Evaluation of rapid QPCR method for enterococci with correlative assessment for molecular markers for sewage contamination in selected environmental water samples from Hawaii.

Problem and Research Objectives

In a consent decree, USEPA agreed to address many criticisms of its current recreational water quality standards which were based on monitoring for two traditional fecal indicator bacterias (FIB) *Escherichia coli* and enterococci by 2012. However, USEPA was not able to properly evaluate the methods and application of alternative fecal indicators such as *Clostridium perfringens*, coliphages and Bacteroidales. As a result, USEPA has proposed to continue to use the same recreational water quality standards and have only added the use of a rapid molecular method (qPCR) for enterococci to be implemented in 2013. The proposed USEPA recreational water quality criteria have raised two potential problems.

The first problem was previously identified and will not be addressed by continuing to use the same recreational water quality standards. The current and future recreational water quality standards are based on the assumption that the source of FIB in recreational waters is sewage and correlated to risk levels for sewage borne infections among swimmers. This was determined by epidemiological studies when the source of contamination was known to be sewage. However, in epidemiological studies, when the source of FIB is environmental rather than sewage, the same risks associated with current recreational water quality standards would not apply. Previous studies have shown that tropical environments favor growth of FIB. This observation is the basis for higher concentrations and persistence of *E. coli* and enterococci in environmental sites (soil, sediments, sand, plants), especially in the state of Hawaii. An identified problem in the application for culture based or qPCR based method to monitor for *E. coli* and enterococci, is that these methods measure all sources of FIB and do not distinguish between sewage sources, animal sources, and environmental sources. The identified need to monitor environmental waters in the state of Hawaii, is to correlate the concentrations of enterococci by culturable and qPCR methods with selected Microbial Source Tracking (MST) assay which shows the presence and concentration of sewage contamination. In addition, the identified need is to assay the same water samples for culturable levels of *C. perfringens* as previous data have shown that monitoring for *C. perfringens* provides more reliable data for presence of sewage than monitoring for *E. coli* or enterococci.

The second identified problem was to establish and validate the reliability of the qPCR method when applied to environmental waters in Hawaii. In this regard, the Hawaii state Department of Health (HIDOH) as well as the City and County of Honolulu (CCH) recognized that they did not have the personnel to validate the qPCR method for enterococci. As a result, these two water quality monitoring agencies have concluded that they would require the assistance of Water Resources Research Center (WRRC) at the University of Hawaii to initially evaluate and establish a reliable/feasible qPCR method that can be applied to water samples obtained from the state of Hawaii. In addition to establishing a method to validate the reliability of the qPCR method, there are three other unknown environmental factors that can influence the qPCR results for enterococci from water samples obtained from a given area. The first environmental factor is related to the levels of inhibition in the water samples that are assayed by the qPCR method. There is evidence of more inhibition when water samples from tropical environments are assayed by the qPCR method. In a recent study conducted at the Boquerón Beach (Puerto Rico), 34% of the samples contained significant levels of PCR inhibitors. The second environmental factor relates to how environmental sources of enterococci, as compared to sewage sources, will react to the qPCR assay method. The third environmental factor relates to the expected ratio of dead to live enterococci populations in water samples obtained from

Hawaii. In summary, it was proposed that a trained molecular microbiologist from WRRC evaluate and establish a reliable qPCR method for enterococci when applied to water samples obtained from the state of Hawaii. It was also recommended that the laboratory personnel from the HIDOH and CCH are trained in this method. As a result, the WRRC microbiologist will be in direct communication with the laboratory personnel from HIDOH and CCH to share information and water samples for assays.

This project will provide an assessment of the rapid qPCR test for enterococci (USEPA Method 1611) in Hawaii and therefore prepare HIDOH and CCH to the changes in the federal water quality criteria. We will work with HIDOH and CCH to provide training as well as provide relevant information to their public outreach programs as requested. Results of the study will enable HIDOH to identify if the USEPA's novel rapid test for enterococci is suitable in Hawaii and if the test should be incorporated into the State's water quality regulations.

Novel molecular tests will improve current microbial water quality monitoring programs and are needed for meaningful water management decisions in Hawaii. While the technology addressed in this project is applicable in all high priority areas identified by the Water Resources Research Institute Program, the project is centered on 'Water Quality' and has the following objectives:

1. Establish and evaluate performance of rapid qPCR test for enterococci (USEPA draft protocol A) in parallel with cultivation based assays in Hawaii.
2. Establish and evaluate qPCR based MST tools in Hawaii.

Methodology

Sample Collection

This research focuses on the island of Oahu. Extension of this research to other Hawaiian Islands has been discussed with the Clean Water Branch (DOH) and is dependent on the outcome of this project. Wastewater influent and effluent samples are currently being collected from the Sand Island Wastewater Treatment Plant (WWTP) as positive controls of human sewage and examined for signal decay. Surface water samples are being collected in locations relevant to the CCH and DOH needs. In this regard we just completed sampling from a sewage spill near Keehi Lagoon, another two sets of samples will be collected after the spill is contained. Ala Wai channel and Palolo Stream are also being sampled. Another ten coastal sites have been identified by DOH for this project and are also being sampled at monthly intervals. A minimum of 70 samples will be analyzed.

Laboratory Analysis

Each water sample is being analyzed by culture based methods for enterococci, *E. coli*, and *C. perfringens* (spores and vegetative cells). Molecular tests are also being used to identify concentrations of enterococci (USEPA method 1611 [USEPA 2012]) and *C. perfringens* (Dumonceaux et al., 2006). Another set of molecular tests, targeting human *Bacteroides* and human polyomavirus groups, are being used to identify human contamination components. We are using standard USEPA approved protocols when available and published protocols for molecular source tracking markers.

Inhibition, positive (standards), extraction blanks and negative controls are being used. We use absolute standard based techniques to quantify molecular signals, except for the calibrator

based ($\Delta\Delta C_t$) method which is used for quantification of enterococci according to the USEPA guidelines (USEPA, 2012). Genomic standards were extracted and quantified from cultured clonal isolates (*Enterococcus faecalis* ATCC29212, *Clostridium perfringens* NCTC8798, *Bacteroides thetaiomicron* ATCC29741) obtained from the American Type and culture collection (ATCC) or in the form of bioball from the bioMérieux Inc. DNA concentration was determined on QBit 2.0 luminometer (Life Sciences) using factory provided standards. Target gene copy numbers were estimated using copy number calculator at the SciencePrimer (http://www.scienceprimer.com/copy-number-calculator-for-realtime-pcr). Human polyomavirus standard was purified and quantified from the cloned synthesized target containing required primer and probe sites at appropriate intervals. All plasmid based standards were linearized using a restriction enzyme (ApaI) and cleaned before use.

Validation of Source Tracking Markers

Human specific source tracking markers (human *Bacteroides*, and polyomavirus) are being validated for the specificity and sensitivity using animal and human fecal samples. Human, rodents, cats and wild pigs are being targeted. Also, concentrations of enterococci in the fecal samples is being determined using cultivation based techniques.

Permits to import micro-organisms (controls), as well as IRB permits to work with human subjects were obtained for this project from January–March 2013.

Principal Findings and Significance

This project is ongoing. The following is a brief summary of our progress.

Sample Collection

Two sets of influent and effluent, two sets of surface water (9 sites each), and one set of coastal samples (10 sites) have been collected. Sample collection is ongoing.

Laboratory Analysis

While cultivation based techniques can be applied routinely without much of an effort for setup, molecular testing requires time to setup and thorough validation of the test is needed before environmental samples can be reliably analyzed. Obtaining the required permits and ordering the components have delayed the project.

QPCR protocols for enterococci, *C. perfringens*, human *Bacteroides* and human polyomavirus have been setup and validated using positive controls. Cell and/or genome based standards demonstrate good efficiency (95%–104%), indicating near perfect doubling of target material in all PCR reactions (Figure 1). The standards were linear over six logs of dilutions and down to 40–50 copies of target per PCR reaction could be reliably quantified. Lower concentrations have not been tested, but in theory up to one copy per reaction should be detectable. We have encountered no problems with extraction blanks or negative controls.

Current ‘surface water’ data, which does not include molecular components, is included in Table 1. It demonstrates high concentrations of *E.coli* and enterococci in freshwater samples, likely originating from environmental pools as *C. perfringens* concentrations were not elevated. The Nimitz culvert site was known to leak sewage from a broken sewer main as indicated by a recent dye tracer study conducted by DOH. While the high enterococci concentrations were

detected in the site, *C. perfringens* spore concentrations were not elevated. This requires further investigation as *C. perfringens* is known to be a good indicator of sewage contamination and the sewer main is still scheduled for repairs. It would be premature to speculate solely based on those two sets of samples, and molecular data analysis has not been completed. The study is ongoing.

Molecular tests on environmental samples will be completed once all samples have been received due to the costs associated with multiple run setups, and to minimize run to run variations.

Validation of Source Tracking Markers

We have finished collecting cat (10) and rodent samples (10 rat, 10 mongoose), and are currently collecting samples from human volunteers and pigs. All collected samples have been analyzed for enterococci concentration, and extraction of DNA has been completed. DNA is stored for molecular tests, which will be completed once all samples have been received due to the costs associated with multiple run setups, and to minimize run to run variations. Concentrations of enterococci varied in rat fecal samples from 555–>23,000, and in cat fecal samples from 34080–>96,000 MPN per 1 g. This variation could be attributed to variability between the individuals, although some variation is likely due to the sample age.

Within the framework of this project, we have strengthened our partnership with the DOH (Clean Water Branch) and CCH (Laboratory Branch, Department of Environmental Services). Opportunities to extend this study to other islands is being discussed with HIDOH. Also, methods and tools validated in this study could be used and transferred to our partner laboratories (DOH, CCH) as needed.

Dr. Mayee Wong, a postdoctoral researcher, was hired in January 2013 partially from the funds of this project. She has been trained in all the methods used in this study and will continue to support this project.

We envision that this study can support our NOAA and Sea Grant funding proposals as well as gain further interest and support from DOH and CCH once this project is completed.

Publication Cited in Synopsis

Dumonceaux, T.J., J.E. Hill, S.A. Briggs, K.K. Amoako, S.M. Hemmingsen, and A.G. Van Kessel, 2006, “Enumeration of specific bacterial populations in complex intestinal communities using quantitative PCR based on the chaperonin-60 target,” *Journal of Microbiological Methods*, 64, 46–62.

USEPA, 2012, Method 1611: Enterococci in Water byTaqMan® Quantitative Polymerase Chain Reaction (qPCR) Assay, Office of Water, Washington DC, Washington, p. 37.

Figure 1. Amplification curves of serially diluted standards for molecular enterococcus (A and B) and human polyomavirus (C and D) test. Insert A also indicates uniform (inhibition free) amplification of inhibition controls (Salmon testes DNA assay).

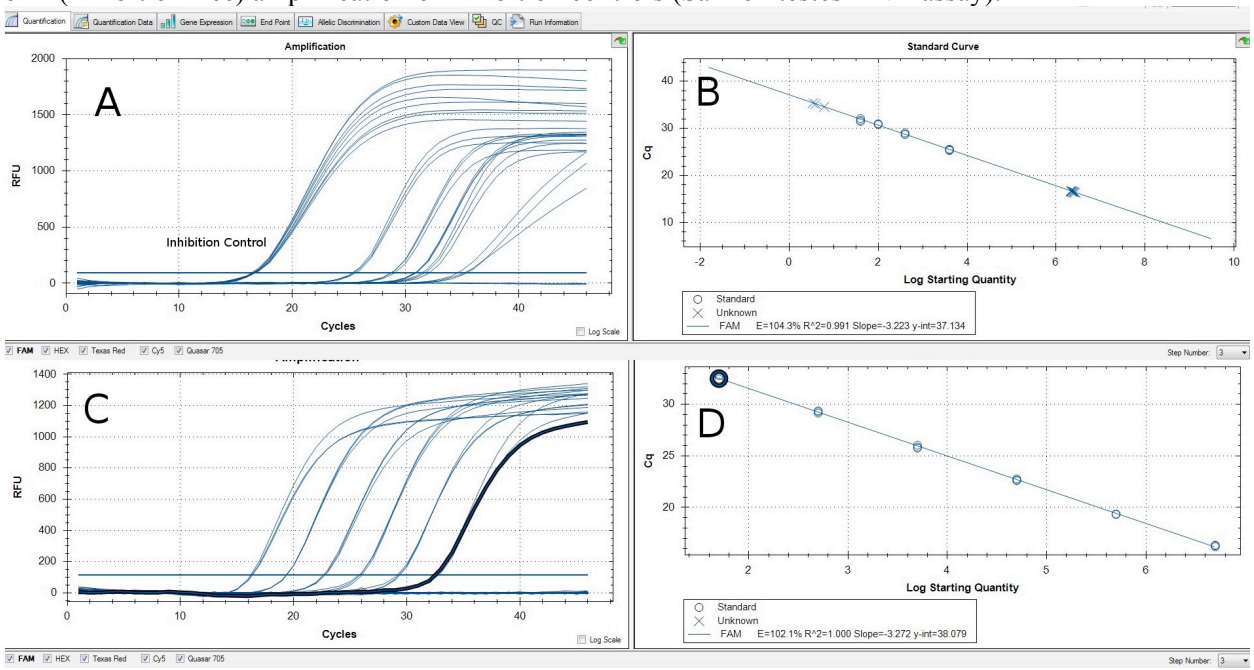

Table 1. Indicator bacteria concentrations in two sets of surface water samples.

	Salinity (ppt)	*E. coli* (MPN/100 ml)	Enterococci (MPN/100 ml)	*C. perfringens* (MPN/100 ml)
Nimitz Road Bridge	24.1-26.6	390-1935	121-613	<1-7
Nimitz Culvert	17.8-21.4	3448-6867	341-1467	<1-15
DAV Bridge	24.4-27.3	512-805	98-355	14
DAV	25.0-28.5	309-420	110-490	8 - 27
Ala Moana Beach	33.0-33.5	41-97	<10-10	<1
Ala Wai Mouth	19.9-28.6	173-6131	97-201	1-4
Ala Wai Canoe Club	7.8-18.8	446-5764	52-691	2-26
Lower Palolo Stream	<0.1	4884-5475	602-12997	<1-49
Upper Palolo Stream	<0.1	4611-3873	697-6488	<1