Monitoring fecal indicator bacteria with alternative real-time PCR instruments to assess health risks associated with recreational water use

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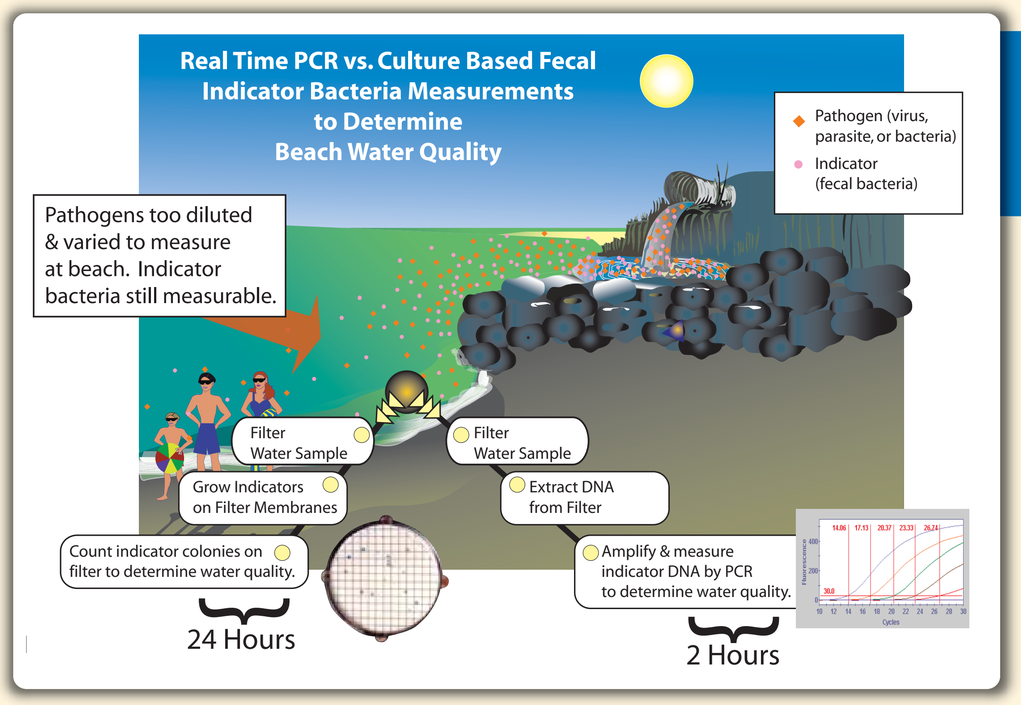
Introduction



THE NEED FOR SPEED: Currently accepted culture-based monitoring methods for fecal indicator bacteria in water such as EPA method 1600 (shown at bottom left in adjacent panel) take at least 24 hr to determine if unacceptable levels of fecal pollution have reached our beaches. Thus we can only tell what the water quality was yesterday. New molecular based technologies such as the real time PCR method (shown at bottom right in panel) have the ability to provide the same water quality measurements in ~2 hr.

REAL TIME PCR GETS THE JOB DONE: The National Epidemiological and Environmental Assessment of Recreational (NEEAR) Waters Study, performed by NERL, NHEERL and CDC in 2003-2004 demonstrated a strong correlation between real time PCR measurements of the fecal indicator bacterial group, *Enterococci* and swimming-related illness rates at 4 Great Lakes beaches. Results of this method can therefore provide a meaningful determination of the water quality at beaches in a timely manner.

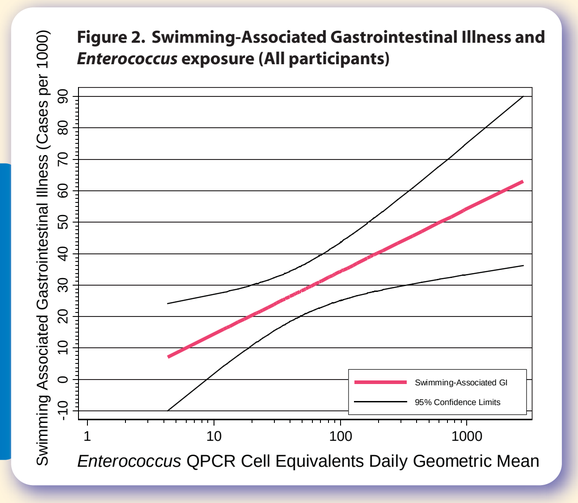
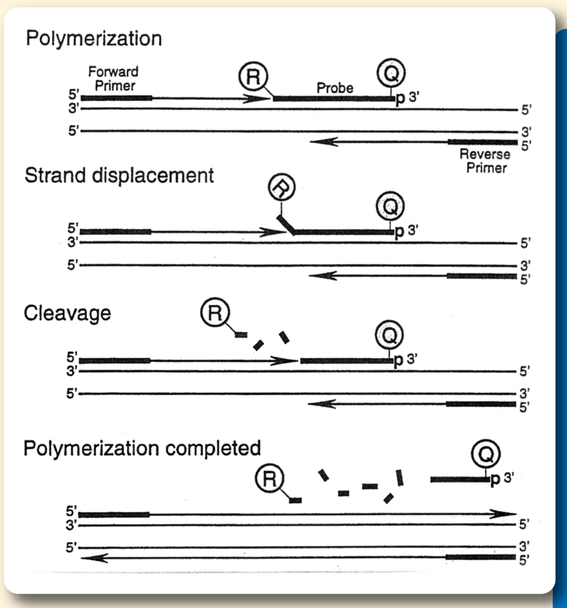


Figure 2. Swimming-Associated Gastrointestinal Illness and *Enterococcus* exposure (All participants)

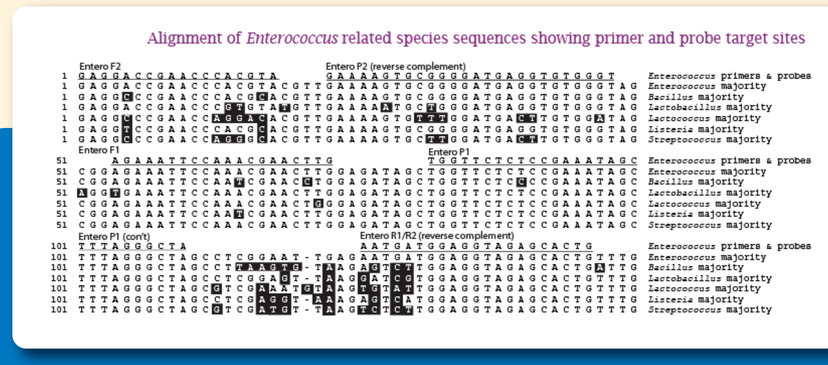
Problem

SO MANY CHOICES OF PCR INSTRUMENTS AND REAGENTS: There are now a number of companies that manufacture real time PCR instruments and reagents. These systems have different features that may be desirable to different end users in terms of sample throughput, analysis speed, portability and cost. Studies are now in progress at NERL-Cincinnati to determine the comparability of results from several different instrument and reagent systems. Systems compared to date include the Applied Biosystems Model 7700 or 7900 with Universal TaqMan MasterMix reagent (96 samples per run, ~2 hr run time), Cepheid Smart Cycler with OmniMix reagent (16-96 samples per run, ~30 min run time) and Applied Biosystems Model 7900 with fast block and Fast Mix reagent (96 samples per run, ~30 min run time)

Methods



REFRESHER ON HOW REAL TIME PCR WORKS: PCR is a technique that makes copies of specific DNA sequences using short flanking primers that are extended by a thermal stable DNA polymerase. This process is repeated in a number of thermal cycles that exponentially amplifies the target DNA. Real time or quantitative PCR detects each target sequence copy as it is made with a fluorescent probe. One specific process for detection shown here uses the nucleolytic property of the Taq polymerase to hydrolyze a short oligonucleotide probe molecule that hybridizes to the target sequence before each new copy is made. This hydrolysis relieves a quenching effect between two dyes on the probe. The resulting fluorescence is detected in real time by the instrument.



OPTIMIZING THE PCR PRIMERS AND PROBES FOR DIFFERENT REAL TIME INSTRUMENTS: Care must be taken in the selection of primer and probe sequences for real time PCR analyses to ensure optimal sensitivity and specificity in the detection of the desired target sequence. The primers and probes used in the NEEAR studies (EnteroF1, R1 and P1 shown above) were designed for optimal performance with Universal TaqMan MasterMix reagent. Our preliminary studies indicated that these primer and probe sequences either showed lower sensitivity or specificity with the other reagent systems. This lead us to redesign the assay with a modified forward primer and probe (Entero F2 & P2) shown above. Computer analyses suggested that this redesigned assay would perform better at the higher temperatures required to maintain specificity with the OmniMix reagent system. We also found that our original Salmon DNA control assay suffered under these higher thermal cycling conditions. We therefore also designed a new control assay for a species related to *Enterococcus* named *Lactococcus lactis*. The primers and probe in this assay recognize the same ribosomal DNA region as those in the redesigned *Enterococcus* assay and thus were expected to perform similarly as a control in real time PCR analyses.

Results

RESULTS FROM THE DIFFERENT INSTRUMENT AND REAGENT SYSTEMS ARE INDISTINGUISHABLE USING THE NEW PCR ASSAYS: The samples analyzed in this study were from three diverse types of surface waters including Lake Michigan freshwater samples, brackish Lake Ponchartrain samples and Gulf of Mexico marine samples. Each of these samples was spiked with known quantities of 10^4 Enterococci cells prior to extraction which were then quantified using both the original primer and probe assay (Entero1) and the redesigned assay (Entero2) -- both with and without either the salmon DNA or Lactococcus DNA control analyses. The results shown here are the log-transformed average measurements of these spiked *Enterococcus* cells in all of the samples (51 in total) which should ideally correspond to the log of the spiked number of cells or 4. The measured numbers are generally lower than expected for all three of these systems when not using the controls (delta CT calculations). This indicates varying degrees of interference by the samples which are most pronounced for the Entero1 assay with the Fast Mix and OmniMix systems. Measurements incorporating the Salmon DNA controls (delta delta CT calculations) tend to give higher than expected results -- probably because this assay is more sensitive to inhibition than the corresponding Enterococcus assays and thus overcorrects. Measurements using the *Lactococcus* controls (delta delta CT calculations) provide the right degree of correction in conjunction with the Entero2 assay for each of the systems. The average results for all three systems were statistically indistinguishable using this target and control assay combination and the precision of the measurements was generally better.

Enterococcus QPCR analysis results using different target and reference assays with Fast Mix, Omni Mix and Taqman Mix reagent systems

Log-transformed Mean (Std. Deviation)
Enterococci QPCR CE in samples spiked with 10e4 cells

Assay (calculation)	Fast Mix	OmniMix	TaqMan Mix
Entero1 (dCT)	3.28 (0.55)	3.75 (0.40)	3.88 (0.35)
Entero1 (ddCT) Salmon DNA ref.	3.70 (0.35)	4.30 (0.36)	4.13 (0.24)
Entero1 (ddCT) Lactococcus ref.	3.33 (0.52)	3.77 (0.40)	4.06 (0.17)
Entero2 (dCT)	3.67 (0.63)	3.89 (0.48)	3.88 (0.22)
Entero2 (ddCT) Salmon DNA ref.	4.41 (0.44)	4.72 (0.44)	4.10 (0.21)
Entero2 (ddCT) Lactococcus ref.	3.99 (0.25)*	4.05 (0.19)*	4.02 (0.18)*

**No significant difference between systems (P>0.05)*

SUMMARY and FUTURE WORK:

Real time PCR is a promising new method for determining the water quality of recreational beaches in a timely manner. Acceptance of this technology will be aided by the availability of choices in instruments and newer PCR reagents that offer even shorter analysis times or higher sample analysis throughput. By slightly modifying the primers and probe used in the original NEEAR study analyses for *Enterococci*, and by employing a new control assay for *Lactococcus* with similar primers and probe, we have shown that the analysis results are comparable from three different instrument and reagent systems. We have also shown that similar analyses for another promising group of fecal indicator organisms in the class Bacteroidetes are comparable on at least two of the different systems (TaqMan and OmniMix, data not shown). These new real time PCR assays and instrument/reagent systems will be used in future analyses of archived NEEAR study water sample filtrates to confirm that their results show the same correlation with swimming related illness rates that has thus far been demonstrated.

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