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Performance of human fecal anaerobe-associated PCR-based assays in a multi-laboratory method evaluation study

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ABSTRACT

A number of PCR-based methods for detecting human fecal material in environmental waters have been developed over the past decade, but these methods have rarely received independent comparative testing in large multi-laboratory studies. Here, we evaluated ten of these methods (BacH, BacHum-UCD, *Bacteroides thetaiotaomicron* (BtH), BsteriF1, gyrB, HF183 endpoint, HF183 SYBR, HF183 Taqman®, HumM2, and *Methanobrevibacter smithii nifH* (Mnif)) using 64 blind samples prepared in one laboratory. The blind samples contained either one or two fecal sources from human, wastewater or non-human sources. The assay results were assessed for presence/absence of the human markers and also quantitatively while varying the following: 1) classification of samples that were detected but not quantifiable (DNQ) as positive or negative; 2) reference fecal sample concentration unit of measure (such as culturable indicator bacteria, wet mass, total DNA, etc); and 3) human fecal source type (stool, sewage or septage). Assay performance using presence/absence metrics was found to depend on the classification of DNQ samples. The assays that performed best quantitatively varied based on the fecal concentration unit of measure and laboratory protocol. All methods were consistently more sensitive to human stools compared to sewage or septage in both the presence/absence and quantitative analysis. Overall, HF183 Taqman® was found to be the most effective marker of human fecal contamination in this California-based study.

1. Introduction

The search for highly specific, sensitive, and cost effective human fecal-associated PCR-based assays has been a major focus of microbial source tracking (MST) research over the last decade. Many new methods have emerged as a result of that effort (Field and Samadpour, 2007; Roslev and Bukh, 2011). It is essential that MST methods be able to confirm the presence of human fecal contamination in environmental waters because of the ubiquity of fecal indicator bacteria (FIB) in non-human sources, knowledge gaps regarding illness risk from recreational exposure to non-human fecal sources (Boehm and Soller, 2011), and the need to prioritize investment in wastewater infrastructure. Previously, library-based methods were in common use, but these were largely supplanted by PCR-based methods following a 2003 MST method evaluation study (Griffith et al., 2003). Until now, a large-scale multiple-laboratory MST method evaluation study has not been conducted since PCR-based methods came to the fore.

The need for confidence in the performance of human fecal-associated MST assays has recently become more urgent. The United States Environmental Protection Agency's new criteria for recreational water quality offer beach managers the possibility of using quantitative microbial risk assessment (QMRA) to set site-specific criteria at beaches where the presence of human fecal pollution has been found sufficiently small through approved MST studies. Therefore it is crucial to robustly characterize the performance of MST methods that may be used to determine whether a beach is contaminated with human fecal pollution.

The most comprehensive, multiple-laboratory PCR-based MST method evaluation study to date is described in Boehm et al. (2013). Several important issues from this study remain open for further exploration in the present work. First, how does alternate classification of detectable but not quantifiable (DNQ) samples – as positive versus non-detect – change assay performance in presence/absence metrics? DNQ data handling often presents a trade-off between sensitivity and specificity, and as there is currently no consensus in the field regarding how to handle DNQ samples, it is important to consider both approaches when judging assay performance. Secondly, how did the assays perform, both qualitatively and quantitatively, under all available challenge filter sample units of measure? Characterization of challenge sample fecal concentrations in different terms (such as 1 mg of wet feces or 1 ng of total DNA) may produce variable performance results, and every available characterization of the samples should be considered in order to comprehensively compare performance among assays. Third, how did the source of "target" sample (human stools, sewage, or septage) influence assay

performance? Several factors may affect the performance assessment of these targets, including different states of decay and potential presence of non-human bacteria in wastewater; thus, it is important to evaluate these "target" sources separately. Lastly, how did the human-associated assays perform with mixed-source samples? The "doubleton" samples all contained a human stool, sewage, or septage "target" source plus a non-human fecal source, and thus offered the unique opportunity to investigate human-associated assay sensitivity in the presence of non-target feces.

The present work seeks to fill the above gaps by considering each of these issues in detail. Accordingly, the goals of this paper are to evaluate the performance of ten human fecal anaerobe-associated PCR-based assays under varying characterizations of: 1) DNQ samples; 2) the challenge filter sample concentration unit of measure; and 3) the human "target" samples; and to evaluate the effect of combining human and non-human fecal sources in a sample.

2. Methods

2.1. Sample creation and analysis

Briefly, 64 blind challenge samples were created by mixing fresh feces (from chicken, cow, dog, deer, goose, gull, horse, human, pig, or pigeon), sewage, or septage in artificial freshwater. All fecal, sewage and septage samples were obtained from various sites across California. The filter set included 19 single-source ("singleton") and 13 mixed-source ("doubleton") samples in duplicate. Each doubleton sample contained human stools, septage, or sewage combined with one non-human fecal source. Detailed methods for the creation of the challenge filter samples can be found in Boehm et al. (2013).

Seventeen laboratories from the United States and the European Union contributed data to the study. The assay naming conventions used here generally follow the original publications (Table 1). The number of laboratories that performed each method is as follows: BacH (1), BacHum-UCD (6), BsteriF1 (4), BtH (1), gyrB (1), HF183 endpoint (7), HF183 SYBR (4), HF183 Taqman® (5), HumM2 (6), and Mnif (5). The laboratories used six different DNA extraction methods: GeneRite DNA-EZ (12), Qiagen DNeasy® (1), Qiagen QIAamp® (1), MP Biomedicals FastDNA™ SPIN (1), MoBio PowerWater® (1), and phenol:chloroform extraction (1). Five laboratories involved in planning the study agreed to standardize their methods of DNA extraction (GeneRite DNA-EZ ST) and quantification (NanoDrop), q/PCR chemistries (Applied Biosystems TaqMan® Universal PCR Master Mix or TaKaRa Ex Taq® with original authors' primer/probe concentrations), and data processing (described in detail in Ebentier et al. 2013). These standardized operating protocols (SOPs) were made available to all participating laboratories, but adherence to the protocols was not required. Details of the laboratory SOPs and supply vendors are provided in the Supplemental Information (Section 1 and Tables S1–S2). All data analyses in the present work were performed in R (v 2.14.0) with RStudio (v 0.96). Details of each analysis are described below.

2.2. Classification of DNQ

The presence/absence sensitivity and specificity metrics were calculated in two ways: once with DNQ (detected, not quantifiable) samples considered positive and a second time with DNQ considered negative. All laboratories' data were analyzed together as one dataset and only the singleton (single-source) samples were included (every doubleton contained both a human and non-human fecal source, so it was not possible to independently evaluate sensitivity and specificity in the doubletons). All positive results for the endpoint assay were considered DNQ. The lower limit of quantification (LLOQ) for qPCR assays was defined for each laboratory as the lowest concentration on the standard curve where amplification was observed in at least 50% of qPCR replicates. The LLOQ values for each laboratory and assay are listed in Table S3. For samples within the range of quantification, the reported copy numbers were used. Samples with a quantification threshold cycle (Cq) greater than the laboratory-specific LLOQ were classified as DNQ regardless of how these samples were originally reported. DNQ samples were assigned a value of 150 copies/filter for quantitative analyses. This value was based on three assumptions: 1) a theoretical minimum detection limit of 3 copies per reaction (Bustin et al., 2009); 2) 2 μl template total DNA per reaction; and 3) 100 μl of DNA extract per filter. Assumptions 2 and 3 were valid for most laboratories and assays. Sensitivity and specificity metrics were calculated using the same equations and benchmarks described by Boehm et al. (2013).

2.3. Challenge filter sample units of measure

The following units of measure were used to normalize the singleton qPCR data: wet mass, total DNA, *Enterococcus* CFU, *Escherichia coli* CFU, *Enterococcus* qPCR (Haugland et al., 2005), *E. coli* 23S qPCR (Chern et al., 2011), and "general" Bacteroidales qPCR by GenBac3 (Siefring et al., 2008), AllBac (Layton et al., 2006), BacUni-UCD (Kildare et al., 2007), *B. fragilis* group (Matsuki et al., 2002), and fecal *Bacteroides* (Converse et al., 2009). The fecal source characterizations presented in Ervin et al. (2013) were used for wet mass, *Enterococcus* CFU, *E. coli* CFU, and *E. coli* 23S qPCR. Total DNA mass data were obtained from the laboratories: a majority (13 of 17) measured total DNA concentrations on each filter with a NanoDrop spectrophotometer. When values for total DNA yield were reported as negative, "too low" or some other indication of data below the detection limit, a value of 1 ng/filter was substituted. Paired measurements (of human assay targets and DNA mass) per filter were used to normalize the data. Some laboratories measured and reported "general" qPCR assay characterizations of the samples, including Entero1A (5), GenBac3 (3), AllBac (1), BacUni-UCD (1), *B. fragilis* group (1) and fecal *Bacteroides* (1). The general assay data were used to normalize the human-associated qPCR data from those laboratories using paired measurements (of human and general assay targets) per filter.

For each of the above units of measure, the assay with the highest gene copy abundance among target samples (human stools, sewage and septage taken together) was considered the most sensitive, and the assay with the largest difference in median gene copy abundance between target and non-target

Table 1 – Summary of original assay developer's publications.

Assay	Reference	Target gene	Oligo names	Reference material (standards)	Test material (target)	Challenge material (non-target)	Challenge quantity (per reaction)	LLOQ (quantity per reaction)	Sensitivity	Specificity
BacH	Reischer et al. (2007)	*Bacteroides* 16S	BacHf, BacH-pT, BacH-pC, BacHr	Plasmid	Human, sewage, cesspits	Cattle, Deer, Chamois, Roe deer, Sheep, Goat, Horse, Fox, Dog, Cat, Pig, Chicken, Turkey, Swan, Duck, Black grouse	1 mg wet wt	30 copies	98%	98%
BacHum-UCD	Kildare et al. (2007)	*Bacteroides* 16S	BacHum160f, BacHum193p, BacHum241r	Plasmid	Human, sewage	Cow, horse, dog, cat, seagull	5000 copies BacUni-UCD	30 copies	100%	87%
BsteriF1	Haugland et al. (2010)	*B. stericoris* 16S	BsteriF1DE, BthetP1, BthetR1	Plasmid	Human, sewage	Cattle, Pig, Chicken, Dog, Cat	1 ng fecal DNA	10 copies	100%	NR[a]
BtH	Yampara-Iquise et al. (2008)	*B. thetaiota-omiron* α-1-6 mannanase	BtH-F, BtH-P, BtH-R	Genomic *B. thetaiota-omicron* DNA	Human, sewage	Dogs, Beef cattle, Dairy cattle, Horses, Swine, Goose, Chickens, Turkeys	1 ng fecal DNA	9.3 copies	100%	100%
gyrB	Lee and Lee (2010)	*B. fragilis* gyrB	Bf904F, Bf923MGB, Bf958R	Genomic *B. fragilis* DNA	Human	Cow, Dog, Pig	10 ng fecal DNA	$1.1*10^2$ copies	100%	97%
HF183 endpoint	Bernhard and Field (2000)	*Bacteroides* 16S	HF183F, Bac708R	Plasmid	Human, sewage	Cat, cow, deer, dog, duck, elk, goat, llama, pig, seagull, sheep	2–4 ng fecal DNA	$1.4*10^{-6}$ g/L dry sewage	88%	100%
HF183 SYBR	Seurinck et al. (2005)	*Bacteroides* 16S	HF183F, HFsybR	Plasmid	Human, sewage	Chicken, cow, dog, horse, pig	2.2 mg wet wt	$2.8*10^2$ copies	91%	NR[b]
HF183 Taqman	Haugland et al. (2010)	*Bacteroides* 16S	HF183F, BthetP1, BthetR1	Plasmid	Human, sewage	Cattle, Pig, Chicken, Dog, Cat	1 ng fecal DNA	10 copies	100%	NR[c]
HumM2	Shanks et al. (2009)	*B. fragilis* hypothetical protein BF3236	HumM2F, HumM2P, HumM2R	Plasmid	Human, sewage	Alpaca, Cow, Goat, Sheep, Horse, Pig, Antelope, Whitetail deer, Mule deer, Moose, Elk, Canadian Goose, Duck, Pelican, Gull, Turkey, Chicken, Marine dolphin, California sea lion, Cat, Dog	1 ng fecal DNA	10 copies	100%	99%
Mnif	Johnston et al. (2010)	*Methanobrevibacter smithii nifH*	Mnif202F, MnifP, Mnif353R	Genomic *M. smithii* DNA	Sewage	Gull, ambient seawater	15 mg wet wt	5 genome equivalents	100%	72%

[a] Not reported; strong cross-reaction with cat, dog.
[b] Not reported; cross-reaction with one chicken.
[c] Not reported; weak cross-reaction with chicken, dog.

samples was considered the most specific. Note that these performance metrics differ from those used in Boehm et al. (2013).

Because performance outcomes can change under different characterizations of fecal concentration, it was necessary to select a primary unit of measure by which to judge quantitative assay performance. We chose to focus on total DNA mass on each challenge filter as measured by NanoDrop spectrophotometry. We defined quantitative benchmarks for sensitivity and specificity based on copies per nanogram of total DNA: an assay was quantitatively sensitive if the median abundance in every target source (human stools, sewage and septage considered separately) was greater than 10 copies/ng, and an assay was quantitatively specific if the interquartile ranges of copies/ng did not overlap between target and non-target sources.

To study the effect of challenge filter sample units of measure on the presence/absence performance metrics, we chose a balanced subset of the data and performed an *in silico* dilution experiment. This subset consisted of assays run by the method developer's laboratory (BacH, BacHum-UCD, BsteriF1, gyrB, HumM2, HF183 Taqman and Mnif). In this subset, the assays were performed under optimal conditions (in the hands of their developer's lab) and the *n* for all assays was the same. Presence/absence method performance in this subset was evaluated using the same challenge filter sample units of measure that the method developers used when the assays were first published (Table 1). This was done by *in silico* dilution or addition of the appropriate amount of fecal material and calculating what the copy numbers would have been based on the observed amplification with the actual challenge filter samples. For this exercise, a limit of detection (LOD) of 10 copies per reaction was applied: amplification below this level was considered negative and anything above 10 copies was considered positive.

2.4. *Doubleton analyses*

To determine the effect of mixed fecal sources on assay performance, sensitivity was evaluated in the doubleton samples with respect to the non-human source present and the estimated relative contributions of total DNA from each source. The proportion of total DNA contribution from each fecal source was estimated using a mass ratio approach. The median NanoDrop measurements on the singleton samples were multiplied by the volumetric proportions used to create the doubleton samples (see Boehm et al. (2013) for sample creation details), and the ratio of target:non-target DNA on each doubleton filter was estimated from those values. Presence/absence sensitivity (with DNQ values considered positive) was calculated for every assay according to doubleton type and compared to the target:non-target DNA ratios.

3. Results

3.1. *Performance by DNQ classification*

None of the assays met the 80% benchmark used by Boehm et al. (2013) and the USEPA (2005) for both specificity and sensitivity when DNQ was considered positive (Table 2). With DNQ negative, BtH, HF183 SYBR and HF183 Taqman met the benchmark for both sensitivity and specificity metrics. Assay sensitivity was high but specificity was low when DNQ results were regarded as positive. All assays except HF183 endpoint and Mnif were at least 80% sensitive with DNQ positive. The only assay that was at least 80% specific with DNQ positive was HF183 endpoint; however, HF183 SYBR, BacH and HumM2 were not appreciably behind the mark at 78%, 77% and 75%, respectively. When DNQ was negative, sensitivity decreased in all assays except BacHum-UCD, and all assays were considered specific except BacHum-UCD, BsteriF1 and Mnif. Note that the results presented in Table 2 use a "per filter" characterization of presence/absence in the challenge filter samples and consider all laboratories' data together as one dataset.

Table 2 – Performance of human-associated assays in singleton samples among all labs, calculated with DNQ (detected, not quantifiable) samples as positive or negative, with presence/absence determined on a per-filter basis.

Assay	Sensitivity			Specificity		
	Human *n*[a]	DNQ+	DNQ–	Non-human *n*[a]	DNQ+	DNQ–
BacH	12	100%	75%	26	77%	85%
BacHum-UCD	72	97%	97%	156	37%	67%
BsteriF1	48	100%	96%	104	44%	61%
BtH	12	100%	92%	26	54%	96%
gyrB	12	92%	50%	26	58%	96%
HF183 endpoint	84	75%	NA	182	96%	NA
HF183 SYBR	48	100%	92%	104	78%	89%
HF183 Taqman	60	100%	95%	130	46%	92%
HumM2	72	93%	67%	156	75%	94%
Mnif	60	78%	60%	130	68%	76%

[a] Values for *n* vary among assays because the methods were performed by different numbers of laboratories; see Section 2.1.

3.2. *Performance by challenge filter sample unit of measure*

The presence/absence specificities of HF183 Taqman and BacHum-UCD under their developer's challenge filter sample units of measure were starkly different from the "per filter" specificity results (both 96% in developers' lab versus 46% and 37% across all labs, respectively, DNQ positive). In general, the assays performed well with their developers' execution and test sample quantities (Table 3). However, in our study the assays often performed worse than reported in their original publications (Table 1), except for BacHum-UCD under its original challenge sample units of measure (Table 3). Interestingly, BacHum-UCD was the only assay that showed excellent sensitivity using the units of 5000 copies of BacUni-UCD per reaction, which was the benchmark used to develop the BacHum-UCD assay (Kildare et al., 2007).

When the challenge filter samples were characterized by total DNA mass and all laboratories' data were analyzed

Table 3 – Sensitivity and specificity of human qPCR assays in singleton samples, calculated using original developer's data generated in this study and the developers' original challenge fecal sample units of measure. Developers' own metrics are shown in bold.

Assay	Sensitivity						Specificity					
	n[a]	1 mg wet mass	15 mg wet mass	5000 copies BacUni-UCD	1 ng DNA	10 ng DNA	n[b]	1 mg wet mass	15 mg wet mass	5000 copies BacUni-UCD	1 ng DNA	10 ng DNA
BacH	12	**100%**	100%	42%	75%	92%	26	**77%**	77%	100%	88%	85%
BacHum-UCD	12	100%	100%	**100%**	100%	100%	26	62%	54%	**96%**	65%	65%
BsteriF1	12	100%	100%	0%	**100%**	100%	26	46%	42%	92%	**77%**	58%
gyrB	12	100%	100%	0%	58%	**75%**	26	69%	58%	100%	100%	**88%**
HF183 Taqman	12	100%	100%	17%	**100%**	100%	26	62%	42%	96%	**96%**	73%
HumM2	12	100%	100%	0%	**58%**	83%	26	92%	81%	100%	**100%**	92%
Mnif	12	100%	**100%**	17%	75%	83%	26	77%	**77%**	100%	81%	81%

[a] Number of target (human stool, sewage or septage) samples in the analysis.
[b] Number of non-target (non-human animal) samples in the analysis.

together, HF183 Taqman was the only assay categorized as both quantitatively sensitive and specific (Fig. 1). The four assays targeting functional genes (BtH, gyrB, HumM2 and Mnif) were less sensitive than the assays targeting the *Bacteroides* 16S rRNA gene, likely due to fewer copies of the functional genes per cell. All assays were considered quantitatively sensitive except BtH, HumM2, and Mnif, while only HF183 Taqman and BtH were considered specific. Dog was a frequent source of false positives: BacH, BacHum-UCD and BsteriF1 had cross-reactivity in dog samples at levels equivalent to that of sewage/septage (BacH, BacHum-UCD) or human stools (BsteriF1). BacH cross-reacted with the fewest number of non-human sources (only dog and deer).

When gene copy abundance of each quantitative assay in the singleton samples was normalized to all available fecal source units of measure, it was clear that which assay performed best was dependent on how the challenge samples and performance metrics were defined (Table 4). BacHum-UCD was

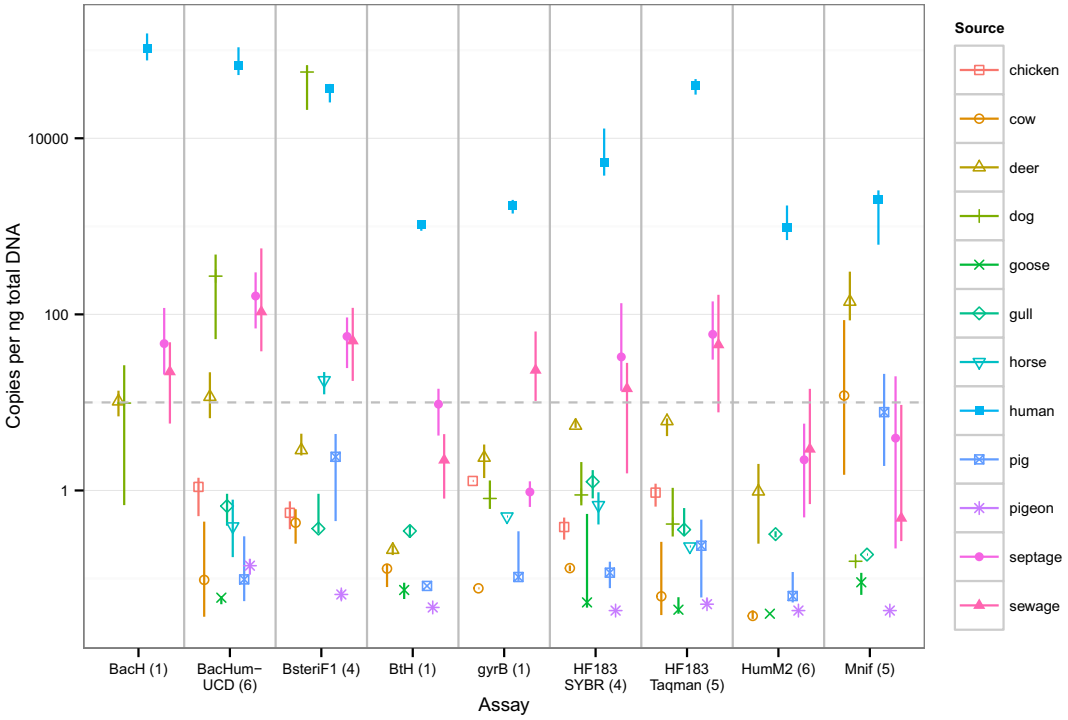

Fig. 1 – Copies per nanogram total DNA in each fecal source for quantitative assays. Each point is the median value for a given source, and the bars represent the interquartile ranges (25th to 75th percentiles). Fecal sources are indicated by a unique combination of color and shape. The solid markers are "target" sources (human stools, sewage or septage). The dashed horizontal line indicates 10 copies per nanogram, which we used as a benchmark of assay sensitivity. The numbers in parentheses after each name on the *x*-axis indicate the number of labs that performed the assay.

Table 4 – Human-associated marker abundance under all available fecal source characterizations. Values are median (standard deviation) of log10-transformed copy numbers across all labs. Underline indicates the most sensitive assay (largest copy abundance in target samples) and bold indicates the most the specific assay (largest difference in median copy abundance between target and non-target) for each unit of measure.

Assay	Source	*n*	MgWet[c]	DNA[d]	ENT MF[e]	ENT qPCR[f]	*E. coli* MF[g]	*E. coli* qPCR[h]	GenBac3[i]	AllBac[j]	BacUni-UCD[k]	Bfrag[l]	FecalB[m]
BacH	Target[a]	12	7.5 (0.2)	1.9 (2)	**2.1 (1.8)**	–	1.5 (0.9)	0.6 (0.9)	–	–	–	–	–
	Non-target[b]	26	2.8 (0.9)	0.9 (0.9)	**−1.5 (1.8)**	–	−1.5 (1.2)	−2.2 (0.9)	–	–	–	–	–
BacHum-UCD	Target	72	7.1 (1)	2.7 (1.7)	3 (1.3)	−0.4 (1.5)	1.9 (1.1)	1 (0.9)	−1.2 (0.4)	–	−0.4 (0.2)	–	–
	Non-target	156	2.4 (1.7)	**0 (1.7)**	**−0.6 (1.3)**	−2.9 (1.3)	−1.3 (1.8)	−2.2 (1.7)	−3.9 (1.9)	–	−3.8 (2.3)	–	–
BsteriF1	Target	48	6.8 (0.2)	2 (1.5)	2.3 (1.1)	−0.3 (1.6)	1.4 (0.6)	0.5 (0.3)	−1.6 (0.5)	–	–	–	–
	Non-target	104	2.9 (2)	0.8 (2)	0.7 (1.4)	−1.8 (2.1)	−1.3 (2)	−2.2 (2.1)	−3.6 (1.8)	–	–	–	–
BtH	Target	12	5.3 (0.1)	1 (1.4)	1.4 (0.9)	−0.2 (1.9)	0.2 (0.9)	**−0.6 (0.5)**	–	–	–	–	–
	Non-target	26	0.9 (0.7)	−0.9 (0.4)	−1.1 (1.2)	−2.8 (1.2)	−3.7 (1.6)	**−4.3 (1.2)**	–	–	–	–	–
gyrB	Target	12	5.2 (0.3)	1.5 (1.4)	0.8 (1.1)	–	0.2 (0.6)	−1.1 (0.5)	–	–	–	−2 (0.7)	–
	Non-target	26	1.2 (0.8)	−0.3 (0.6)	−0.9 (1.5)	–	−2.8 (1.1)	−3.5 (0.8)	–	–	–	−3.4 (1.2)	–
HF183 SYBR	Target	48	5.9 (1.1)	1.7 (1.5)	1.9 (1.4)	−0.9 (1.5)	0.5 (1.3)	−0.2 (1.2)	−2.2 (0.8)	−2.1 (0.3)	–	–	−1.5 (0.5)
	Non-target	104	2.3 (1)	−0.2 (0.8)	−0.9 (1.4)	−3.7 (1.3)	−2.1 (1.4)	−3.2 (1.3)	−5.4 (1.9)	−5.1 (NA)[n]	–	–	NA (NA)[o]
HF183 Taqman	Target	60	**6.9 (0.1)**	**2.2 (1.5)**	2.4 (1.1)	**−0.3 (1.7)**	**1.3 (0.6)**	**0.5 (0.3)**	**−1.7 (0.6)**	–	–	–	–
	Non-target	130	**1.2 (0.9)**	**−0.5 (0.8)**	−1.1 (1.5)	**−4 (1.3)**	**−2.8 (1.4)**	**−3.2 (1.1)**	**−5.1 (2.1)**	–	–	–	–
HumM2	Target	72	5.3 (0.3)	0.9 (1.4)	1.1 (1)	−1.6 (1.7)	0.2 (0.7)	−0.8 (0.5)	−2.9 (0.7)	–	–	–	–
	Non-target	156	0.8 (0.9)	−1.1 (0.7)	−0.9 (0.8)	−3.7 (1.3)	−2.6 (1.2)	−3.2 (0.7)	−6.2 (1.9)	–	–	–	–
Mnif	Target	60	5.7 (0.5)	1.3 (1.6)	2 (1.2)	−2.3 (2.1)	0.2 (1.1)	−0.5 (0.9)	−3.1 (0.8)	–	–	–	–
	Non-target	130	3.4 (1.2)	0.8 (1.3)	1.6 (2.2)	−1.9 (2.2)	−0.7 (1.9)	−1.3 (1.7)	−4.5 (1.3)	–	–	–	–

[a] Human stools, sewage and septage.
[b] non-human animals.
[c] mg wet mass, sewage and septage samples excluded.
[d] ng total DNA by NanoDrop.
[e] EPA method 1600.
[f] Entero1(A) (Haugland et al., 2005).
[g] *E. coli* membrane filtration.
[h] *E. coli* 23S qPCR assay EC23S857 (Chern et al., 2011).
[i] (Siefring et al., 2008).
[j] (Layton et al., 2006).
[k] (Kildare et al., 2007).
[l] *B. fragilis* group specific (Matsuki et al., 2002).
[m] Fecal *Bacteroides* (Converse et al., 2009).
[n] *n* of samples with amplification was too small to calculate standard deviation.
[o] No amplification was observed.

Table 5 – Sensitivity of human-associated assays in singleton human, sewage and septage samples calculated with detected, not quantifiable (DNQ) values as positive or negative on a per-filter basis.

Assay	n[a]	Human		Sewage		Septage	
		DNQ+	DNQ−	DNQ+	DNQ−	DNQ+	DNQ−
BacH	4	100%	100%	100%	50%	100%	75%
BacHum-UCD	24	100%	100%	92%	92%	100%	100%
BsteriF1	16	100%	100%	100%	88%	100%	100%
BtH	4	100%	100%	100%	75%	100%	100%
gyrB	4	100%	100%	100%	50%	75%	0%
HF183 endpoint	28	96%	NA	57%	NA	71%	NA
HF183 SYBR	16	100%	100%	100%	81%	100%	94%
HF183 Taqman	20	100%	100%	100%	85%	100%	100%
HumM2	24	100%	100%	83%	46%	96%	54%
Mnif	20	95%	95%	55%	20%	85%	65%

[a] Number of singleton samples in each target source (varies by number of laboratories running each assay).

the most sensitive assay using the total DNA mass, *E. coli* CFU, *E. coli* qPCR and GenBac3 measurements. BtH was the most sensitive assay using the *Enterococcus* qPCR copy units, but was less sensitive in other quantitative measures. BacH was the most sensitive assay only under the wet mass unit of measure, which was the same fecal unit used to develop that assay. HF183 Taqman was the most specific assay in six of the seven units of measure where it was possible to make a comparison: milligrams of wet feces, mass of total DNA, *E. coli* CFU, *E. coli* qPCR, *Enterococcus* qPCR, and GenBac3. The only fecal source characterization for which HF183 Taqman was not the most specific assay was *Enterococcus* CFU, where BacHum-UCD excelled.

3.3. *Performance by target source*

The sensitivity of each assay differed for each of the three "target" sources: human stools, sewage and septage. In almost every case, sensitivity was greatest in human stools, followed by septage, and least sensitive in sewage samples (the exception was gyrB, which had greater sensitivity in sewage

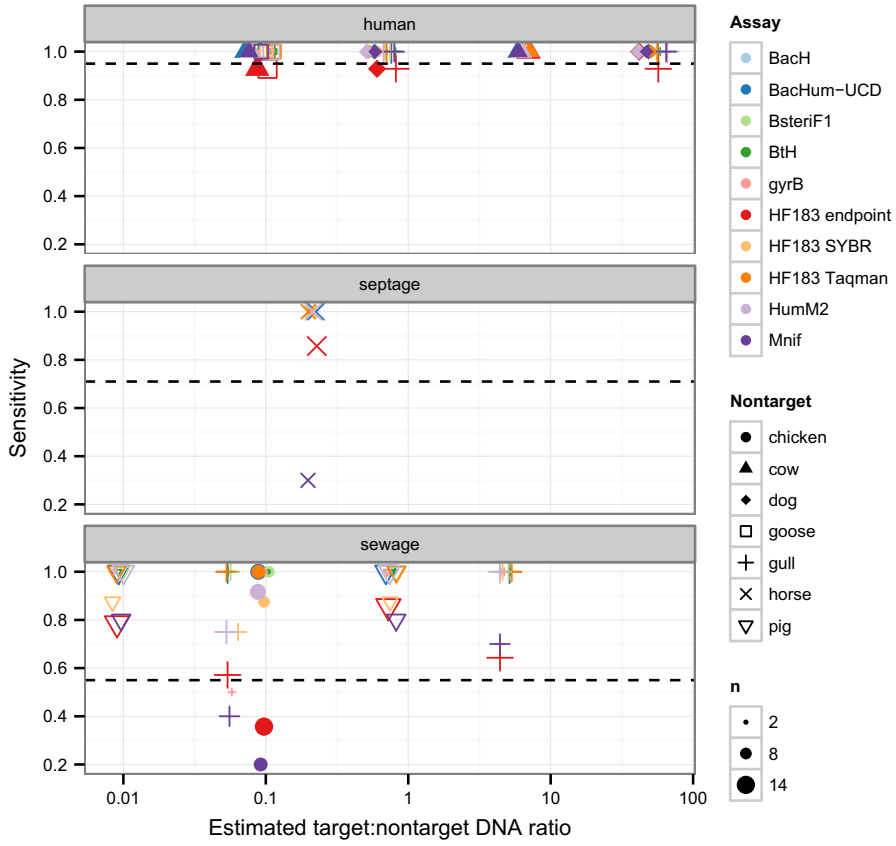

Fig. 2 – Presence/absence per-filter sensitivity (DNQ positive) in doubletons versus the estimated target:non-target DNA ratio. The three target sources present in the doubletons are organized into the horizontal panels. The shape of each point indicates the non-target source in the doubleton, and the assays are differentiated by colors. The size of each point indicates the number of measurements that were used to calculate the sensitivity value for that point, which ranged from 2 to 14. The horizontal positions of the points were "jittered" to make more of the data visible. The dashed lines represent the lowest sensitivity of any assay in the target singletons (DNQ positive).

than septage; Table 5). The presence/absence metrics were also greatly dependent on DNQ classification. With DNQ positive, five assays were perfectly sensitive (100%) to all three target sources: BacH, BsteriF1, BtH, HF183 SYBR and HF183 Taqman. No assay was 100% sensitive to all three targets with DNQ negative, though BacHum-UCD, BsteriF1, HF183 SYBR and HF183 Taqman met the 80% benchmark. In quantitative terms, every assay was orders of magnitude more sensitive (by copies per nanogram of total DNA) to human stools than to septage or sewage (Fig. 1). BacH was the most sensitive of all assays to human stools, and BacHum-UCD was most sensitive of all assays to sewage and septage.

3.4. *Performance in doubleton samples*

In the doubletons containing sewage, a decrease in target:non-target DNA ratio appeared to decrease sensitivity in the samples containing gull feces (bottom panel, Fig. 2). This change was especially noticeable for Mnif, HumM2, gyrB and HF183 SYBR. Sensitivity was also low in the chicken/sewage samples for Mnif and HF183 endpoint. For the doubleton samples containing human stools (top panel), sensitivity remained consistently high; only HF183 endpoint showed a slight decrease in sensitivity at lower target:non-target DNA ratios. The doubletons containing septage consisted of only one sample type: 10% septage:90% horse. For these samples, all assays were considered sensitive except Mnif.

4. Discussion

4.1. *DNQ classification*

The classification of DNQ samples as positive or negative dramatically affected the performance of the assays in presence/absence metrics, and this has important implications for local beach management applications. Changing the DNQ classification from positive to negative decreased sensitivity slightly but improved specificity substantially for all assays (Table 2). The assays that were judged as acceptably sensitive or specific with the presence/absence metrics differed slightly from Boehm et al. (2013) because here we considered only singletons and pooled all labs' data together. However, variable performance was observed among laboratories and this variability may skew the overall DNQ classification results (Fig. S1). The variable results among laboratories may be a product of the different LLOQ values obtained by using different types and quantities of standard reference material, Cq threshold settings, and other variations in method protocols.

The treatment of DNQ samples in the MST literature is mixed. For example, some studies have defined DNQ values as negative (Stapleton et al., 2009), while others have regarded DNQ amplification as a positive detection (Kelty et al., 2012); still others established a lower limit of detection for the qPCR but did not differentiate between LLOQ and LOD (Sauer et al., 2011). For SYBR assays, most groups consider DNQ samples negative, due to the difficulty in validating melt curves for such low amplification. Information on DNQ handling is often not reported at all. To our knowledge, this work and the other manuscripts from the present study (Raith et al., 2013; Sinigalliano et al., 2013) are the first to comprehensively examine the effects of varying DNQ classification on MST assay performance.

In our analysis, we found that assay specificity was superior when DNQ results were treated as negative. One possible explanation is that most human-associated genetic markers are not strictly found in human sources; instead, they are typically found at a higher abundance in human sources (Shanks et al., 2010). Thus, the more sensitive the method is, the more likely it is that cross-reactivity will be observed in the DNQ range. In environmental samples, DNQ measurements may result from dilution or degradation of a human fecal source or from cross-reactivity. Experts in the field have not yet reached consensus regarding how to classify DNQ results obtained in MST field studies (Stewart et al., 2013). In practice, it may be beneficial to perform both a human bacteria-associated assay (highly sensitive, less specific) and a human viral assay (highly specific, less sensitive); however, the optimal method for concentrating human viruses from environmental water samples is yet to be determined (see Harwood et al., 2013).

4.2. *Challenge filter sample units of measure*

The amount of fecal matter on a filter can be described using several units of measure, and we found that changing the challenge filter sample units of measure can change which assays performed best. The relative quantities of fecal material in our challenge filter samples changed considerably among fecal sources when different units of measure were used to describe fecal concentrations. For example, one fecal source may have low *Enterococcus* levels, but a high wet mass compared to another source (Ervin et al. 2013). Accordingly, the assay that performed best on a "per unit" basis depended on which unit of measure was used. Data from all labs were used in this analysis, even though there were differing sizes of datasets and clear lab-to-lab variation (see Section 4.6), and our results should be interpreted with those factors in mind. To our knowledge, the present work and other manuscripts from this study (Boehm et al., 2013; Raith et al., 2013; Sinigalliano et al., 2013) are the first to examine the effects of changing fecal units of measure on the performance outcomes of molecular MST methods.

We focused on total DNA mass as the primary challenge filter sample unit of measure. The amount of fecal matter varied substantially from filter to filter, both within a given fecal source and across sources (Ervin et al., 2013), yet it was not possible to directly quantify the fecal material on each filter for every unit of measure. Total DNA mass was the only unit of measure with measurements on individual filters using the same quantification method from a majority of laboratories (832 total DNA measurements). Further, because total DNA yield varied extensively among laboratories (Figure S2), normalizing to total DNA minimized bias and put the assays on the most level playing field possible (see Kelty et al., 2012).

When the data were normalized to ng total DNA, HF183 Taqman was the only assay categorized as both sensitive and specific. Numerous studies from around the world have demonstrated the success of the original HF183 endpoint PCR assay (e.g. Griffith et al., 2003; Gawler et al., 2007; Ahmed et al.,

2012), and many qPCR assays have been developed to target the same region of the *Bacteroides* 16S rRNA gene (see Supplemental Information). In the present work, the HF183 endpoint assay was much less sensitive to sewage than the HF183 qPCR assays (Table 5), suggesting that a qPCR version of this method is preferable where sewage contamination is a concern.

4.3. *Influence of target source*

Assay sensitivity varied among the three "target" sources (human stools, sewage or septage). While every assay was highly or perfectly sensitive to human stools, success was more varied with sewage and septage sources. There are several possible explanations. Firstly, the sewage and septage challenge filter samples had very low quantities of fecal material compared to the human stool samples (Ervin et al., 2013), which affects sensitivity on a "per filter" basis. Secondly, sewage and septage are mixed sources with fecal inputs from humans as well as other animal species. The mixed nature of these sources could affect sensitivity both in terms of decreased amount of target per unit of fecal material as well as possible cross-reactivity to the non-human inputs. Lastly, the assays in this study were initially designed to be human fecal-associated, not necessarily sewage or septage-associated (though many were validated with sewage samples). Given the differences between stool samples and sewage/septage, there is a need for methods that can discriminate sewage and septage, such as community analysis (Cao et al., 2013). In consideration of the differences among target sources, Table 4 was recreated with the stool and sewage/septage target sources analyzed separately (Table S4).

Almost every assay had lower sensitivity to sewage than septage (Table 5, DNQ negative). Septic tanks may be more hospitable environments for fecal anaerobes than sewerage systems, and thus the microbes targeted by the assays in this study may be more numerous in septage samples than sewage. This hypothesis is supported by the greater copy numbers of general Bacteroidales per total DNA mass observed in septage versus sewage samples (Fig. S3). Further, it has been previously shown that septage has higher concentrations than sewage of *E. coli* uidA, *Enterococcus* 16S rRNA and BtH gene copies (Srinivasan et al., 2011), and that only a small percentage of microorganisms in sewage are fecal-derived (McLellan et al., 2010).

Our findings contrast with those of some of the original assay publications. For example, Kildare et al. (2007) found the BacHum-UCD marker to be less prevalent in human stools than wastewater samples, though gene copy abundance in these sources was not reported. Similarly, researchers in France found HF183 SYBR to be less prevalent in stools than wastewater (Mauffret et al., 2012). In the present study, HumM2 and HF183 Taqman were orders of magnitude less sensitive to sewage but substantially more abundant in human stools than reported by Shanks et al. (2009, 2010).

The discrepancies between our results and those of previous studies may due to differences in the wastewater samples. In the present work, we sampled a relatively small number of treatment plants ($n = 9$), some of which receive industrial wastewater (up to 20% of total input volume and as much as 50% during certain times of day; C. McGee, pers. comm.). Stapleton et al. (2009) found several orders of magnitude fewer gene copies of human *Bacteroides* in industrial wastewater compared to sewage. In addition, the microbial community present in the sewerage infrastructure (biofilms) may be quite different among locations due to a number of factors. Thus it is possible that the microbial profile of the sewage influent used in the present study may vary considerably from those found elsewhere, which could explain some of the contrasting results. Before these methods are employed in local MST studies, management agencies may benefit from performing small studies to establish the assays' sensitivity to the wastewater sources present in their watersheds.

4.4. *Doubletons*

In environmental water samples, there will be numerous sources of bacterial DNA, including multiple fecal hosts and indigenous microbes. The doubleton challenge samples represent an idealized model of very a simple two-host system. In our analysis, we uncovered an interesting effect of gull feces on sensitivity to sewage (Fig. 2). It appears that gull feces decreased sensitivity to sewage in several assays, yet this effect was not observed in the sewage/pig samples or in the human/gull samples. To our knowledge, no other method evaluation studies have tested these assays against a sewage/gull matrix. This finding has implications for application of these assays at beaches with large native seagull populations; however, not every assay was affected and the number of samples in this category was relatively small. Further study is needed before definite recommendations can be made on this issue.

4.5. *Effect of individual laboratory performance*

An important source of variability in assay performance is the effect of individual laboratories, whether due to differing protocols or varying levels of experience with the technology. The sources of inter-laboratory variability in assay performance include: DNA purification approach and efficiency, DNA yield measurements, qPCR chemistry, type of qPCR standard reference material, qPCR instrument, laboratory infrastructure (i.e. spatial separation of tasks) and technician skill level. Even with standardized protocols, laboratories may produce different results (Pan et al., 2010). In the present study, there are several instances of assay performance differing across laboratories (Fig. S1). These differences are often driven by DNQ classification, which reflects the varying LLOQ values among laboratories (Table S3). Issues associated with repeatability among laboratories in this study are explored in depth in Ebentier et al. (2013). It is clear that SOPs – which should include everything from laboratory setup to data handling and stringent quality assurance guidelines – must be established for accurate performance assessment and successful implementation of these methods.

Another important limitation of this work is the imbalance in size of the datasets between assays (ranging from 1 to 7 laboratories), which creates a statistical bias in the performance metrics. This bias is exacerbated by the clear lab-to-lab variability in performance. This bias and variability make it difficult to compare performance metrics across assays, and could be why the overall performance in the present study often does not match the original reports. Method

performance needs to be determined with an unbiased dataset where lab-to-lab variability is not a factor (e.g. Table 3).

5. Conclusions

- HF183 Taqman consistently excelled across numerous performance benchmarks
- In practice, it may be beneficial to use two assays targeting different genes and/or bacterial species, such as HF183 Taqman with BtH or HumM2
- Further work is needed to determine whether the additional uncertainty associated with using multiple human-associated assays adds value to source tracking efforts
- While these assays performed well with fresh fecal pollution sources from California in an artificial water source, several issues still need to be thoroughly addressed prior to implementation in local management settings: persistence and decay, standardization of protocols, performance with reference feces from different geographical areas and animal species, and potential influence of the environmental sample matrix on amplification

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2013.05.060.

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