrics were employed to assess method performance including: 1) sensitivity (true positives/(false negatives + true positives)), 2) specificity (true negatives/(false positives + true negatives)), 3) estimated mean target abundance ($\log_{10}$ copies/target group), and 4) estimated mean non-target abundance ($\log_{10}$ copies/non-target group). For cattle-associated methods, the target group includes only reference samples with cow fecal sources. For ruminant-associated methods, the target group included both cattle and deer reference samples. All other pollution sources were classified as non-target.

Impact of Performance Benchmark Threshold and Fecal Material Test Concentration

Original publications for each method utilized different performance benchmark thresholds to report performance (Table 1). A performance benchmark threshold refers to the unit of measure (i.e., mass of total DNA, wet mass of fecal material, etc.) and cut-off concentration used by a particular laboratory to characterize method performance. An *in silico* approach was used to investigate how these differences may impact sensitivity, specificity, as well as abundance of target and non-target metrics. For this particular analysis, only data from respective developing laboratories were used to eliminate variability introduced due to lack of standardization of protocols and potentially different proficiency levels of laboratory personnel. A developing laboratory was defined as the original laboratory which first reported the tested method in the peer-reviewed literature. Raw data from developing laboratories was available for all qPCR methods in this study and was reanalyzed as follows. First, standard curve quantification cycle (C_q) values for each method were used to generate master calibration models (Sivaganesan *et al.* 2010). Second, reference sample C_q values were used to estimate respective DNA target concentrations ($\log_{10}$ copies/reaction). Estimates were then classified as either a detect or non-detect based on six different benchmark thresholds including: 1) estimates $<0.1 \log_{10}$ copies per reaction = non-detect, 2) estimates $<$LLOQ ($\log_{10}$ copies/reaction) = non-detect where LLOQ is equal to the mean estimate from the lowest calibration

curve standard concentration C_q value reported by each developing laboratory, 3) estimates $<$predicted concentration had 1 ng of total DNA been placed in the reaction = non-detect where the predicted $\log_{10}$ copies of a genetic marker per ng of total DNA was determined by NanoDrop ND-1000 UV spectrophotometer measurements submitted by each developing laboratory, 4) estimates $<$the predicted concentration had 5000 copies of GenBac3 genetic marker (Siefring *et al.* 2008) been placed in the reaction = non-detect, 5) estimates $<$the predicted concentration had 0.1 mg wet mass of fecal material been placed in the reaction = non-detect, and 6) estimates $<$the predicted concentration had 104 MPN enterococci been placed in the reaction = non-detect. Predicted $\log_{10}$ copies per reaction for 5000 copies of GenBac3, 0.1 mg wet mass of fecal material, and 104 MPN of enterococci were inferred based on measurements from replicate samples reported elsewhere (Ervin *et al.* 2013). Predicted concentrations of a given genetic marker per benchmark threshold were estimated using *in silico* dilutions or additions based on the proportions of the benchmark value (i.e., 1 ng total DNA, 0.1 mg wet mass, etc.) to measured quantities previously reported for each reference sample. This proportion was then applied to predict the respective $\log_{10}$ copies of a genetic marker per reaction for a given benchmark threshold approach. This method of data normalization assumes that the fecal proportion between different threshold definitions remains constant within each fecal pollution source when diluted or concentrated *in silico*. To illustrate the impact that fecal material test concentration per reaction has on perceived performance, the specificity of the BacCow qPCR assay was estimated over a range of GenBac3 concentrations ranging from 2.0 to 5.0 $\log_{10}$ copies/reaction using the same *in silico* dilution approach described above.

Data Analysis

Simple statistics including correlation of coefficient determination (R^2) were calculated with SAS software (Cary, NC) and Microsoft Excel. Amplification efficiencies (E) were based on the following equation: $E = 10^{\wedge}(-1/slope) -1$.

RESULTS

Method Performance across All Participating Laboratories

The range of performance metric values reported by participating laboratories is listed in Table 2 for

each method. Sensitivity ranged from 33 to 100% and specificity ranged from 38 to 100%; however, multiple methods showed 100% sensitivity and specificity. Influence of performance benchmark threshold and test concentration C_q values submitted from each developing laboratory for CowM2, CowM3, BacCow, Rum2Bac, and BacR qPCR methods were analyzed using the same calibration model and LLOQ definition. Calibration models for each method exhibited a high level of quality with correlation coefficients (R^2) of 0.99, amplification efficiencies ranging from 0.922 to 1.01, and LLOQ values spanning 0.89 to 1.93 $\log_{10}$ copies/reaction (Table 3). Performance metrics for each qPCR method are reported in Table 4 using the six previously described benchmark definitions. Sensitivity ranged from 0 to 100% and specificity spanned 59 to 100% across methods depending on

performance benchmark definition. The specificity of the BacCow qPCR method was predicted *in silico* over a range GenBac3 genetic marker test reaction concentrations (Figure 1). In addition, the originally reported benchmark test concentration (5000 GenBac3 target copies/reaction) and the estimated mean GenBac3 target copies/reaction calculated from previously reported (Ervin *et al.* 2013) concentrations in replicate samples all reference fecal pollution sources (3.69 $\log_{10}$ copies/reaction) are shown.

DISCUSSION

Standardization of Method Protocols Is Paramount

A large range of specificity and sensitivity values was observed between different participating

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Table 2. Summary of reported performance metric values from practicing laboratories.

Method	N	Sensitivity (%)	Specificity (%)	Abundance[b] (mean Log₁₀ copies/group)	
				Target	Non-target
BacCow	5	100[a]	38 - 88	7.62 - 8.34[a]	3.04 - 4.69
BacR	2	100[a]	58 - 100[a]	6.17[a] - 7.64	<0.1[a] - 1.87
Rum2Bac	1	100[a]	100[a]	6.97[a]	<0.1[a]
CowM2	5	100[a]	97 - 100[a]	4.80 - 5.48	<0.1[a] - 2.69
CowM3	2	100[a]	100[a]	4.52[a] - 5.89	<0.1[a]
CF128	2	33 - 100	88 - 100	NA	NA
CF193	1	67	94	NA	NA

"N" refers to the number of participating laboratories that submitted data for a respective method.

"NA" indicates not applicable.

[a] Denotes value reported by method developing laboratory.

[b] Abundance refers to the mean log10 copies for each collection of reference samples for either "target" or "non-target" classification reported by each participating laboratory.

Table 3. Calibration model statistics for developing laboratory qPCR data.

Method	Instrument	Calibration Equation	R^2	E	ROQ
CowM2	ABI 7900 HT	Y = 40.1-3.52X	0.99	0.922	10 to 1 x 10⁵
CowM3	ABI 7900 HT	Y = 37.6-3.41X	0.99	0.961	10 to 1 x 10⁵
BacCow	ABI PRISM 7900	Y = 40.5-3.28X	0.99	1.017	100 to 1 x 10⁸
Rum2Bac	BioRad CFX96	Y = 41.3-3.38X	0.99	0.974	10 to 1 x 10⁶
BacR	iCycler iQ Real-Time Detection System	Y = 40.5-3.28X	0.99	0.967	9 to 9 x 10⁴

"Instrument" denotes quantitative real-time PCR machine used to generate respective master calibration equation.

"R^2" indicates the coefficient of determination representing the proportion of variability in the data set accounted for by the linear model.

"E" represents amplification efficiency and is equal to $10^{[1/-slope]}-1$.

"ROQ" refers to the range of quantification measured in copies of target DNA for each respective qPCR assay.

Table 4. Performance metric values of ruminant- and cow-associated qPCR methods with different performance benchmark thresholds.

Method	Threshold	Sen (%)	Spec (%)	Animals with false positive	Abundance[a] (mean Log$_{10}$ copies/group)	
					Target	Non-target
CowM2	Raw	100	100	–	3.14	<0.1
	LLOQ	100	100	–	3.14	<0.1
	1 ng	75	100	–	2.25	<0.1
	GenBac3	50	100	–	1.63	<0.1
	Ent 104	100	100	–	3.78	<0.1
	Wet	75	100	–	2.32	<0.1
CowM3	Raw	100	100	–	2.05	<0.1
	LLOQ	75	100	–	2.51	<0.1
	1 ng	50	100	–	1.33	<0.1
	GenBac3	0	100	–	–	–
	Ent 104	100	100	–	2.69	<0.1
	Wet	50	100	–	1.65	<0.1
BacR	Raw	100	85	Chicken, dog, human, septage	3.91	<0.1
	LLOQ	100	100	–	3.91	<0.1
	1 ng	100	97	Chicken	3.10	0.88
	GenBac3	100	100	–	2.49	1.48
	Ent 104	100	97	Chicken	4.88	1.79
	Wet	100	97	Chicken	3.47	2.42
Rum2Bac	Raw	100	97	Septage	5.25	0.80
	LLOQ	100	100	–	5.25	<0.1
	1 ng	100	100	–	4.25	<0.1
	GenBac3	100	97	–	3.35	<0.1
	Ent 104	100	97	Septage	5.72	2.07
	Wet	100	100	–	4.31	<0.1
BacCow	Raw	100	59	Chicken, dog, goose, gull, horse, human, pig, septage	5.50	1.67
	LLOQ	100	79	Chicken, dog, horse, pig	5.50	2.66
	1 ng	100	79	Chicken, dog, horse, pig	5.58	2.68
	GenBac3	100	91	Chicken, gull, pig	3.48	3.26
	Ent 104	100	82	Chicken, horse, pig	6.06	3.19
	Wet	100	85	Chicken, dog, horse, pig	4.65	2.80

"Threshold" indicates the performance benchmark threshold used to establish performance.

"Raw" denotes use of any data generated from respective instrument run.

"LLOQ" signifies use of lower limit of quantification as cut-off for use in performance determination.

"1 ng" indicates 1 ng of total DNA mass per reaction cut-off.

"GenBac3" represents 5000 copies of GenBac3 per reaction cut-off.

"ENT 104" shows 104 MPN of enterococci per reaction cut-off.

"Wet" refers to 0.1 mg of wet fecal material per reaction cut-off.

"Sen" denotes sensitivity equal to total positives/(false negatives + total positives).

"Spec" denotes specificity equal to total negatives/(false positives + total negatives).

[a] "Abundance" refers to the mean log$_{10}$ copies for each collection of reference samples for either "target" or "non-target" classification based on data reported by respective developing laboratory.

laboratories suggesting that differences in nucleic acid isolation, qPCR instrumentation, laboratory proficiency, quality assurance, and/or data analysis protocols can dramatically influence perceived method performance (Table 2). This was most pronounced in the assessment of the BacR qPCR method where reported specificity ranged from 58 to 100% between participating laboratories. Because protocols and data analysis approaches were not standardized across laboratories, it is impossible to deduce whether the range of specificity values is a function of the performance of the method or simply
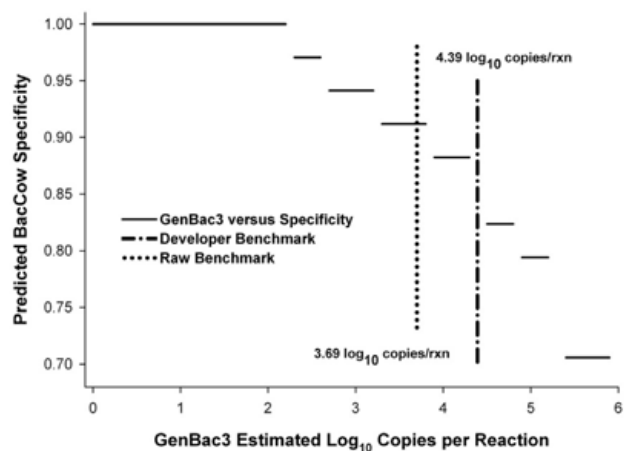
Figure 1. Multiple line plot indicating predicted specificity ranges of the BacCow qPCR method at different GenBac3 test reaction amounts. Vertical lines represent $\log_{10}$ copies of GenBac3 per reaction performance benchmark thresholds for the developing laboratory (dotted line) and initial, unadjusted amounts from reference samples "raw" in current study (dashed line).

variability introduced due to differences in technician skill and data analysis choices. It is interesting to note that the BacR developing laboratory reported 100% specificity, raising an important question: which participating laboratory value reflects the true specificity of the method? Other studies that have investigated inter-laboratory variability of water quality qPCR technologies suggest that when method protocols are standardized, the variability between laboratories is usually very low (Griffith and Weisberg 2011, Shanks *et al.* 2012, Ebentier *et al.* 2013). Thus, it is evident that specific guidelines and standardization of protocols must be established in order for these qPCR methods to perform consistently across laboratories, and that protocols should be established based on optimal conditions described by developing laboratories.

Impact of Performance Benchmark Threshold on Perceived Method Performance

Each developing laboratory initially used a different benchmark threshold (i.e., 1 ng total DNA, 5000 copies GenBac3, etc.) to establish the original performance of their respective methods (Table 1). In other words, different developing laboratories used different amounts of fecal material in test reactions to assess method performance. Comparisons of different performance benchmark thresholds in this study resulted in sensitivity values ranging from 0 to 100%: the largest span of values possible (Table

4). Specificity was also highly variable across some methods not only resulting in large shifts in frequency of predicted false positives, but also changing which non-target pollution sources are responsible for incorrect identifications. For example, using the GenBac3 5000 copies per reaction performance benchmark, data indicates that the BacCow qPCR method cross-reacts with chicken, dog, and gull, however if the 104 MPN enterococci per reaction threshold is substituted, the method no longer cross-reacts with gull. Gulls are resident in most coastal environments and cross-reaction to these animals could be considered a severe liability. Investigation of differences in method performance due to benchmark definition selection not only highlights the potential for dramatically different conclusions of perceived method performance, but also brings to light the important question: which performance benchmark definition should be used? It is likely that there will not be one benchmark threshold that is best for all applications. Instead, a particular method may be considered the best for a given application, but not appropriate in another scenario.

Method Performance Is Influenced by Fecal Material Concentration in a Test Reaction

The concentration of fecal material in reference samples used in this study spanned over five orders of magnitude based on enterococci MPN concentration estimates (Boehm *et al.* 2013, Ervin *et al.* 2013). The notion that there could be a 100,000 times higher concentration of fecal material representative of one animal source compared to another confounds the ability to assess method performance without first normalizing reported results. Data normalization was possible for qPCR based methods based on measurements of enterococci MPN, total DNA mass, and fecal wet mass determined from replicate samples, but not for qualitative end-point PCR approaches. Thus, the performance metrics reported in Table 2 for the CF128 and CF193 end-point PCR methods should be interpreted with caution.

An *in silico* exercise was conducted to demonstrate the impact that the amount of fecal material tested has on specificity. Results indicate that a three order of magnitude shift in the amount of fecal material used for amplification can result in a 20% difference or more in predicted specificity (Figure 1). Several studies report the use of an 80% criterion for establishing an acceptable specificity level for fecal source identification technologies (Boehm *et*

al. 2013, Layton *et al.* 2013); thus, the potential for a 20% shift due to test concentration of fecal material could be problematic. The notion that method performance is dependent on the amount of fecal material in a sample has several implications. First, it will be important to establish the appropriate range of fecal material that should be used to establish method performance. This range should be based on the anticipated amount that will be encountered in environmental samples for a particular application. Second, the best method for a particular application will be dependent on the anticipated amount of fecal material from different pollution sources that will be present in environmental samples.

Trends between Ruminant- and Cattle-Associated qPCR Methods

A closer examination of qPCR protocols and results with different performance benchmark thresholds reveals some interesting trends in ruminant- and cattle-associated methods tested in this study (Table 4). First, qPCR methods that targeted 16S rRNA genes were consistently more sensitive and had a higher abundance of target DNA in ruminants compared to methods that target non-ribosomal genes associated with cattle only. However, the difference between target abundance could be as small as six-fold depending on performance threshold selection suggesting that while there is a clear advantage from a sensitivity perspective for 16S rRNA-based methods, the magnitude of the difference does not always confer a decisive benefit. Second, qPCR methods targeting non ribosomal genes exhibited higher specificity compared to 16S rRNA-based methods in almost all instances. Together these trends illustrate the conundrum often faced by researchers that develop fecal source identification technologies; is it more important to be more sensitive or more specific?

Recommendations for Ruminant and Cattle Fecal Pollution Identification

The primary goal of this study was to characterize the performance of seven previously reported end-point PCR and qPCR methods for the identification of ruminant and cattle fecal pollution in environmental waters. Due to constraints imposed by the preparation of reference samples which resulted in samples containing different amounts of fecal material, an unbiased assessment of end-point PCR methods CF128 and CF193 was not possible. However,

experiments suggest that many of the qPCR-based methods may be suitable for fecal source identification applications. Both CowM2 and CowM3 proved to be cattle-associated and did not cross-react with any non-target pollution sources regardless of performance benchmark threshold. However, CowM2 has a slight advantage over CowM3 based on sensitivity and target abundance metrics making it the better choice relative to the samples in this study. It is more challenging to gauge the performance of the three ruminant-associated methods (BacCow, BacR, and Rum2Bac). All three methods had 100% sensitivity regardless of performance benchmark definition and all three cross-reacted with one or more non-target pollution sources. BacCow proved to be the most abundant in ruminant fecal pollution sources, followed by Rum2Bac and BacR. However, the BacCow method cross-reacted with three or more non-target pollution sources under all test conditions. Thus, the top performing ruminant associated method could be either Rum2Bac or BacR, depending on which animal sources are present in the watershed or beach of interest and what performance benchmark threshold is used. In practice, it may be beneficial to pair a cow-associated method with a ruminant-associated method, especially if cattle are present in the study area.

Even though several methods performed well in this study, it is important to note that factors such as the persistence and decay rate of genetic markers in environmental matrices, potential influence of environmental matrix on sensitivity, performance with a larger fecal pollution reference collection including samples from other geographic locations, as well as correlations to human pathogens will ultimately determine their suitability for widespread FSI applications.

LITERATURE CITED

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

Boehm, A.B., L. Van De Werfhorst, J.F. Griffith, P. Holden, J. Jay, O.C. Shanks, D. Wang and S.B. Weisberg. 2013. Performance of forty-one microbial source tracking methods: A twenty-seven lab evaluation study. *Water Research* 47:6812-6828.

Cabelli, V.J., A.P. Dufour, L.J. McCabe and M.A. Levin. 1982. Swimming-associated gastroenteritis and water quality. *American Journal of Epidemiology* 115:606-616.

Ebentier, D.L., K.T. Hanley, Y. Cao, B. Badgley, A.B. Boehm, J. Ervin, K.D. Goodwin, M. Gourmelon, J.F. Griffith, P. Holden, C.A. Kelty, S. Lozach, C. McGee, L. Peed, M. Raith, M.J. Sadowsky, E. Scott, J. Santodoming, C.D. Sinagalliano, O.C. Shanks, L. Van De Werfhorst, D. Wang, S. Wuertz and J. Jay. 2013. Evaluation of the repeatability and reproducibility of a suite of PCR-based microbial source tracking methods. *Water Research* 47:6839-6848.

Ervin, J.S., T.L. Russell, B.A. Layton, K.M. Yamahara, D. Wang, L.M. Sassoubre, Y. Cao, C.A. Kelty, M. Sivaganesan, A.B. Boehm, P. Holden, S.B. Weisberg and O.C. Shanks. 2013. Characterization of fecal concentrations in human and other animal sources by physical, cutlure, and quantitative realtime PCR methods. *Water Research* 47:6873-6882.

Fayer, R. and E.J. Lewis. 1999. Cryptosporidium parvum in oysters from commerical harvesting sites in the Chesapeake Bay. *Emerging Infectious Diseases* 5:706-712.

Griffith, J.F. and S.B. Weisberg. 2011. Challenges in implementing new technology for beach water quality monitoring: Lessons from a California demonstration project. *Marine Technology Society Journal* 45:65-73.

Harwood, V.J., J.N. Butler, D. Parrish and V. Wagner. 1999. Isolation of fecal coliform bacteria from the diamondback terrapin (*Malaclemys terrapin centrata*). *Applied and Environmental Microbiology* 65:865-867.

Keene, W.E., J.M. McAnulty, F.C. Hoesley, L.P. Wiliams, K. Hedburg, G.L. Oxman, T.J. Barrett, M.A. Pfaller and D.W. Fleming. 1994. A swimming-associated outbreak of hermorrhagic colitis caused by *Escherichia coli* O157:H7 and *Shigella sonnei*. *New England Journal of Medicine* 331:579-584.

Kildare, B.J., C.M. Leutenegger, B.S. McSwain, D.G. Bambic, V.B. Rajal and S. Wuertz. 2007. 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dogspecific fecal Bacteroidales: A Bayesian approach. *Water Research* 41:3701-3715.

Layton, B., Y. Cao, D.L. Ebentier, K.T. Hanley, L. Van De Werfhorst, D. Wang, T. Madi, R.L. Whitman, M.N. Byappanahalli, E. Balleste, W. Meijier and A. Schriewer, S. Wuertz, R.R. Converse, R.T. Noble, S. Srinivasan, J.B. Rose, C.S. Lee, J. Lee, J. Shields, J.R. Stewart, G. Reischer, A. Farnleitner, C.D. Sinagalliano, R. Rodrigues, S. Lozach, M. Gourmelon, L. Peed, O.C. Shanks, J. Jay, P. Holden, A.B. Boehm and J.F. Griffith. 2013. Performance of human fecal associated PCR-based assays: An international source identification method evaluation. *Water Research* 47:6897-6908.

MacKenzie, W.R., N.J. Hoxie, M.E. Proctor, M.S. Gradus, K.A. Blair, D.E. Peterson, J.J. Kazmierzak, D.G. Addiss, K.R. Fox, J.B. Rose and J.P. Davis. 1994. A massive outbreak in Milwaukee of Cryptosporidium infection transmitted through the public water supply. *New England Journal of Medicine* 331:161-167.

McLain, J.E.T., H. Ryu, L. Kabiri-Badr, C.M. Rock and M. Abbaszadegan. 2009. Lack of specificity for PCR assays targeting human Bacteroides 16S rRNA gene: Cross-amplification with fish feces. *FEMS Microbiology Letters* 299:38-43.

Mieszkin, S., J.F. Yala, R. Joubrel and M. Gourmelon. 2010. Phylogenetic analysis of Bacteroidales 16S rRNA gene sequences from human and animal effluents and assessment of ruminant faecal pollution by real-time PCR. *Journal of Applied Microbiology* 108:974-984.

Reischer, G.H., D.C. Kasper, R. Steinborn, R.L. Mach and A.H. Farnleitner. 2006. Quantitative PCR method for sensitive detection of ruminant fecal pollution in freshwater and evaluation of this method in Alpine Karstic regions. *Applied and Environmental Microbiology* 72:5610-5614.

Shanks, O.C., E. Atikovic, A.D. Blackwood, J. Lu, R.T. Noble, J. Santo Domingo, S. Siefring, M. Sivaganesan and R.P. Haugland. 2008. Quantitative PCR for detection and enumeration of genetic markers of Bovine fecal pollution. *Applied and Environmental Microbiology* 74:745-752.

Shanks, O.C., M. Sivaganesan, L. Peed, C.A. Kelty, R.T. Noble, A.D. Blackwood, R.N. Bushon, E.A. Stelzer, J. Kinzelman, T. Anan'eva, C.D. Sinagalliano, D. Wanless, J.F. Griffith, Y. Cao, S.B. Weisberg, V.J. Harwood, C. Staley, K.H. Oshima, M. Varma and R. Haugland. 2012. Inter-laboratory

comparison of real-time PCR methods for quantification of general fecal indicator bacteria. *Environmental Science and Technology* 46:945-953.

Shanks, O.C., K. White, C.A. Kelty, S. Hayes, M. Sivaganesan, M. Jenkins, M. Varma and R.A. Haugland. 2010. Performance assessment of cattle-associated PCR and quantitative realtime PCR assays targeting Bacteroidales genes. *Applied and Environmental Microbiology* 76:1359-1366.

Siefring, S.C., M. Varma, E. Atikovic, L.J. Wymer and R.A. Haugland. 2008. Improved real-time PCR assays for the detection of fecal indicator bacteria in surface waters with different instrument and reagent systems. *Journal of Water and Health* 6:225-237.

Sivaganesan, M., R.A. Haugland, E.C. Chern and O.C. Shanks. 2010. Improved strategies and optimization of calibration models for real-time PCR absolute quantification. *Water Research* 44:4726-4735.

Soller, J.A., M.E. Schoen, T. Bartrand, J.E. Ravenscroft and N.J. Ashbolt. 2010. Estimated human health risks from exposure to recreational waters impacted by human and nonhuman sources of faecal contamination. *Water Research* 44:4674-4691.

US Department of Agriculture. 2012. California livestock review. *National Agricultural Statistics Service* 34:1-6.

Wang, D., S.S. Silkie, K.L. Nelson and S. Wuertz. 2010. Estimating true human and animal host source contribution in quantitative microbial source tracking using the Monte Carlo method. *Water Research* 44:4760-4775.