Inter-laboratory comparison of real-time PCR protocols for quantification of general fecal indicator bacteria

Orin C. Shanks[1], Mano Sivaganesan[1], Lindsay Peed[1], Catherine A. Kelty[1], A. Denene Blackwood[2], Monica R. Greene[2], Rachel T. Noble[2], Rebecca N. Bushon[3], Erin A. Stelzer[3], Julie Kinzelman[4], Tamara Anan'eva[4], Christopher Sinigalliano[5], David Wanless[5], John Griffith, Yiping Cao, Steve Weisberg, Valarie J. Harwood[6], Christopher Staley[6], Kevin H. Oshima[7], Manju Varma[7] and Richard A. Haugland[7]

Abstract

The application of quantitative real-time PCR (qPCR) technologies for the rapid identification of fecal bacteria in environmental waters is being considered for use as a national water quality metric in the United States. The transition from research tool to a standardized protocol requires information on the reproducibility and sources of variation associated with qPCR methodology across laboratories. This study examines inter-laboratory variability in the measurement of enterococci and *Bacteroidales* concentrations from standardized, spiked, and environmental sources of DNA using the Entero1a and GenBac3 qPCR methods, respectively. Comparisons are based on data generated from eight different research facilities. Special attention was placed on the influence of the DNA isolation step and effect of simplex and multiplex amplification approaches on inter-laboratory variability. Results suggest that a crude lysate is sufficient for DNA isolation unless environmental samples contain substances that can inhibit qPCR amplification. No appreciable difference was observed between simplex and multiplex amplification approaches. Overall, inter-laboratory variability levels remained low (<10% coefficient of variation) regardless of qPCR protocol.

Introduction

The application of quantitative real-time PCR (qPCR) technologies for the rapid identification of fecal indicator bacteria (FIB) in environmental waters

[1]*United States Environmental Protection Agency, Office of Research and Development, National Risk Management Research Laboratory, Cincinnati, OH*

[2]*University of North Carolina at Chapel Hill, Institute of Marine Sciences, Morehead City, NC*

[3]*United States Geological Survey, Columbus, OH*

[4]*City of Racine Health Department, Racine, WI*

[5]*National Oceanic and Atmospheric Administration, Atlantic Oceanographic and Meteorological Laboratory, Ocean Chemistry Division, Miami, FL*

[6]*University of South Florida, Department of Biology, Tampa, FL*

[7]*United States Environmental Protection Agency, Office of Research and Development, National Exposure Research Laboratory, Cincinnati, OH*

is being considered for use as a national water quality metric in the United States. Unlike cultivation techniques that require 18 or more hours to generate test results, qPCR methods can provide the necessary information to open or close a beach in less than four hours. Shorter sample processing times represents a significant improvement over conventional culture-based methods, as they would allow watershed and beach managers to assess water quality on the same day. As a result, qPCR methods have been developed to detect and estimate the concentration of key fecal FIB such as enterococci (Ludwig and Schleifer 2000, He and Jiang 2005, Siefring *et al*. 2008) and *Bacteroidales* (Dick and Field 2004, Layton *et al*. 2006, Kildare *et al*. 2007, Siefring *et al*. 2008). These methods have been the subject of many research studies focusing on the density and distribution of genetic markers in primary sources of fecal pollution (Kildare *et al*. 2007, Silkie and Nelson 2009, Shanks *et al*. 2010), the detection and decay of DNA targets in fresh and marine water matrices (Bell *et al*. 2009, Walters *et al*. 2009, Green *et al*. 2011, Schulz and Childers 2011), comparisons between culture and qPCR measurement in paired samples (Frahm and Obst 2003, Haugland *et al*. 2005, Viau and Peccia 2009, Whitman *et al*. 2010), as well as the identification of correlations between genetic marker concentrations and associated public health risk (Wade *et al*. 2006, Wade *et al*. 2008). However, little is known about the reproducibility and sources of variation in these FIB qPCR methods across laboratories.

The lack of information available on inter-laboratory performance is, in part, due to the complexity of these qPCR methods and the lack of consensus among researchers on standardization of protocols. Some areas of contention between researchers include DNA isolation protocols and the use of a simplex (single gene target) or multiplex (multiple gene targets) amplification approaches. Before a FIB qPCR method protocol can be considered for regulatory use, studies must be performed to characterize the advantages and disadvantages of these different protocol options, especially in the context of inter-laboratory variability. For example, the advantages of a multiplex amplification approach become irrelevant if the protocol is too complicated to be performed across laboratories with a high level of reproducibility.

In this study, inter-laboratory variability of two qPCR methods designed to estimate the concentration of enterococci and *Bacteroidales* in ambient water samples is reported based on data generated from eight facilities including federal, state, city, and academic laboratories. Each laboratory followed a predetermined series of protocols to generate comparable data. In addition to using standardized protocols, participating laboratories used the same lots of reference DNA sources, DNA isolation kits, and amplification reagents, as well as the same qPCR thermal cycler instrument model to generate estimates of FIB on replicate filters from spiked and environmental water samples. The above instruments, reagents, and protocols were standardized to help isolate the impact of the DNA isolation step, as well as simplex or multiplex amplification approaches has on inter-laboratory variability.

Methods

Participants

Eight laboratories were selected for participation including the U.S. EPA National Risk Management Research Laboratory (Cincinnati, OH), U.S. EPA National Exposure Research Laboratory (Cincinnati, OH), University of South Florida (Tampa, FL), University of North Carolina at Chapel Hill (Morehead City, NC), U.S. Geological Survey (Columbus, OH), City of Racine Health Department (Racine, WI), National Oceanic and Atmospheric Administration (Miami, FL), and Southern California Coastal Water Research Project (Costa Mesa, CA) and were randomly assigned numbers between one and eight.

Assay Selection

Three qPCR assays were included in the study including Entero1a, GenBac3, and Sketa22 as described in EPA Method A for Enterococci and EPA Method B for *Bacteroidales* [http://water.epa.gov/scitech/methods/cwa/bioindicators/biological_index.cfm#rapid]. Primer and hydrolysis probe sequences are listed in Supplemental Information (SI) Table SI-1 (Supplemental Information can be accessed at ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2012AnnualReport/ar12_19SI.pdf).

Scheme Design and Reagent Sets

Participants received detailed protocols including instructions to complete the multiple laboratory

study. All participants were required to use the following: 1) 50 µl of calibration curve DNA plasmid construct for Entero1a and GenBac3 containing 5 x 10^4 copies per µl prepared by a central laboratory: 2) 500 µl of internal amplification control (IAC) DNA plasmid construct for Entero1a and GenBac3 containing 2 x 10^2 copies per µl prepared by a central laboratory; 3) BioBall™ Multishot 550 *Enteroccocus faecalis* (ATCC #29212; BTF, North Ryde, Australia); 4) BioBall™ Custom HighDose 10K *Bacteroides thetaiotaomicron* (ATCC #29741; BTF, North Ryde, Australia) supplied by a central laboratory; 5) Salmon testis DNA (Sigma-Aldrich; Catalog # D7656 or D1626) prepared by individual laboratories; 6) DNA-EZ DNA purification kit (GeneRite, North Brunswick, NJ; Catalog #K102-02-50); 7) TaqMan® Universal PCR Master Mix (Applied Biosystems; Catalog #4304437); 8) Applied Biosystems StepOne Plus real-time PCR instrument; and 9) Test sample filters M, D, Z, G, and S1-S9 provided in triplicate prepared by central laboratory. Test samples were blinded to all participants except one laboratory (see Test sample preparation). Using the required supplies, participants were instructed to: 1) Generate six individual calibration curves for Entero1a and GenBac3 qPCR assays; 2) Carry out DNA isolation and qPCR amplification protocols for all supplied test samples; and 3) Submit raw data to statistics expert for analysis.

Preparation of Reference DNA Sources

Five different reference DNA sources were used in this study. DNA sources included two plasmid constructs (Integrated DNA Technologies), two BioBall™ preparations, and salmon testis DNA. Calibration curve and IAC DNA plasmid constructs were prepared by a single laboratory. Plasmids were linearized by NotI restriction digestion (New England BioLabs, Beverly, MA), quantified with a NanoDrop ND-1000 UV spectrophotometer (NanoDrop Technologies), and diluted in 10 mM Tris, 0.1 mM EDTA (pH 8.0) to generate 5 x 10^4 copies/µl and 2 x 10^2 copies/µl, respectively. Participating laboratories were responsible for preparing dilutions of 3.17 x 10^3, 1 x 10^3, 3.17 x 10^2, 1 x 10^2, 3.17 x 10^1, and 1 x 10^1 copies/5 µl for the calibration curve standards, as well as a 50 copy/2 µl IAC working stock solution. BioBall™ DNA sources were supplied from the same lot and commercial laboratory. Salmon DNA working stocks containing 10 µg/ml were prepared by each laboratory either by dilution of a commercially available 10 mg/ml solution (Sigma-Aldrich #: D7656) or from lyophilized material (Sigma-Aldrich #: D1626). All stock and working stock solutions were stored in low-retention microtubes and at 4ºC until time of analysis.

Preparation of Stock Solutions and Test Filters

Stock solutions of ambient water samples were prepared from four different marine beach locations along the southern coast of California (Table SI-2) by a centralized laboratory. Some water preparations were spiked with *E. faecalis* (ATCC #29212) and *B. thetaiotaomicron* (ATCC #29741) cultured cells (samples D, Z, and G) or primary effluent collected from a local wastewater treatment facility (samples S4-S9). The number of *Enterococcus* spp. colony forming units (CFU) per 100 ml was estimated for each water test sample preparation prior to filtering using the U.S. EPA Method 1600 mEI agar approach (USEPA 2006). Water test samples ranged in *Enterococcus* spp. concentrations from 4 to 1,545 CFU/100 ml (Table SI-2). Test filters were prepared in triplicate for each participating group. For each test filter, 100 ml of water was filtered through a 47 mm, 0.4 µm pore size polycarbonate filter (Osmonics Inc., Catalog #K04CP04700). Filters were then placed in sterile 2 ml screw cap tubes containing a silica bead mill matrix (GeneRite, Catalog # S0205-50), immediately frozen at -80ºC, and then shipped within 24 hours on dry ice to each participating laboratory.

DNA Isolation

Two different DNA isolation protocols were investigated in this study. Prior to DNA isolation, 10 µg/ml stocks of salmon testis DNA were diluted to 0.2 µg/ml in AE buffer. Six hundred microliters of 0.2 µg/ml salmon testis DNA was added to each bead mill tube. Each tube was then sealed, bead milled at 5,000 reciprocations/minute for 60 seconds, and centrifuged at 12,000x g for 1 minute to pellet silica beads and debris. The supernatant was then transferred to a fresh microtube and centrifuge for an additional five minutes. For the crude extract (CE) approach, 40 µl of crude lysate was diluted in 160 µl of 10 mM Tris, 0.1 mM EDTA (pH 8.0) and stored at 4ºC until DNA amplification. For the purified extract (PE) approach, DNA from the remaining bead mill lysate was isolated using the DNA-EZ kit (GeneRite) according to manufacturer's instructions. Purified

DNA was eluted off the DNA-Sure™ columns with 100 µl of EZ elution buffer™ and stored at 4ºC until DNA amplification.

qPCR Amplification.

The Entero1a and GenBac3 qPCR assays were performed in simplex (*Enterococcus* or *Bacteroidales* DNA target) and multiplex (*Enterococcus* or *Bacteroidales* DNA target + IAC target) amplification formats as previously described (Haugland *et al*. 2010). The Sketa22 qPCR assay was run in the simplex format only for use as a DNA isolation efficiency control as previously described (Haugland *et al*. 2010). All reactions were performed in triplicate on a StepOne Plus real-time PCR sequence detector (Applied Biosystems) with a 25 µl reaction volume in MicroAmp® Optical 96-well reaction plates with MicroAmp® 96-well Optical Adhesive Film (Applied Biosystems). Data was initially viewed with Sequence Detector Software (Version 2.3) and quantification cycle (C_q) values (0.03 threshold for all assays) were exported to Microsoft Excel.

Identification of Amplification Inhibition and Competition

An internal amplification control (IAC) designed to evaluate the suitability of isolated DNA for qPCR-based amplification was performed on each test sample DNA extract with the Entero1a and GenBac3 multiplex IAC qPCR assays as previously described (Haugland *et al*. 2010). The amplification interference criterion for each assay and laboratory was based on repeated experiments measuring the mean C_q of a 50 copy IAC spike in buffer only. Evidence of amplification interference was defined as any observed IAC C_q value in a test sample DNA extract greater than the respective mean C_q + 1.5 for a given assay and laboratory. DNA extracts exhibiting amplification interference were further classified into groups affected by inhibition or competition based on observed competition thresholds, which were calculated separately for the Entero1a and GenBac3 assays, and for each laboratory (see Supplemental Information for details).

Evaluation of DNA Isolation Efficiency.

For each test sample filter, the efficiency of DNA isolation was estimated using a salmon testis DNA control spike and subsequent amplification with the Sketa22 qPCR assay. A DNA isolation acceptance threshold for each participating laboratory was established based on repeated control experiments where laboratory grade water was substituted for ambient water. Any test sample filter DNA isolation with a Sketa22 C_q measurement that differed from the laboratory-specific control mean $C_q \pm 3$ threshold was discarded from the study.

Monitoring for Extraneous DNA

To monitor for potential sources of extraneous DNA during DNA isolation and qPCR amplification, extraction blanks where laboratory grade water was substituted for ambient water were performed in each participating laboratory over the course of the study.

Calculations and Statistics

Outliers were removed from data sets for calibration curve plasmid and BioBall™ cell calibrator standardized sources of DNA. The coefficient of determination (R^2) and amplification efficiency ($E = 10^{(1/\text{-slope})}\text{-}1$) were calculated for each Entero1a and GenBac3 individual fitted calibration curve (n = 24 per laboratory). Individual fitted calibration curves with $R^2 < 0.90$ and/or E values outside the range of 0.70 to 1.30 were discarded from the study. The remaining acceptable quality calibration curve data was then pooled to generate a master fitted calibration curve for each respective laboratory and method variant (Entero1a simplex, Entero1a multiplex, GenBac3 simplex, and GenBac3 multiplex). All fitted curves were constructed using a simple linear regression model. The mean $\log_{10}$ number of cell equivalents per filter for each test sample was estimated using the $\Delta\Delta C_q$ model where cell calibrator and Sketa22 values were allowed to vary by laboratory and instrument run. The *Enteroccocus* spp. and *Bacteroidales* reference numbers are assumed to be constant values of 583 and 10^4 cells respectively, based on manufacturer reports. Test sample C_q values above an assay-specific lower limit of quantification (LLOQ), defined as the Lab #1 (source of centralized reference plasmid DNA) 10 copy calibration curve plasmid standard mean C_q, were not included in quantitative analyses. ANOVA was used to identify statistically significant differences in C_q and cell equivalent estimate data sets. Analysis of covariance was done to compare the slope parameter between fitted master calibration curves. All reported statistical analyses were performed by a single laboratory with SAS (Version 9.2; Cary, NC).

Results

Quality Control Metrics and Data Trimming

In this study, data generation and analysis were performed in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al*. 2009). A summary of quality control metrics and acceptance criteria employed for data validation and analysis are summarized in Table 1. *E* and R^2 performance metrics were expanded beyond recommendations by experts and manufacturer's to allow the inclusion of a wider range of results to estimate inter-laboratory variability. For example, the recommended *E* acceptance range of 0.9 to 1.1 (AppliedBiosystems 2011) was expanded to 0.7 to 1.3. Field blank controls indicated the absence of extraneous DNA molecules in 96.3% of all amplifications. Forty-two instances (81%) were from controls using the PE DNA isolation protocol.

Variability in Reference DNA Sources

Variation in C_q values are reported for reference DNA sources utilized in this study (Table 2). Reference DNA sources were classified as centralized (initial stock originated from single lab) or non-centralized (initial stocks prepared by each lab). The BioBall™ and salmon DNA sources were subdivided into CE and PE DNA isolation groups.

Generation of Pooled Calibration Curves

Six fitted calibration curves were generated for each assay and participating laboratory using both simplex and multiplex amplification approaches. Each fitted curve was evaluated based on R^2 and *E* values (Table SI-3) and low-quality individual fitted curves were discarded from the study. Pooled calibration curves were calculated for each laboratory and qPCR method consisting of high-quality individual calibration curves to derive the slope parameter for the $\Delta\Delta C_q$ model and associated quality metrics (Table SI-4). For pooled fitted curves, *E* and R^2 quality metrics ranged across laboratories from 0.88 to 1.17 and 0.91 to 0.98 for Entero1a and 0.99 to 1.20 and 0.91 to 0.97 for GenBac3, respectively. ANCOVA comparison of pooled fitted curve slope parameters identified a significant difference ($p < 0.03$) between respective simplex and multiplex slopes for laboratories #1, #2 and #3 with Entero1a and lab #1 for GenBac3 (example in Figure SI-1).

Detection of Amplification Interference with Multiplex qPCR

To identify amplification interference, an interference C_q threshold was calculated for each laboratory, assay, and DNA isolation strategy combination (Table SI-5). A nested-ANOVA to compare interference C_q thresholds across laboratories indicated significant differences between CE and PE DNA isolation preparations for laboratories 1, 5, 7, and 8 ($p < 0.05$) with the Entero1a IAC multiplex qPCR assay and lab 7 ($p < 0.05$) with the GenBac3 IAC multiplex qPCR assay. An ANOVA indicated significant inter-laboratory variability ($p < 0.0001$) between IAC ROQ values for Entero1a and GenBac3.

Table 1. Summary of quality control metrics and data trimming. 'Metric' indicates specific quality control utilized. 'Acceptance criteria' lists criterion used for a specific metric to determine inclusion or exclusion of a particular data point. 'Total' denotes the number of measurements subject to respective quality metric. '%' reports the frequency of failed measurements. 'Ref' provides previously published documents used to define quality control metric and acceptance criteria. 'Stdev' denotes standard deviation.

	Metric	Acceptance Criteria	Total	%	Reference
Reference DNA	Outliers	Absolute value of C_q – mean / stdev	7,240	1.1	(Smirnov and Dunin-Barkovskii 1969)
Calibration Curve	Outliers	Absolute value of studentized residual >3	3,580	1.2	(Clark and Sivaganesan 2002)
	E	0.7 to 1.3	384	5.5	(AppliedBiosystems 2011)
	R^2	≥0.90	384	3.4	(AppliedBiosystems 2011)
DNA Isolation	Sketa22 C_q	mean control Sketa22 C_q ±3 C_q	624	10.1	(Haugland et al. 2010)
Amplification Inhibition	IAC C_q	see Table SI-4	448	3.8	(Shanks et al. 2008, Bustin et al. 2009)
Quantification Range	LLOQ	Lab #1 10 copy plasmid mean C_q	2,688	3.9	(Bustin et al. 2009)
Extraneous DNA	Field blanks	Within ROQ	1,408	3.7	(Bustin et al. 2009)

Table 2. Summary of mean C_q and standard deviation ranges for reference DNA sources. Type denotes multiplex (mplx) or simplex (smlx) approach. Plasmid standard represents 100 copy test quantity. IAC denotes 50 copy test quantity of internal amplification control. PE and CE represent crude and purified extractions, respectively. Stdev indicates standard deviation. ND depicts no data available.

Range	Assay	Type	Centralized				Non-Centralized	
			Plasmid Std	IAC	BioBall PE	BioBall CE	Salmon PE	Salmon CE
Mean	Entero1a	Smlx	32.6 - 34.9	ND	29.9 - 30.8	32.4 - 33.6	ND	ND
		Mplx	32.5 - 35.3	33.0 - 35.9	30.0 - 31.7	32.4 - 33.7		
	GenBac3	Smlx	32.8 - 35.8	ND	29.3 - 30.3	31.2 - 32.9		
		Mplx	32.6 - 36.7	32.9 - 35.2	28.7 - 31.3	31.2 - 33.0		
	Sketa22	Smlx	ND	ND	ND	ND	19.0 - 26.3	18.7 - 24.3
Stdev	Entero1a	Smlx	0.37 - 1.02	ND	0.25 - 1.26	0.32 - 0.73	ND	ND
		Mplx	0.31 - 1.21	0.30 - 1.41	0.27 - 1.44	0.24 - 1.08		
	GenBac3	Smlx	0.32 - 1.04	ND	0.37 -3.33	0.22 - 0.81		
		Mplx	0.25 - 0.72	0.24 - 1.07	0.34 - 1.49	0.32 - 1.42		
	Sketa22	Smlx	ND	ND	ND	ND	0.34 - 1.29	0.06 - 2.36

A total of 448 test sample DNA extracts were screened for IAC amplification interference, and interference was detected in 173 (38.6%) DNA extracts. IAC assay interference can arise from two possible sources including inhibition or competition between the IAC and respective FIB DNA target. Based on laboratory-specific competition C_q thresholds (Table SI-5), 90.2% of the DNA extracts (n = 156) exhibiting amplification interference were attributed to competition of which 73.9% were from the GenBac3 method. Overall, only 17 of the 448 (3.8%) test sample DNA extracts showed evidence of amplification inhibition. All but three of the inhibited DNA extracts were from the CE DNA isolation protocol. Fifty eight percent of inhibited DNA extracts (n = 10) were from S2 test sample DNA extracts. Based on amplification interference screening, all data associated with test sample S2 (all laboratories) and CE Entero1a data from lab #4 were discarded from quantification analysis due to evidence of inhibition.

DNA Isolation Efficiency

Acceptance criteria (mean control Sketa22 C_q ± 3 C_q) were allowed to vary by laboratory and DNA isolation protocol. Test sample Sketa22 C_q measurements ranged from 21.6 to 28.9 C_q. Acceptance thresholds ranged from < 22.5 to <30.2 C_q across laboratories. Sixty-three test sample DNA extracts (10.1%) failed the DNA isolation efficiency screen and were discarded from quantification analysis. All failed DNA extracts were prepared by lab #1 (PE test samples M, G, D, and Z) and #3 (PE and CE test samples S1-S9) and attributed to laboratory personnel experimental error based on deviations from standardized protocols.

Lower Limit of Quantification (LLOQ)

The LLOQ threshold for each method variant was 36.4 C_q (Entero1a smlx), 35.7 C_q (Entero1a mplx), 36.1 C_q (GenBac3 smlx), and 35.7 C_q (GenBac3 mplx). A total of 107 (3.9%) C_q values from test samples were greater than the respective LLOQ and discarded from quantification analysis. All instances occurred with samples D, M, and S2. The majority of these instances were from Entero1a measurements (92.5%).

Estimation of Target Concentration in Spiked and Environmental Samples

All spiked and environmental samples were analyzed in triplicate by each participating laboratory to estimate the mean $\log_{10}$ cell equivalents per filter of *Enterococcus* spp. and *Bacteroidales* using the Entero1a and GenBac3 qPCR methods, respectively (Figure 1). When data from all test samples were pooled by assay, DNA isolation protocol, and smlx/mplx approach, an ANOVA indicated significant variability across laboratories for each protocol combination ($p > 0.05$). Thus, potential trends in

DNA isolation protocols, smlx/mplx approaches, and lab-to-lab variability was analyzed on a sample by sample basis. To visualize the degree of inter-laboratory variability for each sample and protocol combination, a percent coefficient of variation (%CV) were determined and plotted (Figure 2). Overall, %CV values exceeded 10% across laboratories only 3 times and all instances were from the same test sample (S9). It is interesting to note that the ambient water used to prepare the S9 stock solution was the same location (see Table SI-2) as the S2 sample, which showed evidence of amplification inhibition by multiple laboratories. %CV values were less than 5% 56 times (58%).

A comparison of mean $\log_{10}$ cell equivalents per filter and variability from CE and PE DNA isolation protocols is shown in Table 3. Results indicate that CE and PE mean $\log_{10}$ estimates were not significantly different across laboratories 90.9% of the time and that variability in CE estimates is less than or equal to PE 79.5% of the time. A similar pattern was observed for mean $\log_{10}$ estimates from mplx and smlx approaches (Table 4) where values were not significantly different across laboratories

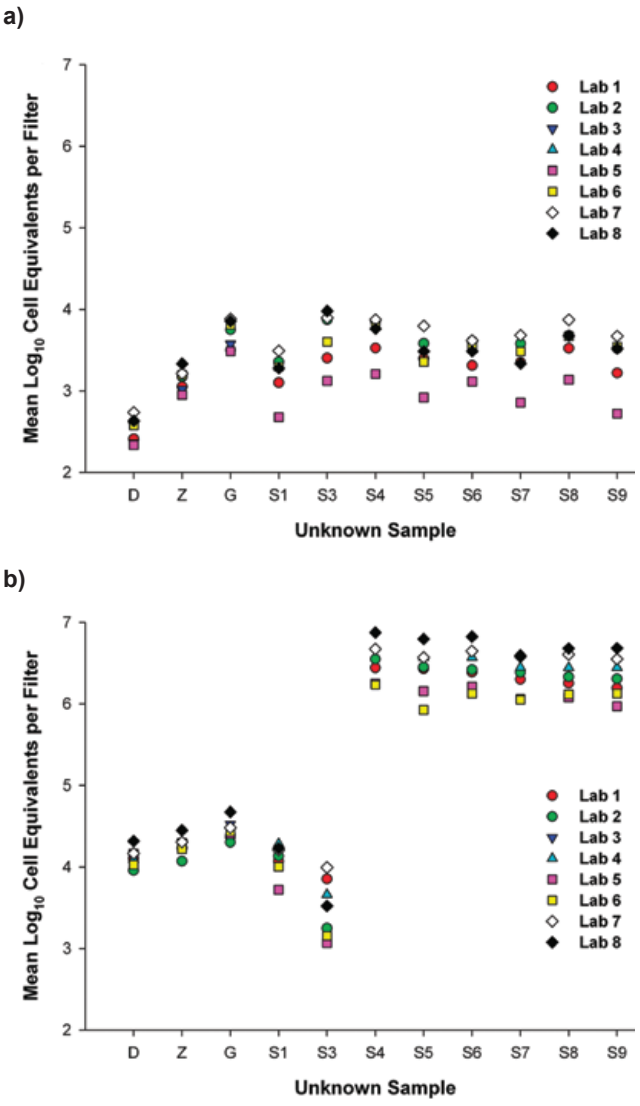

Figure 1. Quantification of *Enterococcus* spp. (Entero1a; A) and *Bacteroidales* (GenBac3; B) by simplex approach in spiked and environmental replicate samples with CE DNA isolation. Concentration estimates reported as mean $\log_{10}$ cell equivalents per sample. Shapes and colors indicate respective participating laboratory data.

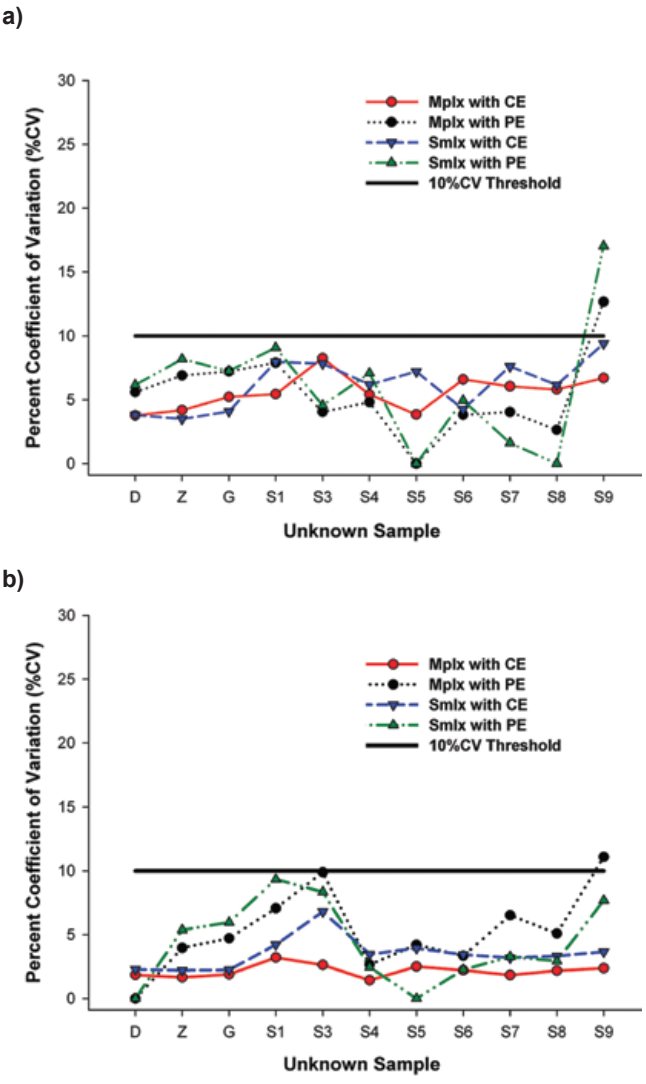

Figure 2. Plot of inter-laboratory percent coefficient of variation (%CV) of mean $\log_{10}$ cell equivalent estimates for each test sample across laboratories for each Entero1a (A) and GenBac3 (B) method variant. The horizontal solid black line indicates the 10%CV threshold. Symbol shape, color, and lines indicate respective protocol combinations.

Table 3. Comparison of impact of crude (CE) and purified extraction (PE) protocols on mean log_{10} estimates and variability by sample. Type indicates mplx or smlx approach. Mean denotes comparison of estimated log_{10} cell equivalents per 100 ml for spiked and environmental samples. Variability denotes comparison of between laboratory variability. CE and PE represent crude extraction and purified extraction protocols. '=' indicates no significant difference, '<' significantly lower, and '>' significantly higher.

Assay	Type	Mean	Variability		
		CE ≠ PE	CE < PE	CE > PE	CE = PE
Entero1a	mplx	Z	G,Z,S9	S1,S4,S6	D,S3,S5,S7,S8
	smlx	.	G,Z,S5,S9	S1,S4,S6,S7	D,S3,S8
GenBac3	mplx	G,Z	G,Z,S4,S6-S9	.	D,S1,S3,S5
	smlx	D,G,Z,S8	G,Z,S1,S6,S9	S4,S5	D,S3,S7,S8

88.6% of the time. In addition, variability in mplx estimates was less than or equal to smlx across laboratories 88.6% of the time.

Discussion

Quality Control Metrics and Data Trimming

The importance of quality control metrics and data trimming for qPCR applications is well recognized (Bustin *et al*. 2009). Parameters such as *E* calculated from a calibration curve slope, R^2 of a calibration curve, evidence for LLOQ, identification of outliers, results of extraneous DNA controls, as well as evidence of acceptable DNA isolation efficiency and absence of amplification interference are required by many peer reviewed journals for data validation and publication (Bustin *et al*. 2009). All of these parameters were defined, measured, and accounted for with each method and assay protocol variant across laboratories in this study and revealed information about the performance of the Entero1a and GenBac3 protocols. For example, only 7% of individual fitted curves failed the *E* and R^2 criteria (Table SI-3). A closer examination of these data shows that 82% of failed curves originated from two laboratories suggesting that calibration curves can be consistently reproduced within an accepted range of variability among the majority of the participating laboratories. The IAC also provides a good example of the utility of quality control metrics where the S2 sample DNA extract accounted for almost 60% of the inhibition interference observations. Presence of inhibition in this sample was corroborated by five of the participating laboratories illustrating the value of the IAC approach. It is also worth noting that all instances of test sample mean estimates below the LLOQ were from only three samples (D, M, and S2), which included the two lowest concentrations of *Enterococcus* spp. measured by membrane filtration Method 1600 (D and M) along with the sample with evidence of amplification inhibition (S2). The inclusion of quality control metrics in this study

Table 4. Comparison of impact of multiplex (mplx) and simplex (smlx) approaches on mean estimates and variability by sample. Type indicates CE or PE DNA isolation protocol. Mean denotes comparison of estimated log_{10} cell equivalents per 100 ml for spiked and environmental samples. Variability denotes comparison of between laboratory variability. mplx and smlx represent multiplex and simplex amplification approaches. '=' indicates no significant difference, '<' significantly lower, and '>' significantly higher.

Assay	Type	Mean	Variability		
		mplx ≠ smlx	mplx < smlx	mplx > smlx	mplx = smlx
Entero1a	CE	Z	.	S9	D,G,Z,S1,S3-S8
	PE	S7,S8	S9	G,S6	D,Z,S1,S3-S5,S7,S8
GenBac3	CE	Z,S6	D,S1,S4,S7-9	.	G,Z,S3,S5,S6
	PE	.	G,S9	S4,S6	D,Z,S1,S3,S5,S7,S8

provides vital information to validate inter-laboratory qPCR data and offers a comprehensive demonstration of how individual control metrics for different steps in a qPCR protocol are combined to increase the confidence in results.

Reliability of Reference DNA Sources

Accurate and reproducible measurement of reference DNA sources is critical for the successful application of any qPCR method including Entero1a and GenBac3. Reference DNA sources are particularly important for water quality qPCR methods because there are currently no certified reference DNA materials available, making it challenging to standardize quantification across a large number of laboratories. The preparation of reference DNA sources for qPCR begins with the determination of the initial reference DNA target concentration. Typically, spectrophotometry or intercalating dyes such as PicoGreen® both of which are reported to introduce variation (Wiseman 2002). In addition, the dilution of reference DNA sources for calibration curve generation is reported to increase variability, especially at lower concentrations (Singer *et al*. 1997).

The $\Delta\Delta C_q$ calibration model employed in this study relies on the ability to reliably measure reference DNA from plasmid DNA constructs (slope determinant and IAC), DNA isolated from BioBall™ preparations (y-intercept determinant), and salmon DNA (DNA isolation control). To help minimize variability across laboratories, initial concentrations of plasmid DNA constructs were determined by a single laboratory and then shipped to participating laboratories. Each laboratory then prepared dilutions to appropriate test concentrations. Although there are numerous reasons to expect some variation in reference DNA source measurements (minor deterioration during shipment, freeze/thaw steps, and dilution preparation), these data represent the best opportunity to characterize inter-laboratory variability related to individual laboratory technical ability. A comparison of C_q standard deviations between participating laboratories indicated that individual laboratory values exceeded 1.5 C_q only 1.6% of the time (2 of 128 instances) and that values were less than 1.0 C_q 84.4% of the time regardless of assay, laboratory, or reference DNA source. In addition, lab-to-lab C_q variances ranged from 0.02 to 1.01 in centralized reference DNA sources. The consistently low variability within and between laboratories observed in this study suggests that reference DNA sources can be highly reproducible, especially when provided by a centralized laboratory in a stable form that requires minimal handling.

Importance of the DNA Isolation Protocol

The DNA isolation protocol determines the concentration and quality of DNA recovered from an environmental sample. Two general strategies are used in water quality testing including bead milling, dilution, and amplification of the resultant crude lysate (CE) or protocols designed to purify and concentrate the DNA target (PE). The CE approach can be performed in as little as five minutes, where PE techniques require anywhere from 30 to 60 minutes to complete. Thus, the CE approach would allow water quality managers to report beach water quality almost an hour faster. A CE protocol also requires less technical training and fewer sample manipulations, which should reduce the frequency of laboratory error and potential cross-contamination of valuable water samples. However, the presence of qPCR inhibitors in the sample matrix can hamper or even prevent amplification (Wilson 1997). Inter-laboratory data in this study illustrates this conundrum where 81% of false positives in field blanks originate from the PE protocol, but 82.4% of DNA extracts exhibiting inhibition were from CE prepared DNA extracts. CE and PE preparations appear to have a minimal impact on estimating Entero1a and GenBac3 genetic marker concentrations in spiked and environmental samples in this study (> 90% agreement amongst laboratories). In addition, variance estimates between CE and PE BioBall™ reference DNA preparations never exceeded 0.51 C_q suggesting that neither approach offers a decisive advantage in terms of variability. Ultimately, the decision to employ a CE approach hinges on the anticipated absence of qPCR inhibitors in the water body of interest. It is also worth noting that both qPCR methods in this study include BSA and salmon testis DNA, two additives reported to combat inhibitory effects (Kreader 1996, Al-Soud and Radstrom 2000). The occurrence of inhibition will most likely be reduced further in future studies as researchers explore the use of engineered polymerases selected to be resistant to common environmental inhibitor effects (Baar *et al*. 2011).

Effect of Multiplexing

Multiplex qPCR amplification entails the simultaneous detection of two more target DNA molecules in the same reaction. A multiplex approach can reduce the cost of sample analysis by combining more than one assay into a single reaction, and it also allows for the inclusion of an internal control to test for amplification interference, a necessary data validation parameter (Bustin *et al*. 2009). Variation in mplx test sample mean estimates were not significantly different ($p > 0.05$) to parallel smlx measurements more than 88% of the time and between laboratory variance for reference DNA sources measured by a mplx approach never exceeded 1.0 C_q. High levels of agreement amongst laboratories combined with low levels of variability suggest that mplx applications are reliable across laboratories if potential competition between FIB target DNA and the IAC is identified and accounted for.

There is always the potential for competition between assays in mplx reactions, especially when both DNA targets use a common set of primers. Competition can result in changes in *E*, LLOQ, and partial or even complete amplification inhibition. Thus, it becomes critical to assess the influence of DNA targets across the range of concentrations used in a particular multiplex application. Detailed experiments were employed to estimate *E* from mplx amplifications with FIB genetic marker concentrations ranging from 10 to 3.17×10^3 copies per reaction in the presence of an IAC 50 copy spike. A comparison of smlx and mplx slopes and competition thresholds (Figure 1 and Table S4) indicated that there can be a significant difference ($p < 0.05$) between smlx/mplx approaches, assays, and laboratories suggesting that these parameters should not be fixed across laboratories or assays during implementation.

Implications for Implementing qPCR-Based Water Quality Monitoring Method

This study characterized inter-laboratory variability of the Entero1a and GenBac3 qPCR methods and we found that mean $\log_{10}$ estimates rarely exceeded 10% CV regardless of method or assay variant (Figure 2). This value is similar in magnitude to differences previously reported for single laboratory replicated qPCR experiments (Haugland *et al*. 2005) suggesting that the two DNA isolation protocols or amplification approaches (simplex and multiplex) investigated in this study do not dramatically increase variability in mean $\log_{10}$ estimates of genetic marker concentration in test samples. While inter-laboratory variability was relatively low in this study, we still observed that two laboratories performed poorly in comparison to other participating groups. Decreased performance appears to be more associated with incorrect implementation of the qPCR protocols rather than inherent method inter-laboratory variability. For example, one of the laboratories was unable to finish sample analysis in a single day and deviated from the standardized protocol by refrigerating crude extracts overnight before completing sample processing. This might not be a concern for purified DNA extracts, but crude lysates can contain nucleases released during bead milling that can rapidly degrade DNA template and potentially reduce sample processing efficiency. Similarly, another laboratory used reconstituted BioBall™ preparations after storing them overnight at 4ºC, rather than preparing a fresh stock daily as directed in the standardized protocol, which may increase variability in cell calibrator measurements. Neither of the above practices are recommended, meaning that the actual inter-laboratory method variability may even be lower than we present. On the other hand, inter-laboratory variability may be underestimated because the eight participating laboratories in this study are professional research groups with more extensive qPCR experience than a typical regional, state, or city facility. In addition, this study focuses on variability associated with technical implementation, using reagents from the same lot and identical thermal cycling instruments. Before any of these protocols can be adopted by a regulatory agency, a single FIB qPCR method protocol must be selected and tested across multiple laboratories. Future inter-laboratory studies must also measure the impact of using different instruments, reagents, and consumables (plasticware), factors that have been shown to exhibit substantial differences in qPCR results between some manufacturers (Saunders *et al*. 2001, Kim *et al*. 2008, Reiter and Pfaffl 2008).

Inter-laboratory experiments also illustrated a number of factors that should be considered in the technology transfer process. First, detailed written procedures need to be developed, as we discovered some differences in qPCR protocol implementation among the eight participating laboratories. Second, there is a need for more training than just publishing

a detailed written protocol, as illustrated by the performance differences even among experienced laboratories. Novice users are likely to require a combination of classroom and hands-on training, even if they already have some familiarity with qPCR. Perhaps most important, experiments reinforce the need for a centralized source of reference DNA materials and the importance of establishing laboratory proficiency benchmarks prior to implementation, raising the question of what is an acceptable amount of variability between laboratories. To include as much inter-laboratory data as possible, broad quality control metric acceptance ranges (i.e., outlier definitions, *E*, R^2, amplification interference threshold, and DNA isolation acceptance range) were employed in this study. Inflated acceptance ranges undoubtedly increased inter-laboratory variability and contributed to the observation that different laboratories can yield significantly different mean $\log_{10}$ cell equivalent estimates from replicate filters. Future investigations designed to characterize the balance between more stringent quality control metrics, inter-laboratory variability, and protocol feasibility are warranted.

This study examined inter-laboratory variability based on the measurement of enterococci and *Bacteroidales* concentrations from standardized, spiked, and environmental sources of DNA using the Entero1a and GenBac3 qPCR methods, respectively. Special attention was placed on the influence of the DNA isolation step and effect of simplex and multiplex amplification approaches on inter-laboratory variability. Results indicated that inter-laboratory variability differences between protocols tested in this study are relatively low and that major differences between laboratories were attributed to experimental error due to deviations in the execution of standardized protocols. These findings should help regulatory agencies decide on a single qPCR protocol, and recognize the importance of standardized protocols, quality control metrics, and proficiency of laboratory personnel.

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Supplemental Information

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