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Comparison of the Host Specificities of Two *Bacteroidales* Quantitative PCR Assays Used for Tracking Human Fecal Contamination▽

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The sewage-associated real-time quantitative PCR (qPCR) assays BacHum and HF183 SYBR were compared for specificity against local fecal sources. Both assays were equally sensitive to sewage, but BacHum showed substantially more false-positive results for cat, dog, gull, and raccoon feces.

The use of conventional PCR and real-time quantitative PCR (qPCR) for host-specific molecular markers, particularly those for genes encoding *Bacteroidales* 16S rRNA, has increased in recent years for detecting fecal pollution in environmental waters. qPCR-based diagnoses of microbiological water quality enable pinpointing sources and quantifying source abundance for prioritizing remediation. However, qPCR-based assays should be specific to avoid costly management decisions based on inaccurate water quality diagnoses.

Bernhard and Field (2, 3) first identified and created a PCR assay for detecting a human-specific *Bacteroidales* marker (HF183). Seurinck et al. (10) then developed a qPCR assay for the HF183 marker (HF183 SYBR), using SYBR green I chemistry and a modified reverse primer. Kildare et al. (8) developed a TaqMan-based qPCR assay to quantify a similar *Bacteroidales* human-specific marker (BacHum). The sensitivity and specificity of the HF183 marker have been reported, primarily using Bernhard and Field primers in PCR (4, 5, 11) but also using Bernhard and Field primers (1) and the Seurinck et al. reverse primer (10) in SYBR green qPCR. In contrast, the sensitivity and specificity of the BacHum marker have not been as widely reported, and the assay has generated variable results (1, 7, 8, 12).

Method performance can vary geographically, and thus testing the specificity of published host-specific markers against local sources is recommended (1, 5, 7). Testing the host specificity of qPCR methods against target source materials is a first-order concern: if there are substantial false negatives or positives against individual host sources, then assays are unlikely to perform well with source mixtures. Here, the HF183 SYBR and BacHum qPCR assays were evaluated against cat (12 individuals), dog (12 individuals), gull (3 composites from >5 individuals per composite), raccoon (1 individual and 4 composites), rat (1 individual and 3 composites), and human (8 individuals) feces, as well as sewage (6 influent from the El Estero wastewater treatment plant [WWTP] and 4 from sanitary sewer manholes in downtown Santa Barbara, CA) and septage (1 from the Santa Barbara Botanic Garden and 2 from residential systems—1 conventional, 1 advanced) samples. Animal feces and sewage samples were aseptically collected in the Santa Barbara, CA, area. The human fecal and 2 residential-system septage samples were from Southern California (Orange County and Malibu, respectively). DNA was extracted from fecal samples using a PowerSoil DNA isolation kit and from sewage and septage samples using a PowerWater DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA), followed by ethanol precipitation and quantification via a Quant-iT double-stranded DNA (dsDNA) broad-range assay kit (Invitrogen, Carlsbad, CA) on a BioTek Synergy 2 plate reader (Winooski, VT).

PCR inhibition was assessed via a salmon testes qPCR assay as published previously (6), with the following differences: dually labeled (FAM-BHQ1 [5′ with 6-carboxy-fluorescein, 3′ with Black Hole Quencher 1]) probe (Eurofins MWG Operon, Huntsville, AL), qPCR MasterMix for probe assay No ROX (Anaspec, Fremont, CA), CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA), and 2.5 μl of diluted DNA template. The qPCR master mix was spiked with salmon testes DNA (0.25 ng/reaction). Four no-inhibition controls were assayed per 96-well plate, along with a 3-log salmon testes DNA standard curve. Samples were assayed in duplicate, and the baseline threshold was set to 200 for data analysis. The average plus 3× standard deviation threshold cycle (C_T) values of the no-inhibition controls ($C_{T\mathrm{ni}}$) were calculated; all sample reactions with an average C_T greater than $C_{T\mathrm{ni}}$ were considered inhibited. qPCR was first performed using 1/5-diluted DNA template. If inhibition occurred, 2-fold dilutions were analyzed until no inhibition occurred. The lowest template dilution without inhibition was used for both the HF183 SYBR and BacHum qPCR assays.

HF183 SYBR qPCR (10) was performed in triplicate using SYBR green I chemistry as reported previously (9), with an iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA). The baseline threshold was adjusted until the coefficient of variation was <3% between assays for each standard. Melt curves were validated for all sample replicates amplifying within the quantification range. BacHum qPCR was performed in triplicate using TaqMan chemistry as published previously (8), except for the following differences: dually labeled (FAM-BHQ) probe, qPCR MasterMix for probe assay No ROX, Bio-Rad CFX96 real-time PCR detection system, 45 cycles, 2.5

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TABLE 1. Comparison of sensitivities and specificities for both human-specific *Bacteroidales* qPCR assays

Fecal source	HF183 SYBR marker[a]				BacHum marker[b]			
	No.[c]	Copies/g wet wt or liter[d]			No.[c]	Copies/g wet wt or liter[d]		
		Range or amt	Mean	Median		Range or amt	Mean	Median
Cat	1/12	2.6×10^3			10/12	2.0×10^3–3.9×10^5	9.7×10^4	4.1×10^4
Dog	0/12				9/12	1.4×10^4–8.9×10^5	2.8×10^5	8.2×10^4
Gull	0/3				1/3	4.4×10^2		
Raccoon	0/5				2/5	1.1×10^4–1.5×10^5	8.1×10^4	8.1×10^4
Rat	0/4				0/4			
Human	5/8	4.9×10^3–5.3×10^8	1.1×10^8	1.7×10^6	8/8	6.4×10^4–5.1×10^8	6.6×10^7	1.3×10^6
Septage	2/3	9.8×10^7–4.9×10^8	3.0×10^8	3.0×10^8	3/3	4.2×10^4–6.5×10^8	2.4×10^8	7.6×10^7
Sewage	10/10	4.0×10^7–2.5×10^9	8.6×10^8	6.7×10^8	10/10	6.0×10^7–8.5×10^9	1.8×10^9	1.1×10^9

[a] Also see reference 10.
[b] Also see reference 8.
[c] Number of samples within the quantification range/number of samples tested.
[d] Range, mean, and median are based on concentrations of samples within the quantification range only.

μl of diluted DNA template. The baseline threshold adjustment was performed as with HF183 SYBR qPCR. For both assays, only samples with two or more analytical replicates in the standard range were reported. Replicates amplifying past the lowest standard were not considered in the analysis, as their concentration could not be accurately quantified.

The HF183 SYBR qPCR assay quantified 100% of the sewage samples, 63% of the human fecal samples, and 67% of the septage samples (Table 1). The variability of HF183 SYBR marker concentrations in individual human fecal samples has been reported previously (7, 8, 10). The HF183 SYBR marker concentration variations in septage could be due to individual sample variability (i.e., two samples were from single-family dwellings, while the third was from a public botanic garden with numerous visitors) or treatment system level. The HF183 SYBR marker was quantified in 8% of cat samples, which is similar to the results from Kildare et al. (14%) (8). The HF183 SYBR marker was not quantified in any of the dog or gull fecal samples, similar to results from other studies (dog [10] and gull [8]), although Kildare et al. (8) reported a few dog fecal samples with the marker. HF183 SYBR markers were also not quantified in any of the raccoon or rat fecal samples.

The BacHum qPCR assay quantified 100% of the sewage, human fecal, and septage samples (Table 1). However, the BacHum marker was also quantified in 83% of cat, 75% of dog, 33% of gull, and 40% of raccoon fecal samples. Rat feces were the only animal source with no detection of the BacHum marker in the quantification range. The amplification of the BacHum marker in dog feces has been shown previously, ranging from 6% (1) to 90% (12). In contrast, Kildare et al. (8) reported no amplification using DNA extracted from either cat or gull feces.

The BacHum marker concentration ranges in cat, dog, and raccoon feces overlapped with those in the human fecal samples (Table 1). To put this in perspective, an environmental sample with 10^4 BacHum marker copies/liter quantified could have been caused by 1 ml of raw sewage, 1 liter of septage, 1 g of human feces, 1 g of cat feces, 1 g of dog feces, or 1 g of raccoon feces (based on lower values from each range). Therefore, it can be argued that contamination by human fecal sources and contamination by nonhuman fecal sources are equally likely at this concentration level.

Validation of any fecal source tracking assay begins with testing against target, as well as nontarget, fecal material: if an assay is nonspecific with individual fecal sources under ideal (i.e., fresh samples without environmental aging) conditions, then it is unlikely to perform well at differentiating sources within environmental water samples. Various studies have compared the specificities of *Bacteroidales* assays for human waste (1, 11), but few (7, 8) have directly compared the HF183 SYBR (10) and BacHum (8) qPCR assays, which use very similar markers. Importantly, the specificity of BacHum was much lower in this study than in the original study (8), and the HF183 SYBR assay appeared superior for identifying human waste in the Santa Barbara, CA, area. This research showed that two qPCR assays used for quantifying similar markers for human waste differed greatly in fecal source specificity. While the use of multiple human-specific assays is recommended to increase confidence in positive values, validation of qPCR assays for their specificity, using local fecal sources of concern, should still be performed prior to adopting a new qPCR-based assay into a source tracking toolbox.

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