Correlation between Quantitative PCR and Culture-Based Methods for Measuring Enterococcus spp. over Various Temporal Scales at Three California Marine Beaches

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Several studies have examined how fecal indicator bacteria (FIB) measurements compare between quantitative PCR (qPCR) and the culture methods it is intended to replace. Here, we extend those studies by examining the stability of that relationship within a beach, as affected by time of day and seasonal variations in source. Enterococcus spp. were quantified at three southern California beaches in the morning and afternoon using two qPCR assays, membrane filtration, and defined-substrate testing. While qPCR and culture-based measurements were consistently and significantly correlated, strength of the correlation varied both among and within beaches. Correlations were higher in the morning (0.45 < p < 0.74 [P < 0.002]) than in the afternoon (0.18 < p < 0.45 [P < 0.021]) and higher when the fecal contamination was concentrated (0.38 < p < 0.83 [P < 0.001]) than when it was diffuse (0.19 < p < 0.34 [P < 0.003]). The ratios of culture-based and qPCR results (CFU or most probable number [MPN] per calibrator cell equivalents [CCE]) also varied spatially and temporally. Ratios ranged between 0.04 and 0.85 CFU or MPN per CCE and were lowest at the beach affected by diffuse pollution. Patterns in the ratios over the course of the day were dissimilar across beaches, increasing with time at one beach and decreasing at another. The spatial and temporal variability we observed indicate that the empirical relationship between culture-based and qPCR results is not universal, even within a beach.

Recreational beach water quality has been assessed using culture-based measurements of fecal indicator bacteria (FIB) for nearly a century. These methods have achieved widespread usage because they are cost-effective and easily implemented and correlate well with health risk (19). However, culture-based methods are slow, requiring 18 to 96 h from sample collection to results, a time frame inappropriate for monitoring beach contamination that is often episodic and of short duration (3, 5, 11).

Quantitative PCR (qPCR) is a new method that eliminates the incubation step by directly measuring genetic material and thereby reducing measurement time to as little as 2 h (15). qPCR-based measurements of FIB have been found to relate well to health risk (20, 21, 22), and the increased speed may provide substantial advantage in health protection. Early applications of qPCR have been successful, but cost and logistical challenges will likely limit application of qPCR to a subset of beaches (7).

As both qPCR and culture-based method will likely be used for beach monitoring in the future, there is a need to understand how often and under what circumstances they yield different results. qPCR does not necessarily produce results comparable to culture-based methods because qPCR measures a genetic, rather than a growth, endpoint. Several studies have found correlations of various strengths between qPCR and culture-based methods (e.g., see references 10, 16, 18, and 24), though qPCR has been found to produce higher values under some circumstances (1, 7, 8, 9, 10, 13). These comparative studies have generally been spatially extensive, based upon a small number of samples collected from a large number of beaches. It remains unclear whether the linear relationship between culture-based and qPCR results, represented by the ratio of culture to qPCR results, is constant or predictable over time. Here, we extend the previous studies by collecting a large number of samples from three beaches to assess the stability of the relationship between enumeration method results over time of day and season.

MATERIALS AND METHODS

Water samples were collected from three southern California beaches: Avalon Bay Beach, Catalina Island; Doheny State Beach, Dana Point; and Surfrider Beach, Malibu. All three beaches are affected by nonpoint sources of contamination. At Avalon, samples were collected at four beach locations at 8:00 a.m., 12:00 p.m., and 3:00 p.m. for 27 days and 31 days between May and September 2007 and 2008, respectively. At Doheny, five beach locations were sampled at 8:00 a.m., 1:00 p.m., and 3:00 p.m. for 8 days in May through July 2007 and 31 days between May and September 2008. At Surfrider, five beach locations were sampled at 8:00 a.m. and 1:00 p.m. for 39 days from May to September in 2009. Surface water discharges are sometimes affected seasonally by naturally occurring sand berms that restrict flow to the ocean at Doheny and Surfrider. These berms were present for all but eight sampling days at Doheny and five at Surfrider.

At all beaches, 25- to 50-liter samples were collected at approximately a 0.5-m depth in 5-gallon buckets that were sterilized with 10% bleach and rinsed with 1% sodium thiosulfate. Samples were immediately mixed in 150-gallon tanks using 0.7-m by 0.7-m stir plates and 20-cm stir bars. After 15 min of stirring, subsamples (approximately 2 liters) were decanted into sterile 4-liter Cubitainers. From these subsamples, 100 or 200 ml was filtered onto 47-mm, 0.4-μm-pore-size polycarbonate filters (HTTP; Millipore Corp., Bedford, MA), which were flash frozen in liquid nitrogen and stored in a −80°C freezer for future qPCR analyses. Two
hundred milliliters was filtered at Avalon in 2008; 100 ml was filtered at the other two beaches and at Avalon in 2007. 

Culture-based analyses of Enterococcus concentrations. Enterococcus spp. were enumerated by membrane filtration (MF) following EPA method 1600 (2). Concentrations were also measured using Enterolert (IDEXX Laboratories, Westbrook, ME) by following the manufacturer’s instructions for seawater, which require a 1:10 dilution of samples in sterile water, and by using the manufacturer-provided most probable number (MPN) table.

DNA recovery for qPCR. Frozen polycarbonate filters were transferred to 2-ml semiconical screw-cap microcentrifuge tubes preloaded with 0.3 g of 0.1-mm zirconia/silica beads (Biospec Corp., Bartlesville, OK). Five hundred or six hundred microliters of AE buffer (Qiagen, Valencia, CA) with 0.2 μg/ml of salmon testis DNA (Sigma, St. Louis, MO) was added to each sample, calibrator, and negative control. The salmon testis DNA served as a specimen-processing control (SPC), used to estimate sample loss during DNA recovery and to identify presumptive PCR inhibition. Tubes were then bead milled in an eight-position minibead beater (Biospec Corp.) for 2 min, followed by centrifugation for 1 min at 12,000 × g. Supernatants were transferred to 1.7-ml microcentrifuge tubes and centrifuged at 12,000 × g for 5 min. Supernatant was transferred to a sterile 1.7-ml microcentrifuge tube, stored at 4°C, and qPCR analyzed within 1 week.

qPCR analyses. Two Enterococcus qPCR analyses were conducted on DNA recovered from replicate filters. Both assays targeted the multiple-copy 23S rRNA gene in an approach similar to that outlined by Ludwig and Schleifer (12). The first used TaqMan chemistry described by Haugland et al. (8) and has been used in several epidemiology and method comparison studies (8, 20, 21). The second was based on Scorpion chemistry, generally following the procedure outlined by Noble et al. (16). This assay was recently used in a large-scale method comparison study (16).

The Scorpion qPCR assays (here referred to as EntScorp) were conducted as 25-μl reaction mixtures using OmnisMix beads (a lyophilized premix with 1.5 units of TaKaRa hot-start Taq polymerase, 200 μM deoxyribonucleoside triphosphates [dNTPs], 4 mM MgCl₂, and 25 mM HEPES with a pH of 8; Cepheid, Sunnyvale, CA), 0.25 μM of the forward primer, 0.25 μM of the probe, and 5 μl of the sample DNA. Reaction mixtures were thermal cycled and monitored in a SmartCycler II (Cepheid). Thermal cycling occurred in two stages: first, 2 min at 95°C, followed by 45 cycles of 5 s at 94°C and 43 s at 62°C. The 25-microliter SPC reaction mixtures were prepared with OmnisMix, a 1.0 μM concentration of each primer, a 0.1 μM concentration of the TaqMan probe, and 5 μl of sample. These reaction mixtures were thermal cycled at 95°C for 2 min, followed by 45 cycles of 15 s at 94°C and 30 s at 60°C.

The TaqMan Enterococcus qPCR assays (here referred to as EntTaq) were conducted following the protocol of Haugland et al. (8). Briefly, 25-μl reaction mixtures were prepared with 12.5 μl of TaqMan universal master mix (Applied Biosystems, Carlsbad, CA), a 5 μM concentration (each) of forward and reverse primers, a 400 nM concentration of probe, 2.5 μl of 2 mg/ml bovine serum albumin, and 5 μl of sample DNA diluted 1:10 in water. Cycling conditions consisted of 2 min at 50°C, 10 min at 95°C, and then 45 cycles of 15 s at 95°C and 1 min at 60°C. The SPC assay was conducted in 25-μl reaction mixtures, using 12.5 μl of TaqMan universal master mix, a 5 μM concentration of each primer, a 400 nM concentration of probe, 2.5 μl of 2 mg/ml bovine serum albumin, and 5 μl of sample diluted 1:10. Thermal cycling conditions were the same as those for the EntTaq assay.

Enterococcus faecalis (American Type Culture Collection 29212) cells were used to create qPCR calibrators. Cell lines were obtained from the ATCC and cultured overnight at 37°C in brain heart infusion broth. Cells were counted spectrophotometrically after 18 h, and cell suspensions were diluted with phosphate-buffered saline (PBS). Calibration standards were prepared by filtering 100,000 cells onto 47-mm, 0.4-μm-pore-size polycarbonate filters. Filters were stored between −70°C and −80°C until sample DNA recovery for qPCR analyses. A four-point duplicate standard curve was run during each reaction using the calibrator and three serial 10-fold dilutions. Amplification efficiency was calculated using the slope of the log standard curve: \( E = 10^{-\text{slope}} \).

For both assays, presumptive sample inhibition and extraction loss were estimated using a TaqMan-based qPCR assay targeting the SPC (8). For the EntTaq assay, cell concentrations were calculated using the threshold cycle (ΔΔCₜ) method described by Haugland et al. (8), which is based on the relative quantity of target DNA in a sample compared to that in a known quantity of target organisms (the calibrator). Results were normalized for DNA recovery by comparing the recovered quantities of the SPC in each sample to the amount of SPC in the calibrator. Samples with more than a 3 Cₜ delay in the SPC were considered inhibited and were diluted 1:5 in sterile water and reanalyzed.

The EntScorp assay results were quantified using the ΔCₜ method outlined by Pfaffl (17) with adjustments for the amplification efficiency.
Like the ΔΔCₚ method, the ratio of the sample Cₚ value to the calibrator Cₚ value was multiplied by the amount of target cells in the calibrator to quantify the total number of calibrator cell equivalents (CCE), but the SPC Cₚs were not used to quantitatively modify results for DNA recovery. Samples with more than a 1.6 Cₚ delay in the SPC were diluted with sterile water and reanalyzed.

**Data analyses.** The ratios between *Enterococcus* concentrations among methods were compared (i) among the three study beaches, (ii) between open- and closed-berm days at Doheny and Surfrider beaches, and (iii) among morning and afternoon samples. Ratios were calculated by dividing the culture result by the qPCR result for each sample. Wilcoxon signed-rank, Kruskal-Wallis, and Friedman tests were used to determine whether differences in the ratios were significant, and Spearman correlations were calculated between log₁₀-transformed method results. When multiple pairwise comparisons were made on a single data set, the significance level was adjusted following Bonferroni.

All concentrations were normalized to CCE, MPN, or CFU per 100 ml. Samples yielding a qPCR nondetect or a measurable concentration below the MF detection limits were assigned a value of 2 CCE or CFU per 100 ml. Samples yielding an Enterolert nondetect were diluted with 10 MPN per 100 ml, because all samples were run at a dilution of 1:10. Because method variability is greater at low concentrations (24), analysis was conducted twice, once using the full data set and once removing samples with concentrations less than 80 MPN, CFU, or CCE per 100 ml.

**RESULTS**

Concentrations of *Enterococcus* spp. varied by method and beach (Fig. 1). Average *Enterococcus* concentrations as measured by Enterolert were similar among the three study beaches, hovering near 30 MPN per 100 ml. Average MF-measured *Enterococcus* concentrations were similar at Avalon and Doheny (30 CFU per 100 ml) but were lower at Surfrider (10 CFU per 100 ml). The EntTaq and EntScorp assays generated similar results regardless of beach. Both qPCR methods had higher average concentrations (200 CCE per 100 ml) at Avalon than either Doheny or Surfrider (30 CCE per 100 ml) at a significance level of P < 0.001.

When results were examined with respect to a single sample standard of 104 CFU, MPN, or CCE per 100 ml, qPCR results agreed with culture results in 76 to 85% of samples at Doheny and Surfrider beaches (Table 1). Agreement was considerably worse at Avalon, where qPCR and culture results would yield the same management decision regarding beach closure only 31 to 43% of the time. These values are relative to 96 to 98% agreement between the two culture-based methods at Surfrider and Doheny and 80% at Avalon. In most cases of disagreement, qPCR results suggested beach closure, while culture results would leave the beach open. It is important to bear in mind, though, that this comparison is based upon the arbitrary assignment of the culture method standard to qPCR results.

Ratios between culture and qPCR results also differed between beaches (Fig. 2), being highest at Doheny and lowest at Avalon.

When data were truncated to remove samples with concentrations below 80 MPN, CFU, or CCE per 100 ml, the ratios were lower at Surfrider and Doheny, sometimes by an order of magnitude (Fig. 3). Ratios were consistent at Avalon using the entire or truncated data set.

**Comparison across time of day.** Concentrations measured by all methods were significantly greater in the morning than later in the day at all sites (see Fig. S1 in the supplemental material). When data were examined by time of day, *Enterococcus* concentrations as measured by qPCR and Enterolert were higher in the morning than in the afternoon by a factor of 5 at Avalon and Doheny. MF-measured concentrations were an order of magnitude higher in the morning.

Ratios between method results were compared among morning and afternoon samples at Doheny and Avalon (Table 2). Surfrider was excluded from this analysis, because no afternoon samples were collected. At Avalon, the ratio tended to increase, often significantly (0.003 ≤ P < 0.067), as the day progressed (i.e., the qPCR results declined relatively faster than the culture results). At Doheny, the ratio was often lower in the afternoon than in the morning, though differences were often not significant (0.01 < P < 0.859). The ratio trends were similar when the data set was truncated.

Correlations between culture and qPCR methods weakened

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**TABLE 1** Percent of the time that management decisions based upon qPCR agree with those based upon culture methods when using the single sample standard of 104 CFU, MPN, or CE per 100 ml

<table>
<thead>
<tr>
<th>Site</th>
<th>MF vs Enterolert</th>
<th>Enterolert vs EntTaq</th>
<th>Enterolert vs EntScorp</th>
<th>MF vs EntTaq</th>
<th>MF vs EntScorp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surfrider</td>
<td>97.6 (333)</td>
<td>85.0 (333)</td>
<td>75.7 (333)</td>
<td>84.7 (333)</td>
<td>76.3 (333)</td>
</tr>
<tr>
<td>Doheny</td>
<td>95.9 (337)</td>
<td>85.2 (337)</td>
<td>81.3 (337)</td>
<td>85.8 (337)</td>
<td>83.1 (337)</td>
</tr>
<tr>
<td>Avalon</td>
<td>80.6 (530)</td>
<td>32.9 (529)</td>
<td>31.5 (530)</td>
<td>43.1 (615)</td>
<td>41.9 (616)</td>
</tr>
</tbody>
</table>

**FIG 2** Ratio of culture-based results to qPCR results at each beach using the entire data set. Box boundaries indicate the first and third quartiles. The median is given as the horizontal line within the box. The whiskers show the 5 to 95% confidence intervals, and samples outside this range are indicated by dots.
TABLE 2 Median ratios of culture and qPCR results

<table>
<thead>
<tr>
<th>Site</th>
<th>Method</th>
<th>All data</th>
<th>Truncated data</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>Culture</td>
<td>qPCR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>result</td>
<td>result</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a.m.</td>
<td>Midday</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p.m.</td>
<td></td>
</tr>
<tr>
<td>Doheny</td>
<td>EntTaq</td>
<td>0.11</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>EntScorp</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td>Avalon</td>
<td>EntTaq</td>
<td>0.76</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>EntScorp</td>
<td>0.58</td>
<td>0.47</td>
</tr>
</tbody>
</table>

a Bolded values indicate a significant difference ($P < 0.05$) in ratios across time of day.

The correlations between culture and qPCR method results were stronger when the berms were open than when they were closed at both beaches (Table 5). At Doheny, correlation coefficients between culture and qPCR results ranged between 0.71 and 0.83 ($P < 0.001$) when the berm was open and 0.20 and 0.27 ($P < 0.003$) when the berm was closed. Correlations were weaker at Surfrider, but the same trend was observed: 0.38 < $P < 0.60$ ($P < 0.004$) when the berm was open and 0.19 < $P < 0.34$ ($P < 0.04$) when the berm was closed.

Inhibition. The frequency of samples that failed SPC tests for potential qPCR inhibition varied by method and in some instances across beaches. When Scorpion method results from all beaches were pooled, 16% of samples were above the 1.6 $C_T$ acceptance threshold for this method and required further dilution. Separating the data by beach, failure rates at Avalon (including 100- and 200-ml samples), Doheny, and Surfrider were 22%, 1%, and 20%, respectively. In contrast, when TaqMan method results were pooled, 7% of samples were above the 3 $C_T$ value. Failure rates were 13% at Avalon (including both 100- and 200-ml samples), 2% at Doheny, and 3% at Surfrider.

DISCUSSION

qPCR consistently yielded higher Enterococcus values than either culture method, likely reflecting that qPCR measures the presence of genetic material while the culture methods measure viable cells. Previous studies have found improved correlations between enumeration methods when fecal contamination is fresh and delivered in a concentrated pulse (8), probably because there is reduced time for decoupling of cellular metabolism and DNA presence (23). This is consistent with ratios between method results being farthest from unity at Avalon, where the fecal source is contaminated groundwater that is filtered through the sand before reaching the beach, thus increasing decoupling time and opportunity (4). It is also consistent with our finding that correlations between qPCR and culture-based results were stronger and ratios closer to unity (when data were truncated) when the berms at Doheny and Surfrider were open and fecal contamination was concentrated.

Time of day affected the relationship between culture and qPCR results inconsistently across beaches. Correlations between methods were strongest in the morning at all beaches, consistent with sunlight serving as an inactivation agent for Enterococcus spp. (6, 14, 23). Because DNA is not as sensitive to sunlight as culturable cells and does not degrade as quickly after UV exposure (23), ratios between culturable cells and CCE were expected to fall over the course of the day. At Doheny, the ratio between culture and qPCR results decreased over the course of the day, as expected. However, the ratio at Avalon increased as the day progressed.

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suggestion that other factors, such as tide, had a larger effect than sunlight (4). When the tide is high or rising, its pressure prevents groundwater from contacting the beach. Low or falling tides allow contaminated groundwater to mix with beach water, giving a fresh pulse of contamination.

The skewness of the qPCR and culture-based measurements of Enterococcus concentrations differed among beaches. At Avalon, Enterococcus concentrations were generally high, but at Doheny and Surfrider, concentrations were often low. Because method variability is greater when concentrations are low (24), we truncated data to ensure that patterns we observed were not driven by these low-concentration samples with high variability. Truncation also eliminated any bias that may have been introduced by assigning Enterolert nondetects a higher concentration (10 MPN/100 ml) than membrane filtration or qPCR nondetects (2 CFU or CCE/100 ml), a difference necessitated by the greater dilution of samples for Enterolert analysis. When data were truncated, correlations between methods were unchanged, but the ratios between culture and qPCR results generally decreased. Patterns in the method ratios over time of day and across beaches were similar, regardless of the exclusion of data at low concentrations. However, truncation of the data set did yield important differences in patterns when comparing data across berm status. This is likely because water quality was good, yielding ratios near unity, on nearly 80% of closed-berm days. On these days, fecal pollution was not simply diffuse but often below detection, and comparisons between method results were not meaningful.

Relationships between methods were sometimes complicated by differences in the qPCR assays. Two qPCR methods were included in this study to identify differences caused by qPCR chemistry. TaqMan assays have been used historically to determine relationships between qPCR results and epidemiological outcomes. Newer Scorpion-based qPCR assays have not been used in epidemiology studies but have the practical advantage of being slightly faster. In this study, the EntScorp assay tended to give slightly higher measurements than EntTaq, even though both assays targeted the same gene. Results from the qPCR assays were always significantly correlated, but the correlation coefficient varied slightly among beaches (data not shown). Because measured qPCR efficiencies were similar (90 to 100%), differences between the assays were potentially due to the quantification method. Results from the EntTaq assay were quantified using the ΔAC_{T} method, which allowed correction for extraction loss and presumptive identification of PCR inhibition using the SPC. In the EntScorp assay, the SPC was used only as a guide for identifying both extraction loss and inhibition. Previous studies on the mean accuracy of Enterooccus spike recoveries from Great Lakes waters using SPC assay-adjusted and unadjusted results indicated that both were within 10% of the expected values, with adjusted estimates being slightly higher than expected and the unadjusted estimates slightly lower (8). Additional studies are needed to determine the ability of the SPC assay and the alternative acceptance threshold values to correctly identify significant qPCR inhibition as well as the comparative effects of inhibitors on the different methods and the accuracy of SPC assay-based adjustments in recovery estimates from the ΔAC_{T} calculation method.

A slight change in the sampling protocol at Avalon may have affected results also. In 2008, 200 ml of sample was filtered for qPCR analyses rather than 100 ml, the volume used in 2007 at Avalon and at the other sites. It is possible that the greater filtration...
tion volume may have resulted in increased concentration of PCR inhibitors and increased error after inhibition corrections.

Implications. Recent work pooling data from 36 sites across the United States has suggested that an empirical relationship can be developed between qPCR and culture-based measurements of FIB, though the authors allow that this relationship may be influenced by local environmental factors (24). Indeed, environmental factors have been shown to affect results from the various methods differently (18). Results from this study demonstrate that the method relationships vary both spatially and temporally and indicate that a single empirical relationship between method results will not be universally appropriate. If qPCR methods for measuring Enterococcus are adopted, new standards will need to be developed or the relationship between the methods will need to be assessed at each beach. At the very least, an understanding of sources of fecal pollution at individual beaches will be required to elucidate meaningful relationships between culture and qPCR results.

ACKNOWLEDGMENTS

We thank Yiping Cao, Darcy Ebentier, Melissa Madison, and Donna Ferguson for their help in coordinating sample collection and processing. We are also grateful to Kevin Oshima for his critical review of the manuscript. This project was supported by the National Institute of Environmental Health Science, U.S. EPA Office of Water, State of California Water Resources Control Board, and the City of Dana Point.

The manuscript has been subjected to the EPA’s peer review and has been approved as an EPA publication. Mention of trade names or commercial sources is for identification purposes only and does not imply endorsement by the United States Environmental Protection Agency water quality guidelines prevent gastrointestinal illness. Epidemiology 19:375–383.


REFERENCES


TABLE 5 Significant Spearman rank correlations (P < 0.05) between log-transformed Enterococcus concentrations as measured by various methods depending upon berm status

<table>
<thead>
<tr>
<th>Site</th>
<th>Berm status</th>
<th>Spearman correlation</th>
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<tbody>
<tr>
<td></td>
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<td>MF vs Enterolet</td>
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<tr>
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<td>Open</td>
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<tr>
<td></td>
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<tr>
<td>Surfrider</td>
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