Same-day Beach Closure Decisions Using Real-time Quantitative PCR Assay: Detection of *E. coli* in Milwaukee Area Beaches

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Abstract

Objective: To compare the real-time quantitative PCR (qPCR) assay with conventional methods for same-day detection of microbial pollution indicators during beach monitoring in Milwaukee.

Study Design: Beach water samples are routinely examined at the City of Milwaukee Health Department Laboratory (MHDL) for the presence and quantitation of *E. coli* using the EPA-approved IDEXX method. We have validated and utilized the qPCR assay for detection of *E. coli* in beach water within 4-6 hours of sampling. During the summer of 2010-2011, the performance of *E. coli* qPCR assay was compared to culture (membrane filtration- EPA 1603) and defined-substrate (Colilert 18) methods by analyzing water samples collected from three frequently used Milwaukee area beaches.

Results: A significant correlation was found between the qPCR (cell equivalents) and conventional methods (CFU/MPN) during this study. Out of 88 samples tested in 2010 and 111 in 2011 (total 199), 75% were in agreement in 2010 and 87% in 2011. Only one warning was missed in 2011 (per EPA threshold standard, >1,000 MPN/100 mL results in beach closure decision) compared to three in 2010. (Figure 5, Table 3)

Conclusions: *E. coli* qPCR assay allows same-day beach closure decisions with follow-up confirmation in 24 hours by Colilert. Further optimization and evaluation of qPCR assay performance is needed to minimize the extent of disagreements between molecular and conventional techniques and to develop more defined water quality standards based on the qPCR assay performance and epidemiological data.

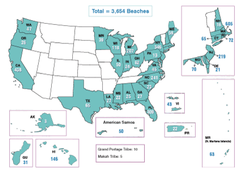
Introduction

Swimming associated illnesses mainly occur as a result of exposure to enteric bacteria, viruses, and protozoa. Fecal indicator bacteria such as *Enterococcus* spp. or *Escherichia coli* are ordinarily harmless microbes that are commonly found in sewage and other sources of fecal contamination. Multi-site epidemiological studies conducted by USEPA and other researchers have established a direct relationship between the density of these indicator bacteria in fresh water beaches and the occurrence of swimming-associated gastroenteritis[1,2,3].

Monitoring of recreational beaches for these fecal indicator bacteria is currently performed using culture-based methods Colilert and Enterolert and utilize defined-substrate technology; EPA's approved method 1600 for *Enterococci* and 1603 for *E. coli* utilize the membrane filtration technique and involve quantification by Most Probable Number (MPN) using serial dilution. These traditional culture-based methods are easier, inexpensive and allow detection in 18-24 hours. Because microbial water quality can change rapidly, guidelines based on indicator organisms that require 18 to 24 hours to develop are likely to result in both unnecessary beach closings and the exposure of swimmers to poor quality water. A recent study estimated that up to 40% of beach closures are in error [4,5].

Here we present the data from the summer of 2010 and 2011 studies monitoring of area beach water quality using Colilert and molecular methods (Table 1 and 2). The studies involved assessing equivalency with traditional water quality monitoring methods through simultaneous processing of water samples using both conventional and molecular methods (Figure 3).

Figure 1a: **2010 swimming season, all thirty coastal states, five territories, and two tribes reported their beach monitoring and notification data to EPA**



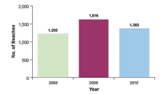
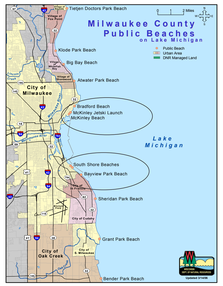


Figure 1b: **Number of beaches with notification actions 2008-10**

Figure 2: **Milwaukee beach study sites include 3 popular Lake Michigan beaches located in Milwaukee County: Bradford Beach, McKinley Beach and South Shore Beach**



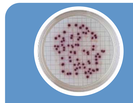
Materials and Methods

Water Sampling
- Approximately 300 mL collected in two sterile containers are transported to the lab and refrigerated within 1 hour

Sample Processing
- Water collected from same site the same day was pooled and mixed before analysis

Calibration Standards and Quality Control
- *Escherichia coli* cell suspension: Prepared for spiking calibrator samples and preparing DNA standard curve
- Sample processing controls (SPC) consisting of *Lactococcus lactis* cells: Prepared for spiking calibrator and test samples prior to extracting DNA
- *E. coli* and *L. lactis* stock cell suspensions containing approximately 10^7 cells per mL were made by diluting fresh cultures in PBS and storing them at –80°C

Various Methods Used at MHDL



Culture method: 24 hrs

Growth of *E. coli* colonies on selective media after membrane filtration

Modified mTEC agar: *E. coli* colonies appear as a red to magenta colony

(results obtained were in comparison to Colilert results)

Colilert-18: Enzyme-Substrate reaction: 18-22 hrs

Colilert: *E. coli* possesses enzyme β-D glucuronidase, which cleaves a flurogenic substrate (MUG) in quanti-trays resulting fluorogen release

(current EPA recommended) method- gold standard)

Molecular method: 4-6 hrs

Quantitative Polymerase Chain Reaction (qPCR) (after membrane filtration):

Detection of *E. coli* and/or *Enterococcus* DNA

qPCR Assay Set up

DNA Extraction
- For *standard curve:* Crude DNA extracted by bead-beating was purified using commercially available Zymo DNA Clean and Concentrator kit.
- For *blank, calibrator and test samples:* DNA extractions were performed using commercially available Zymo Research Fecal DNA kit.

qPCR Setup
- Master mix: *E. coli* and *Lactococcus* SmartBeads (Biogx, Inc.)
- Templates: No template control (NTC) included all qPCR reagents plus nuclease-free water. Two calibrators consisted of DNA extracted from filters spotted with 10-µl of a suspension of 1 x 10^7 cells/ml.
- Real-time PCR Instrument: Cepheid Smart Cycler II
- Thermal cycling conditions: Stage 1: 95°C for 2 min; Stage 2: 45 two-temp cycles at 95°C for 15 s and 62°C for 43 s (optics ON). Cycle threshold (Ct) fluorescence value was manually adjusted to 8 units.

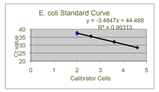
Results

qPCR Data Analysis and Calculations

Standard Curve (Ct values *Vs* Calibrator Cells): The generated Ct values were subjected to regression analysis against the log10-transformed Cell Equivalents (CE) per reaction

Amplification factor, AF = $10^{(-1/\text{slope value})}$ = 1.94

Calculations: Used comparative cycle threshold (C_t) method to estimate target *E. coli* in the beach water sample by determining the relative quantity of target *E. coli* cells present in an unknown beach water sample compared to the quantity of target *E. coli* cells present in known quantity in a calibrator sample



Target cells in sample	Sample type	Ct, target (*E. Coli*)	Ct, SPC (*L. lactis*)	ΔCt	ΔΔCt	Measured Cells in Test Sample Extract ($AF^{\Delta\Delta Ct}$ x cells in calibrator sample)
100,000	calibrator	22.7	19.8	2.9	.....	
Unknown	test	32.3	20.1	12.2	9.3	0.002105 x 100000 = **210**

Inhibition if > 1.5 C_t (0.5 log) difference between sample filtrate and calibrator Ct value for the Sample Processing Control (SPC); qPCR repeated using diluted extracts.

Table 1: **Percentage agreement between qPCR and Colilert results at three beaches in 2010**

Beach water tested	Bradford	McKinley	South Shore
Number of samples tested	38	12	38
Number of sample results in agreement	33 (87%)	8 (67%)	25 (66%)
Number of sample results not in agreement	5 (13%)	4 (33%)	13 (34%)
[1]Warnings missed	2	0	1
[2]Advisories missed	2	2	8
C.E. value > 200 but MPN < 235	1	1	1
C.E. value > 1000 but MPN < 1000	0	1	3

Table 2: **Percentage agreement between qPCR and Colilert results at three beaches in 2011**

Beach water tested	Bradford	McKinley	South Shore
Number of samples tested	43	25	43
Number of sample results in agreement	37 (86%)	22 (88%)	38 (88%)
Number of sample results not in agreement	6 (14%)	3 (12%)	5 (12%)
[1]Warnings missed	0	0	1
[2]Advisories missed	2	1	2
C.E. value > 200 but MPN < 235	4	2	2
C.E. value > 1000 but MPN < 1000	0	0	0

[1]Advisories were based on MPN per 100 mL between 235 and 1000 as compared to CCE per 100 mL between 200 and 1000.
[2]Warnings were issued when MPN/CCE per 100 mL was equal to or above 1000.

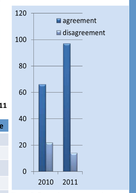
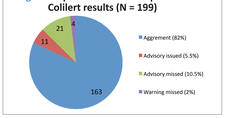


Figure 3: **Percentage agreement between qPCR and Colilert (2010-2011)**

Table 3: **qPCR and Colilert comparison study results summary for year 2010 and 2011**

Study Year	2010	2011
Number of samples tested	88	111
Number of sample results in agreement	66(75%)	97(87%)
Number of sample results not in agreement	22 (25%)	14 (13%)
Warnings missed	3	1
Advisories missed	12	5
C.E. value > 200 but MPN < 235	3	8
C.E.value > 1000 but MPN < 1000	4	0

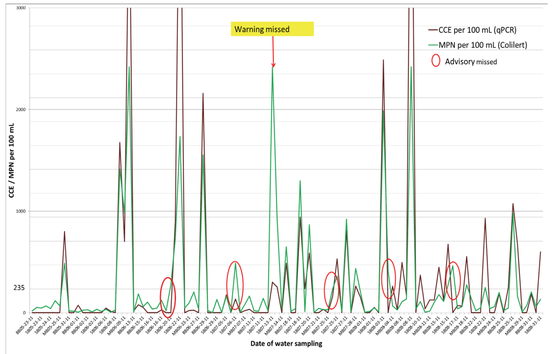
Figure 4: **qPCR results as compared to Colilert results (N = 199)**



Colilert Results	qPCR results: Open	Advisory	Close
Open	126	7	0
Advisory	13	22	7
Close	0	4	15

Table 4: **Beach water quality results from qPCR and Colilert-18 methods**

Figure 5: **2011 Beach testing qPCR data as compared to culture based method**



Limitations

- Ratio of viable and non-viable bacteria varies in different environments
- Environmental factors (e.g. wind direction, rainfall, and sewage overflow)
- Proper collection of water sample (representative sample preferred, by pooling water samples from different sites on same beach)
- Beach to beach variation (e.g. more inhibition seen in samples collected from South Shore Beach)
- Inhibition in PCR reaction (e.g. chemicals, excess nucleic acid from other species)
- Cost of reagents and labor for multiple methods
- No standardized cut-off values for cell equivalents (per 100 mL)

Current MHD Beach Closure Decisions Based on Colilert
(Next day)

Post advisories when *E. coli* levels ≥ 235 MPN per 100 mL → **Beach closure** when *E. coli* levels ≥ 1000 MPN per 100 mL → Closing or posting advisories due to heavy rainfall or sewer overflows, or other environmental indicators

Proposed MHD Beach Closure Decisions Based on Preliminary qPCR Findings
(Same day)

Post advisories when *E. coli* levels ≥ 200 CCE per 100 mL

Beach closure when *E. coli* levels ≥ 1000 ± 100 CCE per 100 mL

Conclusions

- Same-day beach closure decision
- qPCR results can be interpreted as prelim results (presumptive positives > 200 CE/100 mL; 1000 ± 100 CE)
- Follow up confirmation in 24 hours by Colilert for enumeration of *E. coli* in beach water
- Further optimization and evaluation of qPCR assay performance is needed to minimize the extent of disagreements between molecular and conventional techniques
- Need to develop more defined water quality standards based on the qPCR assay performance and epidemiological data

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