QPCR Determined Fecal Indicator Bacterial Densities in Marine Waters from Two Recreational Beaches

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Abstract

The use of real-time qPCR to determine fecal indicator bacteria (FIB) densities is currently being investigated by the U.S. EPA. The present recreational water quality guidelines, based on culturable FIB, prevent same day determinations of water quality whereas results from the qPCR method can be available within several hours. Epidemiological studies at POTW-impacted freshwater beaches have shown a strong correlation between qPCR determined *Enterococcus* densities and swimming-related illness rates. This study provides an initial assessment of qPCR estimated *Enterococcus*, *Bacteroidales*, *E. coli* and *Clostridium* densities in marine water from two recreational beaches sampled over one summer. The estimated geometric mean cell densities per 100 ml of marine water from both beaches across sampling visits were 3.28×10^1, 1.71×10^1, 7.37×10^2, and 9.26×10^2 for *Enterococcus*, *Bacteroidales*, *E. coli* and *Clostridium*, respectively. These cell equivalent density estimates, determined using whole cell calibrator samples by a comparative cycle threshold (CT) approach, did not correspond with the relative target sequence density estimates of the different FIB in the samples which gave geometric means of 1.28×10^3, 2.35×10^4, 3.04×10^2, and 1.03×10^4 for *Enterococcus*, *Bacteroidales*, *E. coli* and *Clostridium*, respectively. This discrepancy was determined to be attributable to differences in recovery of target sequences from cells of the different organisms. QPCR analyses using whole cell calibrator samples provides a simple approach for comparing both total cell and target sequence density estimates of different FIB groups in water samples.

Introduction

Recreational water quality is determined by fecal indicator bacteria (FIB) levels which assess the potential risk of exposure to disease causing pathogens. Epidemiological studies, which have shown positive correlations between measured densities of FIB groups and illness rates in recreational waters impacted by publicly owned treatment works (POTW), have led to U.S. EPA's current recommendation on acceptable water quality guidelines based on the densities of *Enterococcus* spp. or *Escherichia coli* (*E. coli*) in freshwaters and *Enterococcus* only in marine waters (USEPA, 1986).

Development of quantitative polymerase chain reaction (qPCR) has enabled the detection of microorganisms within a few hours. Many studies have reported on the use of PCR to detect other bacterial groups associated with fecal material, however, analysis of FIB by qPCR have been limited to date. The aim of this study was to:

1. Compare the qPCR estimated densities of *E. coli*, *Bacteriodales* and *Clostridium* spp. with *Enterococcus* from water and sand samples collected from two POTW impacted marine recreational beaches.
2. Examine temporal and spatial variability of the different FIB groups at the two beaches
3. Examine the relationship between estimated qPCR target sequence copies and qPCR-estimated cell densities for each of these FIB groups.

Notice - Although this work was reviewed by EPA and approved for publication, it may not necessarily reflect official Agency policy.

Methods

Sample Collection

Fairhope Beach (Alabama, USA) and Goddard Beach (Rhode Island, USA)

- June - September 2007
- Weekly on Saturday, Sunday and holidays
- 8 AM, 11 AM and 3 PM from 3 transects, parallel to shoreline ~ 60 meters apart
- Depths of 0.3 and 1 meters.
- 25g (ww) sand samples collected ~ 1 meter from lowest water level at 8 AM only using 2 x 12 inch sterile stainless steel soil auger liner

Filtration

- 0.4 µm pore size (47 mm diameter) polycarbonate membrane filter
  - 50ml or 100ml of marine water samples
  - 20ml supernatant from addition of 1xPBS used to resuspend microorganisms in sand samples

DNA Extraction

- Filters transferred into extraction tubes containing 0.3 g of glass beads.
- 600 µl of 0.2 µg/ml Salmon DNA in AE buffer added and bead milled for 60 seconds at maximum speed.
- Centrifuged 1 minute and supernatant diluted 5-fold.

Taqman® quantitative PCR

- ABI Model 7900 DNA thermal cycler
- Primers and probes
  - *Enterococci* – Ludwig and Schleifer, 2000
  - *Bacteroidales* – Siefring *et al*., 2007
  - *E. coli* – This study
  - *Clostridium* spp. – Rinttilä *et al*., 2004
  - *O. keta* (salmon)– Haugland *et al*., 2005

Estimation of FIB densities

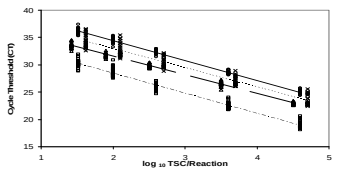
- Calibrator cell equivalents (CCE) calculated from CT values (Haugland *et al*., 2005)
- Mean Target Sequence Copies (TSC) per calibrator cell = $(10^{(y - b)/m} \cdot v)/n$

  where, y = Mean CT value
  b = Intercept from master standard curve
  m = Slope from master standard curve
  v = Extract volume in PCR reaction
  n = Number of calibrator cells
- Calibrator sequence equivalents (CSE) = CCE x Mean TSC per calibrator cell

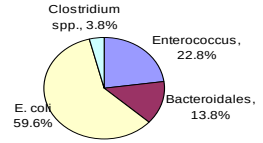
Results

Summary of estimated log10 CCE densities and variability of the different FIB groups in marine beach water and sand

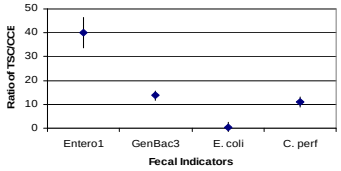
		Fairhope Beach				Goddard Beach			
		Enterococcus	*Bacteroidales*	*E. coli*	*Clostridium* spp.	*Enterococcus*	*Bacteroidales*	*E. coli*	*Clostridium* spp.
Combined Depths	Mean per 100 mL	1.53	3.47	3.00	3.07	1.50	2.68	2.65	2.84
	Within Visit SD[a]	1.14	0.78	0.61	0.54	1.46	1.43	1.00	0.77
	Between Visits SD[a]	1.28	0.97	0.72	0.58	1.85	1.75	1.42	1.23
0.3M	Mean per 100 mL	1.90	3.71	3.22	3.19	1.76	2.82	2.72	3.14
	Within Visit SD[a]	1.18	0.58	0.65	0.43	0.91	0.74	0.74	0.52
	Between Visits SD[a]	1.25	0.90	0.84	0.49	1.09	1.14	0.83	0.77
1.0M	Mean per 100 mL	1.15	3.24	2.79	2.95	1.23	2.54	2.59	2.54
	Within Visit SD[a]	1.05	0.74	0.49	0.58	1.01	0.86	0.67	0.68
	Between Visits SD[a]	1.25	0.82	0.55	0.64	1.24	1.16	0.79	0.82
Sand	Mean per g	0.15	0.74	1.85	2.27	0.80	0.54	2.64	2.53
	Within Visit SD[a]	1.30	1.25	0.78	0.67	1.09	1.18	0.71	0.47
	Between Visits SD[a]	1.42	1.45	0.79	0.60	1.43	1.33	1.17	0.93



Master standard curves of log10 target sequence copies per reaction of each indicator generated from 13 independent runs. Regression lines from top to bottom of figure represent *E. coli* (y = -3.64x + 41.70), *Enterococcus* (y = -3.50x + 39.62), *Bacteroidales* (y = -3.40x + 38.70) and *Clostridium* spp. (y = -3.66x + 35.77). (*Enterococcus* and *Bacteroidales* concentrations offset by ±0.1 log10 copy for display purposes)



Comparison of the frequency (%) of *Enterococcus*, *Bacteroidales*, *E. coli* and *Clostridium* qPCR negatives in samples from both beaches.



Ratio of Fecal Indicator Bacteria Geometric Mean Target Sequence Copies to Calibrator Cells Equivalents.

Summary

- FIB densities at both beaches
  - Significantly greater at 0.3 m than at 1 m sampling depth
    - Similar to culturable FIB (Wymer *et al.*, 2004)
    - From resuspension of FIB in sand into water column by waves?
    - Densities in sand and in 0.3 m water depth samples not better correlated than at 1 m water depth.
      - Correlations in sand and water samples not observed at both beaches.
  - *Bacteroidales* and *Clostridium* spp. > *E. coli* > *Enterococcus* CCE densities
    - Between > within visit variability at the 6 sampling locations for all FIB.
    - Densities not well-correlated with each other over time
      - Differences in degradation rates in surface water?
      - Influences by nonpoint sources?
  - *E. coli*: High CCE densities but low CSE densities
    - High frequency of qPCR negative samples is a limitation to *E. coli* analyses in epidemiological studies at marine beaches
    - QPCR assay based on single copy *uidA* gene
    - Assay targeting multi-copy rRNA gene similar to the other assays may increase sensitivity of method
- Enterococcus: Low CCE densities but high CSE densities
- Ratio of cells to sequences may vary in environmental samples but determination of both CCE & CSE may be informative.
- Relative CCE & CSE quantification approaches do not depend on a knowledge of absolute target sequence recovery efficiency from cells and facilitate standardization of method results between laboratories.

EPA United States Environmental Protection Agency

References

Dick LK and Field KG. 2004. Appl. *Environ.* Microbiol. 70(9): 5695-5697
Haugland RA, Siefring S, Wymer L, Brenner K and Dufour A. 2005. Water Res. 39:559-568.
Khan IU, Gannon V, Kent R, Koning W, Lapen DR., Miller J, Neumann N, Phillips R, Robertson W, Topp E, van Bochove E, and Edge TA. 2007. J. Microbiol. Methods. 69(3): 480-488.
Ludwig W and Schleifer KH. 2000. Syst Appl Microbiol. 23(4):556-562.
Rinttila T, Kassinen A, Malinen E, Krogius L, and Plava A. 2004. J. Appl. Microbiol. 97: 1166-1177.
Siefring S, Varma M, Atikovic E, Wymer L, and Haugland R. 2007. J Water Healh, 6(2): 225-237.
USEPA. 1986. EPA 440/5-84/002. Criteria and Standards Division, U.S. Environmental Protection Agency, Washington, DC.