Factors affecting the relationship between qPCR and culture-based enumeration of *Enterococcus*

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Abstract

Several studies have reported a good relationship between quantitative polymerase chain reaction (qPCR) methods for beach water quality monitoring and the culture-based methods they are intended to replace, but these studies were not designed to investigate reasons for discrepancies when they occurred. Here we processed 306 samples using two culture-based methods (EPA 1600 and IDEXX) and two qPCR methods (Taqman and Scorpion), all processed in duplicate, to examine what portion of the discrepancies could be attributed to variability within method or variation within method class. The EPA 1600 and Taqman qPCR agreed regarding beach management decisions for 86.6% of the samples, but qPCR indicated that beach warnings should be issued when EPA Method 1600 did not for 12.1% of the samples. This discrepancy was reduced to 11.7% after accounting for differences between replicates within method, and reduced to 7.7% after accounting for differences between methods within class. For these remaining 7.7% of samples, both qPCR methods indicated that warnings should be issued while both culture methods indicated they should not, suggestive that the differences were attributable to qPCR measuring a DNA endpoint while culture methods measure a growth endpoint.

Introduction

Beach water quality monitoring is presently conducted using culture-based methods that have achieved widespread usage because they are cost effective, easily implemented, and correlate well with health risk (Wade *et al.* 2003). However, sample processing for culture-based methods requires 18 to 96 hours, which means that health warnings are issued days after the samples are originally collected, inconsistent with beach contamination that is often of short duration (Leecaster and Weisberg 2001, Boehm *et al.* 2003). EPA is presently planning adoption of quantitative polymerase chain reaction (qPCR) as a new measurement method (Boehm *et al.* 2009) that reduces processing time to as little as two hours by directly measuring genetic material and eliminating the time-consuming incubation step (Noble and Weisberg 2005). qPCR-based measurements have been found to relate well to health risk (Wade *et al.* 2006, 2008, 2010; Colford *et al.* 2012) and initial beach applications of qPCR have been successful (Griffith and Weisberg 2011).

Several studies have found that qPCR and the culture-based methods they are intended to replace correlate well, though qPCR produces higher values under some circumstances (Haugland *et al.* 2005, He and Jiang 2005, Morrison *et al.* 2008, Lavender and Kinzelman 2009, Noble *et al.* 2010, Whitman *et al.* 2010, Abdelzaher *et al.* 2010). This has been largely

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attributed to differences in target, as qPCR measures a genetic rather than a growth endpoint (Noble *et al.* 2010). However, there are many other factors that can lead to such differences, including method variability and calibration bias (Shanks *et al.* 2012, Cao *et al.* 2013). Most of the comparison studies have been conducted processing a single replicate from each of a single culture based and qPCR method, making it difficult to differentiate among these potential sources of variability.

Here we extend those previous method comparison studies by simultaneously processing two qPCR and two culture based methods, each in duplicate, for a series of marine beach samples. The data are then used to examine several hypotheses to determine causes of method disagreement, including within method variability, variability across methods within class and differences in target endpoint.

Table 1. Sampling sites and agencies that conducted the sample collections.

Collecting Laboratory[a]	Station Name	Longitude	Latitude
OCPHL	Newport Dunes	-117.892	33.61595
OCPHL	Corona del Mar	-117.876	33.59315
OCPHL	15th Street	-117.924	33.60563
OCSD	Newland Street	-117.981	33.6417
OCSD	Magnolia Street	-117.975	33.63838
OCSD	Brookhurst Street	-117.966	33.63311
SOCWA	San Juan Creek	-117.683	33.46195
SOCWA	North Beach Doheny	-117.689	33.46139
SOCWA	Mid North Beach	-117.685	33.46171

[a] OCPHL: Orange County Public Health laboratory; OCSD: Orange County Sanitation District; SOCWA: South Orange County Wastewater Authority.

Methods

Sample and Laboratory Analysis

Nine Orange County, CA, sites were sampled daily from July 6 to August 31, 2010. Eight of the sites were open coast beaches, while the ninth was a high salinity embayment site located in Newport Bay (Table 1). Samples were collected between 7 and 9 A.M. at approximately 0.5 m depth on incoming water in acid washed 1.5 L polypropylene bottles and then filtered through polycarbonate 47 mm, 0.4 μm pore size filters (100 ml per filter) for qPCR analysis. Water from the same sample was also filtered through 47mm mixed cellulose HA filters (10 ml and 1 ml volume filtered) for membrane filtration (MF) culture analysis.

Each sample was analyzed for *Enterococcus* spp. in duplicate for each of two qPCR methods and two culture based methods. The culture methods were membrane-filtration following EPA Method 1600 (APHA 2005) and Enterolert® (IDEXX Laboratories, Westbrook, ME) following manufacturer's instructions. The two qPCR assays targeted the multiple copy 23S rRNA gene in an approach similar to that outlined by Ludwig and Schleifer (2000). The first used TaqMan® chemistry following EPA Method A (2010). The second was based on Scorpion® chemistry following Noble *et al.* (2010). The Orange County Sanitation District, the South Orange County Wastewater Authority and the Orange County Health Department were responsible for collecting samples from three sites each and performing the culture methods. The Southern California Coastal Water Research Project Authority performed the qPCR assays.

qPCR calibrators were prepared using *Enterococcus faecalis* (American Type Culture Collection #29212) cells. Cells were cultured overnight at 37°C in 1% brain heart infusion broth in phosphate buffered saline (PBS) following standard protocol (USEPA 2010). Cells were counted spectrophotometrically after 18 hours and cell suspensions were diluted with PBS. Calibration standards were prepared by filtering 100,000 cells onto 47 mm, 0.4 μm pore size polycarbonate filters. Filters were stored at -80°C until sample DNA recovery for qPCR analyses. A four-point duplicate standard curve was included in each qPCR run using the calibrator and three serial 10-fold dilutions.

Results for both assays were calculated using the comparative C_t method as per Noble *et al.* (2010) and expressed as cell equivalents (CE). Samples for which the C_t value for the salmon DNA control assay was >1.7 C_t higher than the value from the calibrator were rerun after 1:5 dilution with DNA free water (Griffith and Weisberg 2011, Cao *et al.* 2012). Sixty-five samples failed this inhibition criteria even after dilution and were excluded.

Data Analysis

Results for qPCR and culture methods were compared in two ways. The first was using ANCOVA to examine whether the slope of the relationship between culture and molecular methods

differed significantly from that between methods within class. ANCOVA was also used to examine whether the intercept of the relationship between mEI and each qPCR method was non-zero, which would be indicative of target bias. *Enterococcus* concentrations were log transformed and all values less than 33 ENT/100 ml, which is the approximate qPCR detection limit (Haugland *et al.* 2005), were excluded from the ANCOVA.

The second approach was categorical analysis, assessing agreement between Taqman qPCR and EPA Method 1600, the method it is intended to replace, relative to the 104 CFU/100 ml threshold used for beach management decisions in California. This was initially done comparing a single replicate of each, and was repeated using the average of the two replicates for each method. The additional methods were then incorporated into a categorical analysis to assess the percent of samples for which there were within method-class differences compared to those for which both methods within a class indicated the same beach management decision and both methods of the other method-class indicated the opposite.

Results

Slopes of the three regression lines (IDEXX vs. EPA1600, Taqman vs. EPA1600, and Scorpion vs. EPA1600) were not significantly different in the ANCOVA, but the intercepts were (Table 2). qPCR overestimated *Enterococcus* relative to membrane filtration on average by 0.7 log unit, while IDEXX overestimated membrane filtration by 0.5 log unit.

Taqman qPCR and EPA 1600 results agreed with respect to California's beach management decision threshold for 86.6% of the 239 samples that passed the inhibition QC criteria (Table 3). qPCR indicated a beach posting should occur when EPA 1600 did not for 12.1% of the samples. In contrast, only 1.3% samples exceeded the warning threshold for the culture method when qPCR did not.

Table 2. Slope and intercept estimates for ANCOVA comparing relationship between IDEXX vs. EPA 1600, Taqman vs. EPA 1600, and Scorpion vs. EPA 1600. A single slope is provided since slopes of the three regression lines were not significantly different (p=0.70).

	Estimate	Standard Deviation
Slope	0.80	0.06
Intercept for IDEXX	0.51	0.14
Intercept for Taqman	0.21	0.07
Intercept for Scorpion	0.18	0.07

Table 3. Agreement relative to California's beach warning decision threshold between EPA 1600 and Taqman qPCR when comparing a single replicate of each method and when comparing the average of the two replicates for each method (n=239).

	EPA 1600 <104 (%)	EPA 1600 ≥104 (%)
Single Replicate		
qPCR<104	82.0	1.3
qPCR ≥104	12.1	4.6
Averaged Replicates		
qPCR<104	82.8	0.8
qPCR ≥104	11.7	4.6

Only a small portion of the discrepancy could be attributed to within method variability. When the comparison between Taqman qPCR and EPA 1600 was repeated using the average of the two replicates, the percentage discrepancy was only reduced to 11.7% (Table 3). When considering each method separately, within-method variability with respect to the beach management decision was minimal for EPA 1600, but approximately 3, 5, and 6% for IDEXX, Taqman and Scorpion qPCR methods, respectively (Table 4).

Table 4. Agreement relative to California's beach warning decision threshold between duplicate measurements within each method.

	Rep 2 <104 (%)	Rep 2 ≥104 (%)
EPA 1600 (n = 239)		
Rep1 <104	93.7	0.4
Rep1 ≥104	0.4	5.4
IDEXX (n = 239)		
Rep1 <104	90.8	2.1
Rep1 ≥104	0.8	6.3
Taqman (n = 239)		
Rep1 <104	80.8	3.3
Rep1 ≥104	2.1	13.8
Scorpion (n = 234)		
Rep1 <104	82.9	3.4
Rep1 ≥104	2.6	11.1

Table 5. Agreement within and across method classes relative to California's beach warning decision threshold (n=234).

	EPA 1600 <104 & IDEXX<104 (%)	Culture Mixed (%)	EPA 1600 ≥104 & IDEXX ≥104 (%)
Taqman <104 & Scorpion <104	80.8	0.4	0.9
qPCR Mixed	4.3	0.4	0.4
Taqman ≥104 & Scorpion ≥104	7.7	0.9	4.3

A larger portion of the discrepancy could be attributed to differences between methods within a class (Table 5), leaving 7.7% samples for which both qPCR methods indicated a warning should be issued while both culture methods indicated it should not. In contrast, for only 0.9% samples both culture methods indicated a warning should be issued when both qPCR methods did not. Most of the within class differences were associated with the qPCR methods, which differed for 5.1% of the samples compared to only 1.7% of the samples for culture methods.

Discussion

Our finding that qPCR overestimated *Enterococcus* relative to EPA Method 1600 is consistent with findings of previous comparative studies (Haugland *et al.* 2005, Lavender and Kinzelman 2009, Byappanahalli *et al.* 2010, Whitman *et al.* 2010). We focused on comparison of EPA 1600 with Taqman qPCR as the former is the predominant method presently in use and the latter is the method EPA is presently proposing for adoption as the molecular alternative, but our findings that qPCR would yield a greater number of beach warnings, if using the current 104 *Enterococcus*/100 ml California management threshold, than culture methods is unchanged regardless of which combination of methods within a class were compared.

The most compelling explanation for the higher qPCR values is that culture methods measure metabolically active cells, while qPCR also measures stressed and dead-but-intact cells. One challenge in reaching this conclusion, though, is distinguishing measurement target difference from calibration error. A whole cell calibrator was used to relate gene copy number to cell equivalents and results would be systematically offset if the calibration was flawed (Cao *et al.* 2013). There is also the potential for the calibration material, which comes from one *Enterococcus* species, to contain different gene copies per cell than environmental cells that may represent a wide range of *Enterococcus* spp. However, these alternative explanations seem unlikely because the cell counts of the calibration material was verified using traditional methods, as well as by microscopy. Although it is known that *E. faecium* have 1.2 times number of gene copies per genome than *E. faecalis* (4 copies of the 23S rRNA gene per genome), potential qPCR overestimation caused by this species difference is minimal compared to observed (Table 6; Supplemental Information (SI) Table SI-1 available at ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2012AnnualReport/ar12_17SI.pdf).

Our comparison of qPCR results was based on data generated using the dCt calibration model, but the original Taqman method development was based on ddCt in which values are adjusted based on performance of the internal control (Haugland *et al.* 2005). We repeated the analysis after recalculating the qPCR methods using the ddCt model, which caused the percent of samples disagreeing between EPA 1600 and Taqman to double (Table 6). It also increased the number of samples in which both qPCR methods indicated a warning should be issued when both culture methods should not from 7.7 to 13.7%. Results from Cao *et al.* (2012) suggested

Table 6. Agreement relative to California's beach warning decision threshold between membrane filtration and Taqman qPCR using ddCt calculation model when comparing a single replicate of each method (n=290).

	EPA 1600 <104 (%)	EPA 1600 ≥104 (%)
qPCR <104	72.8	0.7
qPCR ≥104	22.1	4.5

against use of the ddCt calculation method because response differences between the internal control and the target lead to qPCR overcorrection, but the choice between these two alternative calculation models is still under debate (Cao *et al.* 2013, Haugland *et al.* 2012).

While overestimation relative to culture methods can be an impediment to adoption of qPCR, it is preferable to the alternative. Previous efforts indicate that when culture methods are used for posting beaches, approximately 70% of the postings were incorrect due to the incubation time delay (Leecaster and Weisberg 2003). Health departments are more concerned with underestimation because it fails to protect swimmers (Griffith and Weisberg 2011), particularly since *Enterococcus* measurements are the screening tool for alerting them to a potential problem. In contrast, false positives may be overprotective, but they usually trigger adaptive sampling that would allow the health departments to refine their warnings. It is also unclear whether qPCR is truly overprotective, as Wade *et al.* (2006, 2008) found health risk relationships with qPCR equivalent to that of culture based methods. Moreover, several studies have found that the survival of viruses is more similar to that of DNA than it is to culture-based measurements of *Enterococcus,* further supporting adoption of rapid methods for microbiological monitoring (He and Jiang 2005).

It is also important to place overestimation relative to existing methods in context of other sources of error. This study was conducted as part of a technology transfer project (Griffith and Weisberg 2011) in which the collecting laboratories also processed the samples for the Scorpion qPCR assay and supplemental data checking procedures were employed to assess the rate of data recording errors. Four such recording errors were identified that would not have been discovered in typical monitoring applications. This is consistent with a previous evaluation that found data recording errors occurred for nearly 10% of samples in routine monitoring, often by an order of magnitude because of failure to account for sample dilution in the calculations (Griffith *et al.* 2006). This is comparable in frequency, and greater in magnitude, than the differences observed between culture and qPCR methods.

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Supplemental Information

Supplemental Information is available at ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2012AnnualReport/ar12_17SI.pdf