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Comparison of *Enterococcus* density estimates in marine beach and bay samples by real-time polymerase chain reaction, membrane filtration and defined substrate testing

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ABSTRACT

Currently, densities of *Enterococcus* in marine bathing beach samples are performed using conventional methods which require 24 h to obtain results. Real-time PCR methods are available which can measure results in as little as 3 h. The purpose of this study was to evaluate a more rapid test method for the determination of bacterial contamination in marine bathing beaches to better protect human health. The geometric mean of *Enterococcus* densities using Enterolert® defined substrate testing and membrane filtration ranged from 5.2 to 150 MPN or CFU/100 mL and corresponding qPCR results ranged from 6.6 to 1785 CCE/100 mL. The regression analysis of these results showed a positive correlation between qPCR and conventional tests with an overall correlation (r) of 0.71. qPCR was found to provide accurate and sensitive estimate of *Enterococcus* densities and has the potential to be used as a rapid test method for the quantification of *Enterococcus* in marine waters.

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1. Introduction

Epidemiological studies have indicated that swimming in natural recreational waters that are contaminated with fecal pollution can lead to gastroenteritis (Cabelli et al., 1982; Cabelli, 1983; Wade et al., 2006). Based on this correlation, the US Environmental Protection Agency (USEPA) requires the monitoring of recreational waters across the United States for bacterial indicators of fecal pollution. *Enterococcus* spp. in marine waters and *Enterococcus* spp. or *Escherichia coli* in fresh waters are the currently approved fecal indicator species. These organisms can be found in the intestinal tract of warm-blooded animals and are good indicators for fecal pollution (Wade et al., 2006). These organisms are not pathogenic, however, they are considered to be surrogates for the presence of bacterial and viral pathogens in fecal material.

Currently, there are approved methods such as membrane filtration (MF), Most Probable Number (MPN) and Defined Substrate Technology (DST) tests for measuring densities of *Enterococcus* spp. and *E.coli* in recreational waters (USEPA, 2002; Budnick et al., 1996). Although these methods have been refined over the years, the results are not available for at least 18–24 h. Due to the fluctuating nature of microbial communities, this delay makes it difficult for decision-making regarding beach closures or swimming restrictions (Boehm et al., 2002). The guidelines on indicator organisms that require 18–24 h to develop may lead up to an error rate of 41% for beach closing and advisories (Kim and Grant, 2004). Gastrointestinal illnesses of up to 1479,200 are estimated to occur annually from contaminated beaches in Los Angeles and Orange Counties, California (Given et al., 2006).

On October 10, 2000, Congress enacted the Beaches Environmental Assessment and Coastal Health Act (BEACH Act) that amended the Clean Water Act (CWA) to improve the quality of coastal recreational waters. The Act seeks new indicators to protect human health in recreational waters (USEPA, 2000). Since 2000, studies using molecular approaches, such as real-time quantitative polymerase chain reaction (qPCR) to quantify *Enterococci* as an indicator for water quality, have been conducted (Haugland et al., 2005; Morrison et al., 2008; Liu et al., 2008). Primer sets and probes are available along with protocols which allow the qPCR instrument to detect and quantify Enterococci within 3 h. The use of qPCR has shown promise as an alternative technology for monitoring water quality at recreational beaches

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(Haugland et al., 2005; Wade et al. 2006). qPCR technology was used to determine water quality and swimming advisories at bathing beaches in Orange County California during the summer of 2010 (http://www.sccwrp.org/ResearchAreas/BeachWaterQuality/RapidIndicators/RapidMethodsDemonstration.aspx).

The purpose of this study was to compare *Enterococcus* densities using qPCR technology compared to conventional microbiological techniques (membrane filtration and defined substrate tests (Enterolert®)). This study used marine beach and bay waters to evaluate qPCR as a technique for determination of recreational water quality. The sensitivity, accuracy and precision of qPCR were examined using ocean and bay sites with varying levels projected for *Enterococcus* densities. Evaluation of qPCR variability was an important objective of this study. qPCR results were directly compared to split samples analyzed using membrane filtration and defined substrate tests. qPCR technology would allow for more timely decisions related to bathing beach water quality because results can be obtained on the same day that the samples are collected as opposed to the 18–24 h that needed now.

2. Materials and methods

2.1. Sample collection

Samples were collected from 20 locations in Ocean and Monmouth Counties, New Jersey once every 2 weeks between 6/18/07 and 8/20/07 (Fig. 1). Sampling sites were selected based on historical microbiological monitoring data. The primary objective of the experimental design was to establish sampling areas with a gradient of microbial densities to compare the sensitivities of the methods. The study sites were comprised of 17 bathing beaches and three environmental sampling areas, of which 10 were classified as ocean coastal and 10 embayments. The sampling areas were comprised of 10 ocean coastal and 10 embayments. Environmental stations, i.e., non-bathing beach stations, were included in the study design because the established bathing beach sampling areas typically do not exhibit *Enterococcus* densities near or above the single sample maximum water quality criterion of 104 CFU/mL.

Samples were collected in sterile HDPE containers in an area with a stabilized water depth between the sampler's lower thighs and chest. The sample container (250 or 500 mL sterilized HDPE wide mouth jars, Nalgene® or equivalent) was placed approximately 8–12 inches below the water surface with the lid and stopper still attached. With the collector's arms extended to the front, the container was held near its base and downward at a 45-degree angle. The cap was removed and the container filled in one slow sweeping motion. The mouth of the container was kept ahead of the collector's hand and the container recapped while it is was still submerged. The cap remained submerged during sample collection. Sample remaining from microbiological analysis was used for turbidity and salinity analyses. A total of four independent (true) replicate samples were collected at each station and MF, defined substrate, and qPCR Enterococcus density estimates were measured from each replicate sample bottle.

Following collection, all samples were placed in coolers with ice during transport to either Ocean County Utilities Authority (Bayville, NJ) or Monmouth County Health Department laboratory (Freehold, NJ) and stored at 1–5 °C prior to filtration in the laboratory. Sample filtration was performed, and MF and defined substrate tests were initiated within 6 h of collection. The filters for the qPCR analysis were frozen immediately after filtration onto the polycarbonate filter paper at −20 °C until transport to the USEPA Region 2 Laboratory (Edison, NJ) for qPCR analysis. The turbidity and pH of each water sample were determined by standard methods (American Public Health Association, 1999). Salinity was measured via Conductance Bridge (YSI, Model 85) or refractometer. All samples were collected in the morning (between 6:00 and 9:00 am). There were additional samples collected in the afternoon at three stations in Ocean County on 7/30/08 and one station in Monmouth County on 8/6/08. All samples were collected from a single point from each designated location with the exception of one sampling event across a transect of three sampling areas established at Myron Wilson Bay, Monmouth County. At this transect, additional sampling stations approximately 40 m away on each side of the existing sampling station were established. Samples were collected at this transect on 8/6/07 at approximately 8:00 am, and repeated at 2:00 pm.

2.2. Microbiological procedures

Monmouth County used EPA Method 1600 using mEI agar plates to enumerate *Enterococcus* (USEPA, 2002). Volumes of 10 mL from each water sample were filtered on 47-mm diameter, 0.45 μm pore size (Millipore Corp., Billerica, MA). The filters were incubated on plates of mEI agar for 24 h at 41 ± 0.5 °C before determining colony numbers. *Enterococcus* by MF was expressed as Colony Forming Unit (CFUs) per 100 mL of water. Monthly verification tests of 10 typical and 10 atypical colonies were conducted on each batch of water samples collected over the nine week study period. Each lot of mEI agar was tested for quality using pure cultures of target and non-target organisms. Sterility of the filters and phosphate-buffered water used for rinsing the filtration apparatus was also tested with each batch of samples received by the laboratory. BioBalls™, TCS Biosciences, LTD, which contain a certified number of bacterium, were used routinely for determination of Ongoing Precision and Recovery.

Enterolert® is a defined substrate tests that provides an MPN result based on the presence or absence of fluorescence in 101 individual wells. Defined substrate testing was used for all samples collected in Ocean County, NJ. A 1:10 dilution of the test water sample was prepared (90 mLs of sterile Buffer plus 10 mL of sample) in a sterile container. A package of powdered Enterolert® regent was then added to the container and the sample solution was mixed and poured into a Quantitray, a sterile plastic disposable panel containing 101 wells. The tray was then mechanically sealed after distribution of the mixture into the wells and incubated for 24 h at 41.0 ± 5 °C. Enterolert® uses a nutrient-indicator to detect *Enterococcus*. The tray was placed under a 365-nm-wavelenth UV light with a 6 W bulb as supplied by IDEXX, Westbrook, Maine, and the number of positive wells was enumerated. Any fluorescence in a well was considered a positive reaction for that well and MPN tables were used to determine the density of *Enterococcus* per 100 mL of sample. Ongoing Precision and Recovery samples were performed on a monthly basis in addition to the required media viability checks. The sterility of the sample containers and sterile water were checked prior to first use. Enterolert® is an EPA approved defined substrate test method for wastewaters and ambient waters. A strong positive correlation with membrane filtration has been established for both fresh and marine water samples using this method (Budnick et al., 1996). Membrane filtration and defined substrate test results from Monmouth and Ocean County samples were treated similarly and compared directly to the qPCR results.

2.3. qPCR procedures

A volume of 50 mL for each test sample were filtered through a 0.4 μm, 47 mm diameter polycarbonate filter fitted in a pre-sterilized disposable 250 mL filter funnel within 6 h of collection. The filter paper was folded in half and folded longitudinally 2–3 more times before being placed into a 2.0 mL polycarbonate preloaded

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