THE USE OF REAL-TIME PCR FOR VALIDATION OF FECAL COLIFORM AND ENTEROCOCCI AS INDICATORS OF HEALTH RISK IN THE WESTERN COASTAL REGION OF THE U.S.-MEXICAN BORDER REGION

PROJECT NUMBER: EH-04-02

RICHARD M. GERSBERG, SAN DIEGO STATE UNIVERSITY
MICHAEL ROSE, SAN DIEGO STATE UNIVERSITY

NARRATIVE SUMMARY
The aim of this study was to develop a quantitative assay for measuring the hepatitis A virus (HAV) load in ocean water samples. Viruses are often present in very low concentrations in water samples, and therefore require an extremely sensitive method such as polymerase chain reaction (PCR) for monitoring. Real-time PCR is one of the recent developments in PCR technique. The method is capable of detecting up to a single copy of the target gene and provides quantitative data of the target amplicon. In addition, the method is rapid, highly robust, and amenable to automation. All these features of real-time PCR make it an ideal tool for the present study (Dhar, Roux, and Klimpel 2002).

Specifically, this project will:

1. Use real-time PCR methodology for the measurement of HAV and enterovirus levels in the surf zone at three sites in the U.S.-Mexican border coastal region, including the mouth of the Tijuana River, the surf zone at Imperial Beach, California at the end of Seacoast Drive, and the beach at Playas de Tijuana in Baja California.

2. Measure the densities of the conventional indicators of microbial water quality, fecal coliform bacteria and enterococci, as well as virus levels.

3. Statistically correlate the viral densities measured at the sites above with those of fecal coliforms to better understand the quantitative relationship (and predictive power) between indicator densities and actual pathogen levels in waters impacted by contaminated runoff from Mexico.

The outcome of the above objectives will be the establishment of a quantitative relationship between viral pathogen and bacteria indicator levels for coastal waters in the border region.

HAV was successfully detected in six of seven Tijuana River mouth samples and in five of seven wet weather Imperial Beach (IB) pier samples at levels (uncorrected for

recovery efficiency) ranging from 59 to 16,960 genome copies/L (Figures 3 and 4). Enterovirus was detected in all seven river mouth samples and six of seven wet weather pier samples at levels (uncorrected for recovery efficiency) ranging from two to 2,254 genome copies/L (Figures 3 and 4). Neither virus was detected in any of the six samples taken at the IB pier during dry weather (Figure 3).

The lowest viral concentrations detected in the seawater samples via real-time reverse transcription (RT)-PCR and confirmed by sequencing were 3.3 and 1.4 genome copies per PCR reaction for HAV and enterovirus, respectively (Figures 3 and 4). These values corresponded to minimal detection limits of 6.8 genomes/L for HAV and 1.8 genomes/L for enterovirus (Figures 3 and 4).

Escherichia coli (E. coli) was detected in 13 of 14 wet weather samples and four of six dry weather samples with levels ranging from 41 to 601,000 most-probable-number (MPN)/100 mL and 10 to 20 MPN/100 mL, respectively (Figures 3 and 4). Enterococcus was detected in 12 of 14 wet weather samples and one of six dry weather samples with values ranging from 72 to 754,000 MPN/100 mL and 10 MPN/100 mL, respectively (Figures 3 and 4). There was a statistically significant correlation between *E. coli* densities and both HAV (R^2=0.66, $p<0.0001$) and enterovirus (R^2=0.49, $p<0.0001$) levels (Figure 7 and Table 4). There was also a significant association between enterococcus and both HAV (R^2=0.59, $p<0.0001$) and enterovirus (R^2=0.43, p=0.0003) levels (Figure 7 and Table 4). Finally, there was a significant relationship (R^2=0.41, p=0.0004) between levels of HAV and enterovirus. For the correlation analysis, non-detectable levels of virus and bacteria were assigned the value of one-half of the limit of detection.

Table 5 show the enterovirus levels detected at the Mexican beaches. It is important to note that at two of the beaches, Playas Municipal in Ensenada, Baja California and Playas Tijuana, Baja California, both HAV and enteroviruses were detected even when the indicator level was not elevated. Highest levels of enterovirus (715 genome copies/L) were found at the beach at San Antonio, while high levels of HAV were found at both Playas de Ensenada and Playas de Tijuana (2951 and 1836 genome copies/L, respectively).

Twelve of fourteen wet weather samples (86%) exceeded the California state water quality standard for one or both of the bacterial indicators, *E. coli* and enterococcus (Figures 3 and 4). In contrast, there were no bacterial exceedances for any of the dry weather samples (Figure 3). Regression analyses of the viral densities (as measured by real-time RT-PCR) and indicator levels showed a significant correlation between the densities of both bacterial indicators and levels of HAV ($R^2>0.59$, $p\leq0.0001$) and enterovirus ($R^2>0.43$, $p\leq0.0003$) (Figure 7 and Table 4). These results suggest that bacterial indicator levels may be predictive of the levels of viruses at the Tijuana River mouth and Imperial Beach pier.

A multiple regression analysis showed that HAV levels were directly (positively) related ($p<0.05$) to levels of the fecal indicator bacteria, *E. coli* and enterococcus, enterovirus

levels, river flow rate, and precipitation, and inversely related to water temperature (Table 4). Levels of enterovirus, on the other hand, were only significantly associated with levels of *E. coli* and enterococcus, HAV levels, and water temperature (Table 4). There was no correlation between water tide levels and levels of either virus. Collectively, these variables were able to predict HAV levels in a sample 33% to 66% of the time ($p<0.002$), and enterovirus levels 32% to 49% of the time ($p<0.003$) (Table 4).

Although the association (concordance percentage) with viral levels was slightly higher for *E. coli* than enterococcus, these differences were not significant ($p>0.05$), suggesting that both bacterial indicators were similarly able to predict levels of virus. A series of large-scale epidemiological studies carried out by the United States Environmental Protection Agency (EPA) (1986), found that among the indicator organisms, only two, *E. coli* ($r=0.51$) and enterococci ($r=0.81$), exhibited a strong correlation to swimming-associated gastroenteritis. Based on these findings, the EPA's (2002) draft of the *Implementation Guidance for Ambient Water Quality Criteria for Bacteria* recommended criteria for marine waters solely based on enterococci. The results from this study suggest that *E. coli* might also be a suitable indicator of viral contamination in sewage-contaminated marine waters.

This research resulted in the first-ever quantification of levels of the specific pathogens HAV and enteroviruses in ocean waters at the U.S.-Mexican border. The results show a good correlation between the conventional fecal indicator bacteria and these viral pathogens, and strongly support the health authorities' use of the fecal indicators to gauge the human health risk for bathing in contaminated ocean waters such as Imperial Beach, California.

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INTRODUCTION

For decades, flows of raw sewage from the city of Tijuana, Baja California have entered the Tijuana River and posed a threat to public health at the marine recreational beaches in the coastal South Bay communities of San Diego, California (Conway et al. 1985; Ganster 1999). In this region of the U.S.-Mexican border, where the incidence of a variety of endemic diarrheal diseases (as well as infectious hepatitis) has been shown to be significantly elevated as compared to the non-border region of the U.S. (Doyle and Bryan 2000), the discharge of sewage into coastal marine waters of California is of increasing concern.

Fecal coliform bacterial densities have long been used as the basis for setting microbiological quality standards for protecting public health in both freshwater and marine waters where body-contact recreation occurs. However, for nearly as long, there has been criticism of the use of fecal coliform as an indicator, particularly in marine waters where it has long been recognized that the survival rate of many viruses that cause gastroenteritis is much longer than that of fecal coliforms (Fattal et al. 1983). Moreover, since water quality standards for the United States are based on the relationship between health effects and coliform levels at U.S. bathing beaches, such relationships may be very different for less developed countries. Accordingly, in the coastal waters of southern San Diego County, which are contaminated by sewage discharges and runoff from Mexico, risk models using data generated for the United States may significantly under predict the actual risk of human disease.

Until very recently, the technology was not available to measure viruses in water directly and quantitatively. However, a new and powerful technique called real-time polymerase chain reaction (PCR) has become available, and is now used in the U.S.-Mexican border region to validate the predictive power of the coliform and enterococci indicator system for marine waters contaminated by sewage flows originating in Mexico. The method is rapid with respect to current water quality monitoring procedures, with an entire processing time of less than 12 hours. In addition, this method has the potential to offer greater sensitivity and quantitative ability than any other method currently available. To the best of the researchers' knowledge, such a PCR-based validation of

the coliform/enterocci indicator system for predicting viral pathogen levels in marine waters has never been done.

RESEARCH OBJECTIVES

The major objective of this proposal is to use the powerful new technique called real-time PCR to validate the predictive capability of the coliform and enterococci indicator system for marine waters in the region of the U.S.-Mexican border. The method is rapid with respect to current water quality monitoring procedures, with an entire processing time of less than 12 hours. In addition, this method has the potential to offer greater sensitivity and quantitative ability than any other method currently available. With further optimization of viral concentration procedures, the applicability of this method to high throughput reproducible assays could be developed for direct measurement of virus levels (such as the hepatitis A virus [HAV]), thus allowing a more accurate assessment of disease risk from swimming in runoff-impacted coastal waters.

Specifically, this project will:

1. Use real-time PCR methodology for the measurement of HAV and enterovirus levels in the surf zone at three sites in the U.S.-Mexican border coastal region, including the mouth of the Tijuana River, the surf zone at Imperial Beach at the end of Seacoast Drive, and the beach at Playas de Tijuana in Mexico.

2. Measure the densities of the conventional indicators of microbial water quality, fecal coliform bacteria and enterococci, as well as virus levels.

3. Statistically correlate the viral densities measured at the sites above with those of fecal coliforms to better understand the quantitative relationship (and predictive power) between indicator densities and actual pathogen levels in waters impacted by contaminated runoff from Mexico.

The outcome of the above objectives will be the establishment of a quantitative relationship between viral pathogen and bacteria indicator levels for coastal waters in the border region.

RESEARCH METHODOLOGY/APPROACHES

Viruses are often present in very low concentrations in water samples, and therefore require an extremely sensitive method such as polymerase chain reaction for monitoring. The polymerase chain reaction (PCR) technique is one of the recent developments that allows for the specific detection of viral pathogens in environmental samples. The method is capable of detecting up to a single copy of the target gene and can provide quantitative data of the target amplicon. In addition, the method is relatively rapid, highly robust, and amenable to automation.

PCR for Detection of Hepatitis A Virus in Environmental Samples

HAV has been detected via conventional reverse transcription PCR (RT-PCR) in river water samples (Pina et al. 2001), ground water samples (Abbaszadegan, Stewart, and LeChevallier 1999), urban canal samples (Kittigul et al. 2000), and sewage samples (Divizia et al. 1998; Morace et al. 2002; Pina et al. 2001). HAV was detected in 17% of various storm water samples in San Diego County by conventional RT-PCR (Deardorff 2001). HAV detected using conventional RT-PCR has also been attempted in ocean water samples with degrees of detection varying between 0% (Gilgen et al. 1997; Katayama, Shimasaki, and Ohgaki 2001; Miossec et al. 2000; Schvoerer et al. 2000; Tsai et al. 1994) and 63% (Griffin et al. 1999). In southern California, researchers failed to detect HAV in surf zone coastal waters; however, HAV was detected in treated sewage samples, while utilizing conventional RT-PCR and vortex flow filtration (Tsai et al. 1993).

Real Time PCR

Real-time PCR records each cycle of amplification to capture the logarithmic phase of the reaction, before a limiting reagent causes the reaction to plateau. By measuring the fluorescence of a binding dye called SYBR Green, the amount of initial template complementary DNA (cDNA) can be calculated with respect to a standard curve. The advantages of real-time PCR are greater sensitivity than conventional PCR, with the ability to quantify results, rather than the positive/negative results obtained with conventional PCR. In addition, real-time PCR is rapid, reproducible, and amenable to high throughput utility.

More recently, real-time RT-PCR has been used for the amplification of viral pathogens. The advantages to real-time RT-PCR include greater sensitivity than conventional RT-PCR, less labor intensiveness allowing for higher throughput, as well as extremely accurate and reproducible quantification (Heid et al. 1996). Real-time RT-PCR (using TaqMan® chemistry) has been used to quantify HAV serum levels during different phases of disease, with a sensitivity of five copies (Costa-Mattioli et al. 2001). Real-time RT-PCR (also using TaqMan®) was used for the quantification of enteroviruses (Coxsackievirus A9, Coxsackievirus A16, and Poliovirus Sabin type 1) in seawater from the Florida Keys. Nine out of fifteen samples tested positive, and a viral concentration of 9.3 viruses/mL was determined. This viral concentration did not take into account the percent recovery of virus from the vortex flow filtration process (Donaldson, Griffin, and Paul 2002)

Sampling Sites

Twenty ocean water samples were collected from two locations: 0.2 km north of the Tijuana River mouth, and the south side of the Imperial Beach (IB) pier in San Diego, California (Figure 1). Seven rain events were sampled during the wet season (late October through April) in these locations for a total of fourteen samples. Each sample was collected following a rain event, which was defined as precipitation of 0.5 cm or more. This definition is based on the San Diego County Department of Environmental Health general advisory, which is issued after 0.5 cm or more of rainfall, and warns the public of possible water contamination by urban runoff. In addition, six dry weather

samples were collected during the dry season (May through early October) from the IB pier in order to determine the microbial water quality at the time of highest recreation use. During the dry season, samples were not taken from the mouth of the Tijuana River since the flow of the Tijuana River at this time is negligible or even zero. Samples ranged from 400 milliliters to eight liters and were collected between 2003 and 2005.

Virus Concentration

Each sample was processed within one to two hours of collection following a published protocol by Katayama, Shimasaki, and Ohgaki (2001). Seawater samples were filtered at a constant rate via a vacuum pump through a series of Whatman filters (of 11 micrometer [µm] and 2.5 µm pore size) to reduce particulate matter. Although it is well understood that viruses can adsorb to particles iggn the environment, removal of particulates is necessary for PCR assays. Samples were then applied to a type HA 0.45-µm negatively charged membrane (Millipore, Burlington, Massachusetts). The negatively charged filter was washed with 200 mL of 0.5 millimoles (mM) sulfuric acid (H_2SO_4) to remove cations, and the virus was eluted from the filter with 10 mL of 1 mM sodium hydroxide (NaOH), into a tube containing 0.1 mL of 50 mM H_2SO_4 and 0.1 mL of 100 X TE buffer (Sigma-Aldrich, St. Louis, Missouri). The filtrate was then concentrated to 450 µL volume by centrifuging the samples in a Centriprep Concentrator (YM-30, Millipore) at 1,500 x g for 15, 10, and five minutes. Total RNA was extracted from the 450 µL filtrate using TRI Reagent® (Molecular Research Center Inc., Cincinnati, Ohio) and the RNA pellet was dissolved in 40 µL of TE buffer (pH 8.0).

Quantitation of HAV by SYBR Green Real-Time RT-PCR

Procedures for cDNA synthesis and SYBR Green real-time RT-PCR were performed as described by Brooks, Gersberg, and Dhar (2005), except a BioRad iCycler real-time thermocycler was used instead of the Applied Biosystems GeneAmp 5700 Sequence Detection System for real-time RT-PCR. The cDNA was synthesized using random hexamer primers and the GeneAmp® Gold RNA PCR Core kit (Applied Biosystems, Foster City, California) in a total reaction volume of 40 µL. The cDNA samples were diluted 1:10 and 1:100 with DNase, RNase-free water containing sonicated herring sperm DNA (5 nanograms [ng]/mL) as carrier DNA (Leutenegger et al. 1999). The SYBR Green real-time RT-PCR amplification was carried out in a 25 µL reaction volume that contained 7.1 µL of 2X iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, California), 300 nanomoles (nM) each of the forward and reverse primers (Table 1), and 1 µL of undiluted stock or diluted cDNA. Each sample had three replicates to ensure the reproducibility of results. The thermal profile for SYBR Green real-time RT-PCR was 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 1 minute.

Quantitation of Enterovirus by Molecular Beacon Real-Time RT-PCR

The enterovirus real-time RT-PCR was accomplished using a One Step RT-PCR Kit (Qiagen, Valencia, California) and an MGB Alert™ Enterovirus Real-Time PCR Kit (Nanogen, San Diego, California). The enterovirus kit contained a 20X primer mix as well as a 20X MGB Eclipse Probe (Table 1) directed toward the 5' untranscribed region (UTR) of enteroviruses (coxsackie A and B, echoviruses, polioviruses, and

enteroviruses 68-71). The RNA samples were diluted 1:10 and 1:100 with DNase, RNase-free water containing sonicated herring sperm DNA (5 ng/mL) as carrier DNA (Leutenegger et al. 1999). Each 50 µL reaction mixture contained 17 µL of RNAse-free water, 10 µL of 5X Buffer, 10 µL of 5X Q-Solution, 2.0 µL of dNTP Mix, 2.0 µL of Enzyme Mix (all components of the Qiagen kit), 2.5 µl of 20X forward/reverse primer mix (Table 1), 2.5 µL of 20X MGB Eclipse Probe (Table 1), and 4.0 µL of undiluted or diluted template RNA.

Samples were run in duplicate on a BioRad iCycler real-time PCR system. The real-time PCR conditions were as follows: reverse transcription for 30 minutes at 50ºC, polymerase activation for 15 minutes at 95ºC, 50 cycles of denaturation for 10 seconds at 95ºC followed by annealing/detection for 30 seconds at 56ºC and extension for 30 seconds at 76ºC, and a final extension step for 10 minutes at 76ºC.

Cloning and Sequencing of HAV and Enterovirus cDNA
Samples found positive for HAV and enterovirus by real-time RT-PCR were taken for cloning and sequencing. A 247 HAV cDNA was amplified by conventional RT-PCR following a published protocol (Brooks, Gersberg, and Dhar 2005). The primers for HAV amplification are given in Table 1. Amplified cDNAs were separated by electrophoresis in a 2% agarose gel and eluted from the gel using a Qiagen QIAQuick Gel Extraction Kit (Qiagen, Inc., Valencia, California). In order to clone the enterovirus cDNA, real-time RT-PCR amplicons of enteroviruses were run in a 2% agarose gel, and gel-purified using a Qiagen QIAQuick Gel Extraction Kit. The enterovirus and HAV gel-purified cDNAs were cloned into a TOPO cloning vector (Invitrogen, Carlsbad, California). Plasmid DNA was isolated from recombinant clones and three to five clones were sequenced for each sample using the vector-derived T7 primer.

Sequence Alignment and Phylogenetic analysis
Nucleotide sequences of HAV and enterovirus clones were BLAST searched and identified based on similarity to GenBank database entries. Multiple alignments and phylogenetic analyses were performed using MEGA version 3.0 by Kumar, Tamura, and Nei (2004). Kimura's two-parameter distance was calculated using transitions and transversions and a neighbor-joining tree was built. The confidence of reconstructed clusters was tested by bootstrapping with 1000 replicates.

Generation of HAV and Enterovirus Standard Curves by Real-Time RT-PCR
An HAV standard curve was generated using plasmid DNA ($8x10^7$ copies/µL) of a recombinant clone containing a 247 bp cDNA insert derived from HAV strain HM-175 (VR-2089; ATCC, Manassas, Virginia). Serial dilutions (from 8 to $8x10^4$ copies/ µL) were prepared in TE buffer (Sigma-Aldrich). Plasmid containing enterovirus cDNA ($1x10^7$ copies/µL) was obtained from Nanogen and a dilution series (10^1 to 10^4 copies/µL) was prepared in TE buffer. Real-time RT-PCR was performed in triplicate for each dilution of HAV and enterovirus plasmids. Standard curves were created by plotting the log of the number of HAV and enterovirus genome copies versus their corresponding cycle threshold (C_T) values and creating a best-fit line through these points (Figure 2). The C_T is defined as the PCR cycle at which an increase in the fluorescence above the baseline

signal is first detected. The C_T value is inversely related to the genome copy number. Using the standard curves, HAV and enterovirus levels in the Tijuana River mouth and Imperial Beach pier samples (Figures 3 and 4) were calculated with the following equations. Concentrations were calculated assuming 100% efficiency of cDNA synthesis from viral RNA, similar to other published work (Deffernez et al. 2004; Mohamed et al. 2004; Haramoto et al. 2005).

HAV genome copies per Liter =

$$\frac{\left(\left(1\times10^{\left[(CT-36.8)/-3.6\right]}\right)(\text{dilution factor})(40)(40/19.5)\right)}{\text{Liters of seawater}} \quad (1)$$

Enterovirus genome copies per Liter =

$$\frac{\left(\left(1\times10^{\left[(CT-38.2)/-3.4\right]}\right)(\text{dilution factor})(10)\right)}{\text{Liters of seawater}} \quad (2)$$

Calculation of HAV and enterovirus recovery efficiencies

Two one-liter seawater samples were seeded with known titers of virus prior to filtration: one with poliovirus 2 (VR-301, W-2 strain; ATCC, Manassas, Virginia) and the other with HAV (VR-2089, Strain HM 175, clone 1; ATCC) (Table 2). The same amount of each virus was also spiked directly into a paired concentrated seawater sample following filtration, but before RNA extraction. Real-time PCR was performed and copy numbers were determined using the standard curves (Table 2). The recovery assay was performed twice for each virus and the HAV and enterovirus recoveries were calculated by dividing the number of virus genome copies in the filtered samples by the number of copies in the unfiltered samples (Table 2).

Detection of E. coli and enterococcus bacteria

To determine *E. coli* and enterococcus fecal bacterial levels (Figures 3 and 4), 100 mL water samples were collected and processed within two hours of collection. Up to three 10-fold serial dilutions of each water sample were applied to the Colilert 18® and Enterolert® test methods (IDEXX Laboratories, Westbrook, Maine) according to the manufacturer's instructions. The detection limit for this method was 10 most-probable number (MPN)/100 mL for both bacteria.

RESEARCH FINDINGS

Recovery of Spiked HAV and Poliovirus from Seawater

In order to determine the efficiency of the virus extraction and concentration protocol, seawater samples were seeded with known amounts of HAV or poliovirus on two occasions, and virus levels were quantified using the real-time standard curves (Figure 2). The mean percent recovery was 12% for HAV and 71% for poliovirus (Table 2).

Quantitation of HAV and Enterovirus Levels in Seawater Samples
Real-time RT-PCR was performed to detect and quantitate levels of HAV and enterovirus in 20 samples from the Tijuana River mouth and IB pier. Samples that were positive for either virus using real-time RT-PCR were further confirmed by sequencing, and were then quantitated using the standard curves (Figures 2, 3, and 4). Concentrations were calculated assuming that no viral genomes were lost during the synthesis of cDNA (Deffernez et al. 2004; Mohamed et al. 2004; Haramoto et al. 2005). Some samples had to be diluted to 1:10 or 1:100 in order to get successful amplification. For each sample, the value from the dilution that exhibited the highest number of genome copies (i.e. showed the least inhibition) was used in Figures 3 and 4.

HAV was successfully detected in six of seven Tijuana River mouth samples and in five of seven wet weather IB pier samples at levels (uncorrected for recovery efficiency) ranging from 59 to 16,960 genome copies/L (Figures 3 and 4). Enterovirus was detected in all seven river mouth samples and in six of seven wet weather pier samples at levels (uncorrected for recovery efficiency) ranging from two to 2,254 genome copies/L (Figures 3 and 4). Neither virus was ever detected in any of the six samples taken at the IB pier during dry weather (Figure 3).

The lowest viral concentrations detected in the seawater samples, via real-time RT-PCR and confirmed by sequencing, were 3.3 and 1.4 genome copies per PCR reaction for HAV and enterovirus, respectively (Figures 3 and 4). These values corresponded to minimal detection limits of 6.8 genomes/L for HAV and 1.8 genomes/L for enterovirus (Figures 3 and 4).

Sequencing of HAV and Enterovirus cDNA from Seawater Samples
Successful HAV amplification was obtained for four of six samples collected during the 2003-2004 rainy season (Figures 3 and 4). Multiple alignments of the HAV sequences (Figure 5) showed 100% similarity to the VP1-VP3 genes of three strains (accession numbers AY441441, AY441442, and AY441443), previously isolated from the same region. A BLAST search showed that these sequences were significantly similar to other entries in the GenBank database (~93% with the wild type isolate, accession number M14707; ~94% with isolate MBB, accession number M20273; and ~97% with isolate M2, accession number AY974170). A total of four nucleotide changes were found at positions 69, 77, 128, and 134 among the HAV samples amplified from the ocean water (Figure 5). Out of four nucleotide changes, the mutation at position 69 resulted in an amino acid change from alanine to threonine. The remaining three nucleotide changes did not alter the amino acid sequence (Figure 5).

Successful enterovirus amplification was obtained for five of six samples collected during the 2003-2004 wet weather season (Figures 3 and 4). Three to five clones were sequenced for each sample for a total of 20 clones. A BLAST search using the 151 nucleotide sequence showed that all the clones had a similarity to the 5'-untranslated region (UTR) of enteroviruses in the database entries. Nine enterovirus types were identified among the clones sequenced (Table 3). A neighbor-joining tree constructed from an alignment of the 151 base nucleotide sequence of the 5'-UTR revealed two

major clusters (Figure 6). The larger clade contained echoviruses 6, 11, and 30, as well as coxsackievirus A5 and enterovirus B. The smaller clade included polioviruses 1, 2, and 3, as well as enterovirus 90. The most prevalent enterovirus was echovirus 30, which was isolated from three out of the six samples. Poliovirus 2 was detected in two samples and the remaining types were each detected in one sample (Figure 6).

Relationship Between Bacterial and Viral Indicators in Seawater Samples
Escherichia coli (E. coli) was detected in 13 of 14 wet weather samples and four of six dry weather samples with levels ranging from 41 to 601,000 MPN/100 mL and 10 to 20 MPN/100 mL, respectively (Figure 3 and 4). Enterococcus was detected in 12 of 14 wet weather samples and one of six dry weather samples with values ranging from 72 to 754,000 MPN/100 mL and 10 MPN/100 mL, respectively (Figures 3 and 4). There was a statistically significant correlation between *E. coli* densities and both HAV (R^2=0.66, $p<0.0001$) and enterovirus (R^2=0.49, $p<0.0001$) levels (Figure 7 and Table 4). There was also a significant association between enterococcus and both HAV (R^2=0.59, $p<0.0001$) and enterovirus (R^2=0.43, $p=0.0003$) levels (Figure 7 and Table 4). Finally, there was a significant relationship (R^2=0.41, $p=0.0004$) between levels of HAV and enterovirus. For the correlation analysis, non-detectable levels of virus and bacteria were assigned the value of one-half of the limit of detection.

Levels of Virus and Bacterial Indicators at Beaches in Mexico
Table 5 show the enterovirus levels detected at the Mexican beaches. It is important to note that at two of the beaches, Playas Municipal in Ensenada and Playas Tijuana, both HAV and enteroviruses were detected even when the indicator level was not elevated. Highest levels of enterovirus (715 genome copies/L) were found at the beach at San Antonio, while high levels of HAV were found at both Playas de Ensenada and Playas de Tijuana (2951 and 1836 genome copies/L, respectively).

CONCLUSIONS

Primary recreational water contact (wading, swimming, surfing, snorkeling, and scuba diving) represents the greatest public health threat when waters are contaminated with sewage (Fujioka 1997). However, non-point source pollution rather than sewage is the source of most coastal water pollution (Novotny 1988), where the concentrations of fecal indicators are no longer reliable predictors of health risk (Calderon, Mood, and Dufour 1991). Contaminated Tijuana River water that flows into the ocean at the U.S.-Mexican border is a result of both point (wastewater treatment plant effluent) and non-point (storm water runoff from urban and rural areas) sources of pollution (Cross, Schiff, and Schafer 1992). Therefore, the recreational waters from the Tijuana River mouth to the Imperial Beach pier represent a very unique environment for the investigation of the relationship between fecal indicators and viral contamination. Similarly, sources of indicators are generally never known in coastal waters (Fujioka 1997; Hardina and Fujioka 1991) making this investigation applicable to marine waters in general. There is an urgent need for more accurate monitoring of recreational water quality in order to protect public health.

The aim of this study was to develop a quantitative assay for measuring the HAV load in ocean water samples. Viruses are often present in very low concentrations in water samples, and therefore require an extremely sensitive method such as polymerase chain reaction for monitoring. Real-time PCR is one of the recent developments in PCR technique. The method is capable of detecting up to a single copy of the target gene and provides quantitative data of the target amplicon. In addition, the method is rapid, highly robust, and amenable to automation. All these features of real-time PCR make it an ideal tool for the present study (Dhar, Roux, and Klimpel 2002).

The goal of the present study was to quantify levels of HAV genomes and other indicators of fecal pollution in coastal ocean waters in the vicinity of the U.S.-Mexican border. Relatively little is known regarding the levels of human enteric viruses in southern California coastal waters near the U.S.-Mexican border. In one study, among samples collected from 12 beach locations from Malibu, California to the border of Mexico, 33% (four of 12) of marine samples were positive for adenoviruses (Jiang, Noble, and Chu 2001). Most-probable-number concentration estimates indicated that there were 880 to 7,500 adenoviruses per liter of water. These marine sites were located outside of river discharge points, and the authors noted that bacteria indicators did not correlate with the presence of viruses. Using real-time PCR, Brooks, Gersberg, and Dhar (2005) detected HAV in all eight samples taken during rain events from either the mouth of the Tijuana River (near the U.S.-Mexican border) or the nearby surf zone at Imperial Beach, California, at levels ranging from 90 to 3,523 copies/L and 347 to 2,656 copies/L, respectively. These relatively high levels of HAV measured during wet weather were attributed to the inadequate sewage collection infrastructure in the region of Tijuana, Mexico.

The main conclusions of this study are presented below.

In the present study, 86% and 100% of wet weather samples collected from the surf zone adjacent to the Tijuana River mouth were positive for HAV and enterovirus respectively. Concentrations of HAV in these samples ranged from 213 to 16,960 genomes/L (geometric mean = 3.1) and values of enterovirus ranged from two to 2,254 genomes/L (geometric mean = 1.8) (Figure 4). Due to the close proximity of this sampling site to the Tijuana River, these levels were anticipated to be higher than levels at the Imperial Beach pier. Levels of HAV and enterovirus were significantly higher ($p=0.05$) for the Tijuana River mouth than those samples collected at the same time from the IB pier (Figures 3 and 4).

HAV and enterovirus were detected in 71% and 86% of wet weather IB pier samples, respectively. HAV concentrations in these samples ranged from 59 to 1,955 genomes/L (geometric mean = 2.0) and enterovirus levels ranged from two to 194 genomes/L (geometric mean = 0.9) (Figure 3). Concentrations of HAV and enterovirus were below the limit of detection for all six samples collected at Imperial Beach during the dry weather season (Figure 3). All of the dry weather samples contained fewer than 6.8 genomes per liter of HAV and 1.8 genomes per liter of enterovirus (Figure 3).

HAV cDNA obtained from the four positive 2003-2004 wet weather samples was cloned and sequenced and a BLAST search identified three highly similar HAV strains (Figure 5). All three strains were at least 98% identical. Ticehurst, Cohen, and Purcell (1988) reported that different human HAV strains of diverse geographic origin were remarkably closely related. The isolates in this study were significantly similar to isolates from southern Italy (~99% with isolate IT-DAL-00, accession number AJ505803), Argentina (~97% with isolate Arg873, accession number AF452067), and Japan (~97% with isolate FH3, accession number AB020569) (Figure 5). Unlike this study in which the virus types were fairly evenly distributed among the samples, a previous study on the Venice Lagoon (Rose et al. 2006) found that a single strain of HAV (accession number AY441443) was present in a majority of the samples.

Nine different enterovirus types were isolated from the five positive 2003-2004 wet weather samples (Table 3). A neighbor-joining tree grouped the enterovirus isolates into two major clades (Figure 6). One clade contained echovirus 6, 11, and 30, coxsackievirus A5, and enterovirus B whereas the second clade contained poliovirus 1, 2, and 3 and enterovirus 90. This is in general agreement with previously published enterovirus phylogeny (Muir et al. 1998). While the enterovirus types in this study were relatively evenly distributed (Figure 6) among the samples, a previous study on the Venice Lagoon (Rose et al. 2006) found that a single virus, poliovirus 2, was present in a majority of the samples. Likewise, Donaldson, Griffin, and Paul (2002) found that coxsackievirus A9 was the dominant enterovirus type. Since the researchers found poliovirus 2 (the same type of enterovirus as the positive control), it could be argued that this resulted from a contamination event in the laboratory. However, this is unlikely because the negative controls run in parallel with positive samples were consistently negative by both PCR and sequencing.

Twelve of fourteen wet weather samples (86%) exceeded the California state water quality standard for one or both of the bacterial indicators, *E. coli* and enterococcus (Figures 3 and 4). In contrast, there were no bacterial exceedances for any of the dry weather samples (Figure 3). Regression analyses of the viral densities (as measured by real-time RT-PCR) and indicator levels showed a significant correlation between the densities of both bacterial indicators and levels of HAV (R^2>0.59, p≤0.0001) and enterovirus (R^2>0.43, p≤0.0003) (Figure 7 and Table 4). These results suggest that bacterial indicator levels may be predictive of the levels of viruses at the Tijuana River mouth and Imperial Beach pier.

Levels of both HAV and enterovirus were detected at two beaches in Mexico, Playas Municipal in Ensenada and Playas Tijuana, even when the indicator level was not elevated. The highest levels of enterovirus (715 genome copies/L) were found at the beach at San Antonio, while high levels of HAV were found at both Playas de Ensenada and Playas de Tijuana (2951 and 1836 genome copies/L, respectively).

A multiple regression analysis showed that HAV levels were directly (positively) related (p<0.05) to levels of the fecal indicator bacteria, *E. coli* and enterococcus, enterovirus levels, river flow rate, and precipitation, and inversely related to water temperature

(Table 4). Levels of enterovirus, on the other hand, were only significantly associated with levels of *E. coli* and enterococcus, HAV levels, and water temperature (Table 4). There was no correlation between water tide levels and levels of either virus. Collectively, these variables were able to predict HAV levels in a sample 33% to 66% of the time ($p<0.002$), and enterovirus levels 32% to 49% of the time ($p<0.003$) (Table 4).

Although the association (concordance percentage) with viral levels was slightly higher for *E. coli* than enterococcus, these differences were not significant ($p>0.05$), suggesting that both bacterial indicators were similarly able to predict levels of virus. A series of large-scale epidemiological studies carried out by the United States Environmental Protection Agency (EPA) (1986), found that among the indicator organisms, only two, *E. coli* ($r=0.51$) and enterococci ($r=0.81$), exhibited a strong correlation to swimming-associated gastroenteritis. Based on these findings, the EPA's (2002) draft of the *Implementation Guidance for Ambient Water Quality Criteria for Bacteria* recommended criteria for marine waters solely based on enterococci. The results from this study suggest that *E. coli* might also be a suitable indicator of viral contamination in sewage-contaminated marine waters.

RECOMMENDATIONS FOR FURTHER RESEARCH

Further investigation is necessary to extend real-time RT-PCR methods to detection and quantification of a range of viruses in ocean water. The sensitivity, quantitative ability, and high throughput utility offered by real-time RT-PCR to monitor recreational waters is unparalleled by current methods. Further improvements need to be made to increase viral recovery from water samples and reduce inhibitor recovery during the viral concentration process. Viral concentration methods should also aim to decrease the number of steps in order to decrease viral loss and increase time efficiency. Lastly, further epidemiological studies should be performed to address the human health risk associated with recreational coastal waters that receive urban runoff. Information gained from such epidemiological studies is necessary for an accurate assessment of the health risk associated with this type of recreational water contamination.

RESEARCH BENEFITS

This research resulted in the first-ever quantification of levels of the specific pathogens HAV and enteroviruses in ocean waters at the U.S.-Mexican border. The results show a good correlation between the conventional fecal indicator bacteria and these viral pathogens, and **strongly support the health authorities' use of the fecal indicators to gauge the human health risk for bathing in contaminated ocean waters such as Imperial Beach, California.**

Publications that have been published or submitted as a result of this project include:

Brooks, H.A., Gersberg, R.M. and A.K. Dhar. 2005. Quantification of hepatitis A virus in seawater via real-time RT-PCR. J.Virol. Methods 127 (2) : 109-118.

Gersberg, R.M., Rose, M., Robles, R. and A.K. Dhar. In Press. Hepatitis A virus and enteroviruses in coastal marine waters near the U.S.-Mexico border and correlation with fecal-indicator Bacteria. Appl. Environ. Microbiol.

This project contributed significantly to the education and training of a number of graduate students including those who co-authored the publications referenced above.

ACKNOWLEDGEMENTS
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APPENDIX

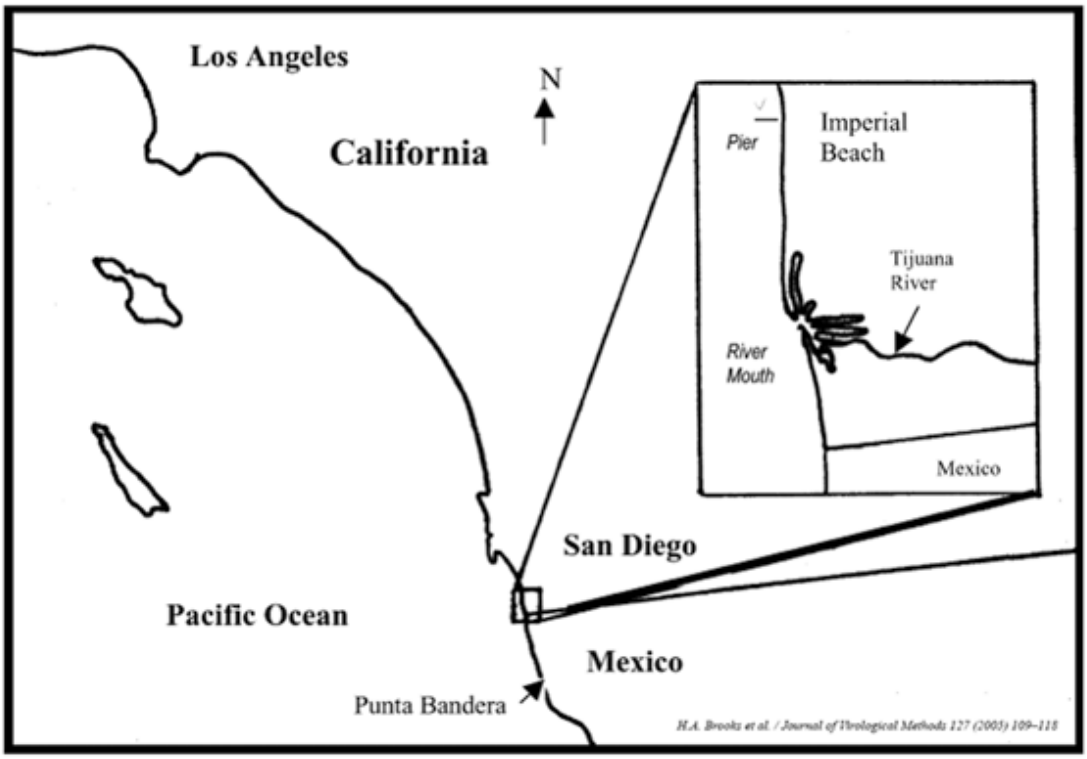

Figure 1. Sample collection sites

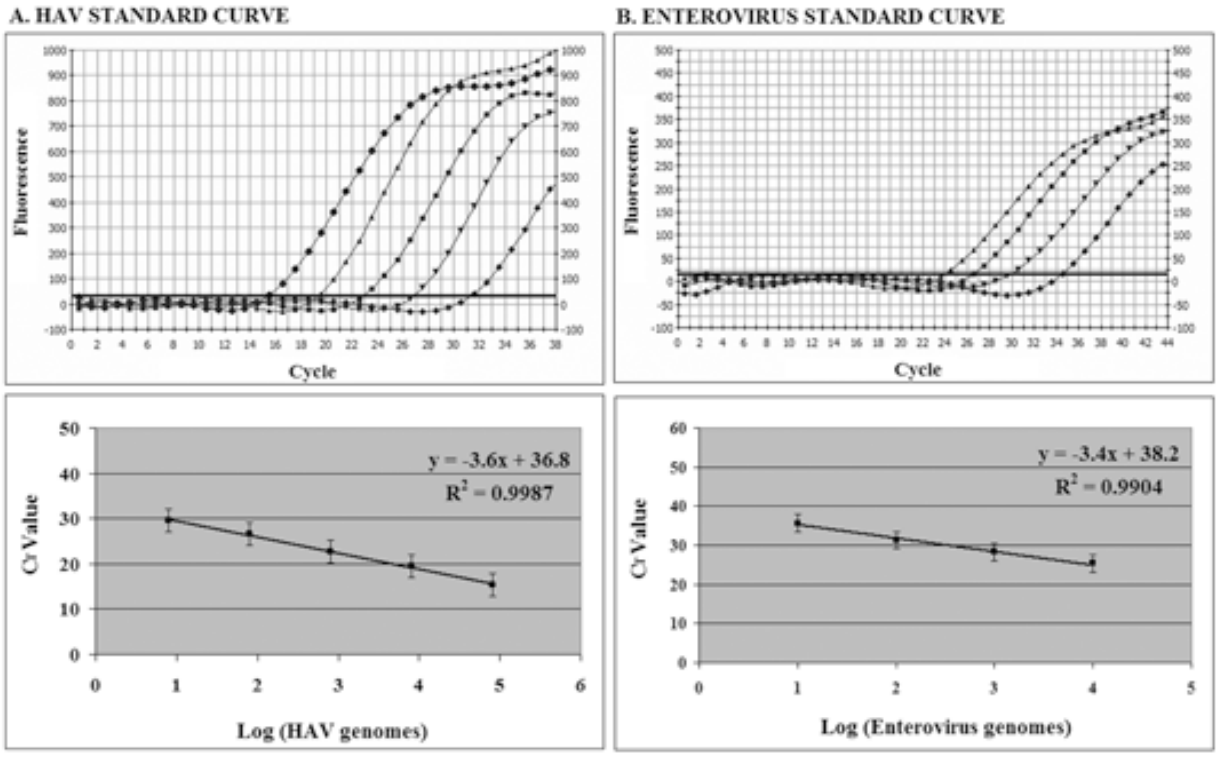

Figure 2. HAV and enterovirus standard curves, as well as the log of the number of HAV and enterovirus genome copies versus their corresponding cycle threshold (C_T) values and lines of best fit

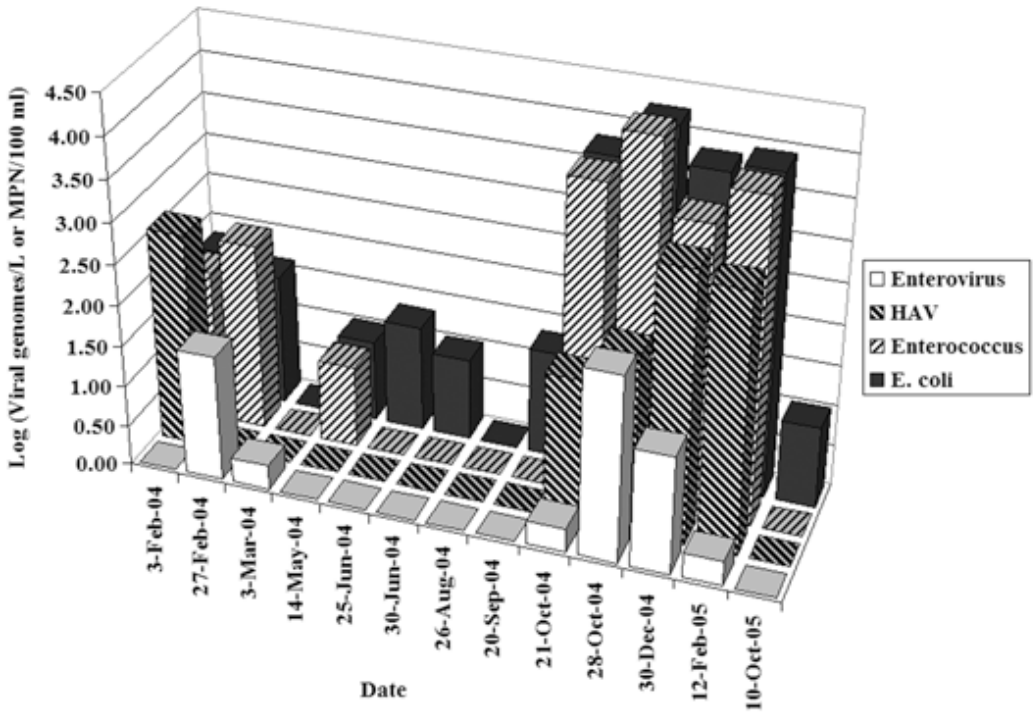

Figure 3. Enterovirus, HAV, enterococcus, and *E. coli* levels in Imperial Beach pier samples, February 2004 through October 2005

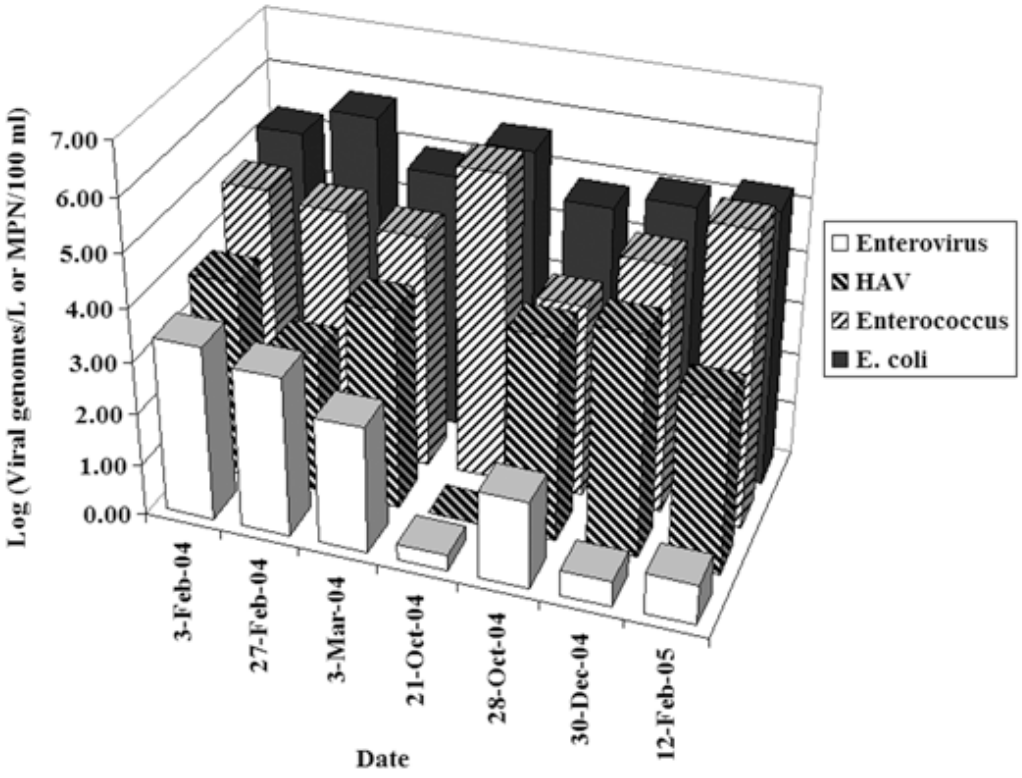

Figure 4. Enterovirus, HAV, enterococcus, and *E. coli* levels in Tijuana River mouth samples, February 2004 through February 2005

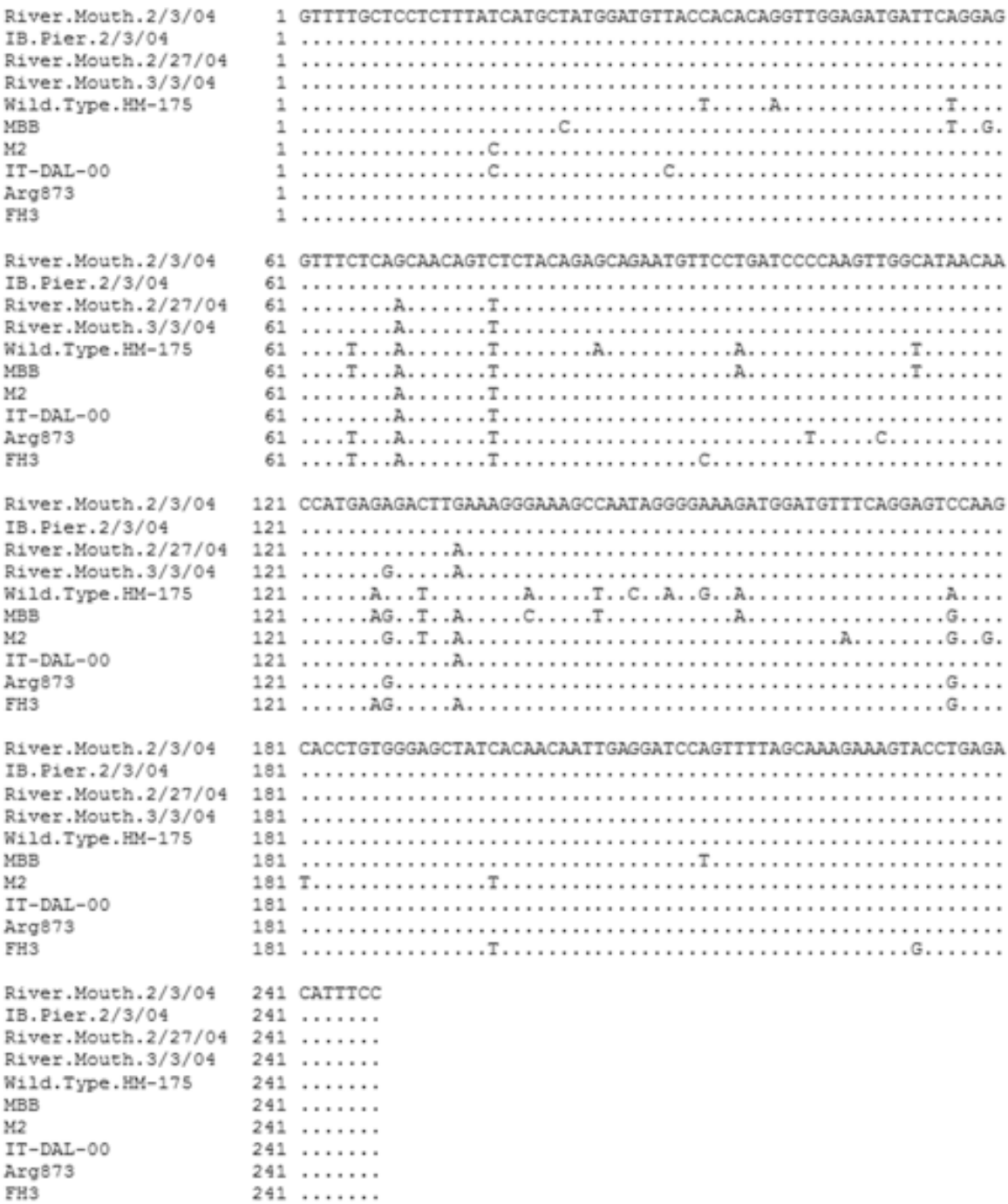

Figure 5. Sequencing of HAV and Enterovirus cDNA from Seawater Samples

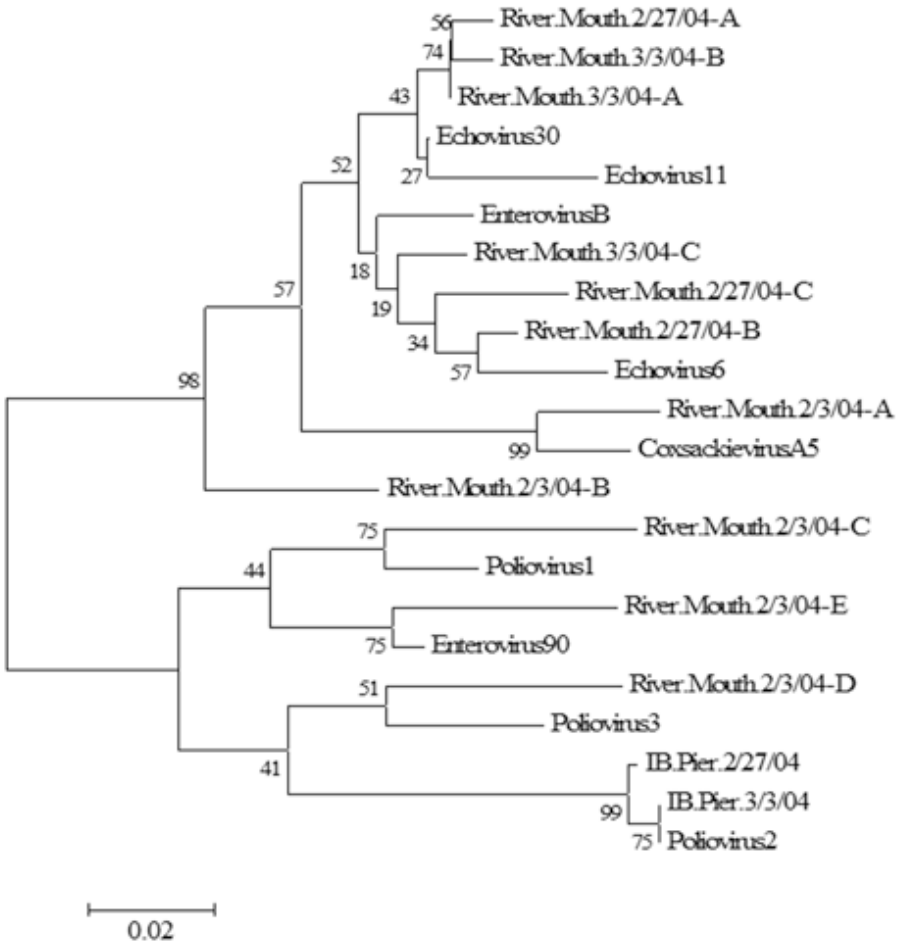

Figure 6. Enterovirus phylogeny

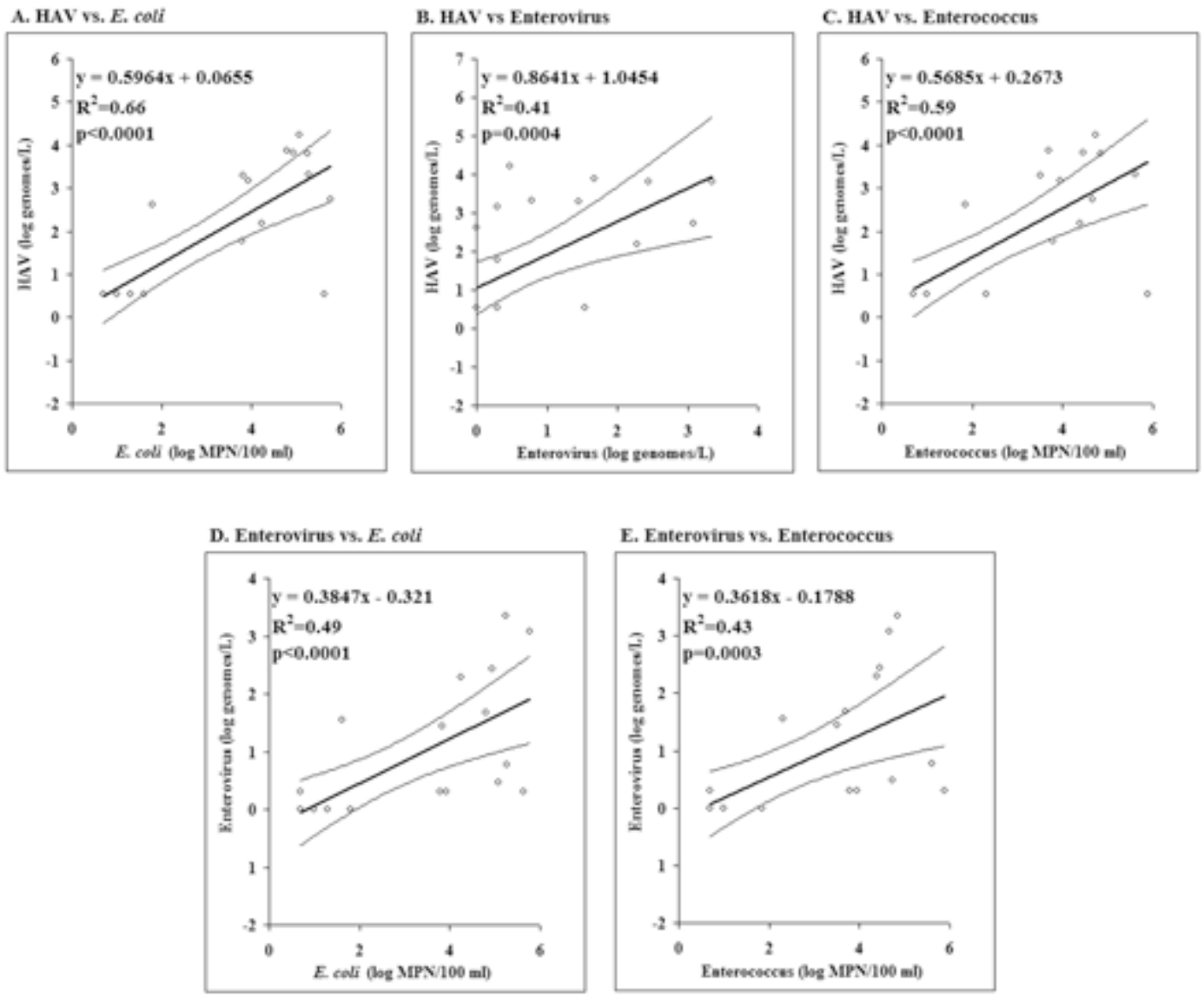

Figure 7. Correlation analyses for statistical significance

Table 1

RT-PCR	Primer name	Primer sequence (5'–3')	(%) GC	Amplicon size (bp)	Reference
Conventional					
HAV	HEPA1	Forward: GTT TTG CTC CTC TTT ATC ATG CTA TG	39	247	Brooks et al. (2005)
	HEPA2	Reverse: GGA AAT GTC TCA GGT ACT TTC TTT G	40		
Real-time					
HAV	HAV1FWD	Forward: TAC AGA GCA GAA TGT TCC TGA TCC	46	76	Brooks et al. (2005)
	HAV3RVS	Reverse: TCC CCT ATT GGC TTT CCC TT	50		
Enterovirus	EV1FWD	Forward: GGC CCC TGA ATG CGG CTA AT	40	151	MGB Alert™ Real-Time PCR Kit (Nanogen)
	EV1RVS	Reverse: CAA TTG TCA CCA TAA GCA GCC A	55		
	Probe	MGB-EDQ-CTT TGG GTG TCC GTG T-Q14-FAM*	44		

* MGB=Minor Groove Binder, EDQ=Eclipse Dark Quencher, FAM=6-Carboxy Fluorescein

Table 2

Sample	Spiked Virus (Genomes)	Recovered Virus (Genomes)	Mean Recovery
Seeded HAV	12,568-32,271	1,760-3,240	12%
Seeded Poliovirus 2	591-775	451-516	71%

Table 3

Viral Type	Designation	Genbank accession number
Human poliovirus 1 isolate CHN-Jiangxi/89-1, complete genome	Poliovirus1	AF111984
Human poliovirus 2 genomic RNA, complete sequence	Poliovirus2	D00625
Poliovirus type 3 20800/Turkey/81 5' untranslated region	Poliovirus3	L76411
Human echovirus 6 patient BE02-4084 5' UTR	Echovirus6	AY343049
Human echovirus 11 strain Pz/87 5' UTR	Echovirus11	AF447476
Human echovirus 30 patient BE02-3831 5' UTR	Echovirus30.	AY343042
Human coxsackievirus A5 gene, isolate:P-2201/CA5/Kanagawa/2003	CoxsackievirusA5	AB126201
Human enterovirus 90 isolate F950027, complete genome	Enterovirus90	AY773285
Human enterovirus B strain EV30_18733_02 5' untranslated region	EnterovirusB	AY271469

Table 4

	Hepatitis A Virus (HAV)		Enterovirus	
	Concordance (%)	P-Value	Concordance (%)	P-Value
E. coli	65.8	<0.001*	49.4	<0.001*
Enterococcus	59.0	<0.001*	43.1	<0.001*
HAV	n/a	n/a	41.0	<0.001*
Enterovirus	41.0	<0.001*	n/a	n/a
Water Temperature[a]	48.9	<0.001*	31.8	0.003*
River Flow[b]	40.5	0.001*	4.97	0.274
Precipitation[c]	32.5	0.002*	10.4	0.108
Water Level (Tide)[d]	3.97	0.329	7.23	0.184

[a] Average temperature 24 hours preceding sampling was used.

[b] Average river flow rate on sampling days was used.

[c] Total rainfall 24 hours preceding sampling was used.

[d] Water level at time of sampling was used.

* An asterisk Indicates the value is significant ($p < 0.05$).

Table 5. Levels of hepatitis A virus (HAV) and enteroviruses at selected beaches in Mexico

Sample ID	Location	Date	Rain (inches)	Sample Size (L)	Bacterial Count (MPN/100 ml)	Bacterial threshold exceeded ‡	Mean Enterovirus Concentration (Copies/L)	Mean HAV concentration (Copies/L)
Mex1	Playas Tijuana	2/11/2005	1.64	1.0	N/A (E. Coli) N/A (enterococcus)	N/A N/A	0.08	1836
Mex2	Playas Tijuana	2/24/2005	1.31	1.0	65000 (E. Coli) 1000 (enterococcus)	Yes Yes	0.97	ND
Mex3	Playas Ensenada	6/29/2005	0.00	3.5	17329 (TC) 72 (E.Coli) <10 (enterococcus)	Yes No No	0.01	2951
Mex4	Playas Tijuana	6/29/2005	0.00	3.8	146 (TC) 10 (E.Coli) <10 (enterococcus)	No No No	0.83	43
Mex5	San Antonio	6/29/2005	0.00	0.4	1299700 (TC) 214200 (E.Coli) 7400 (enterococcus)	Yes Yes Yes	715	ND
Mex6	Playas Ensenada	8/9/2005	0.00	1.4	<10 (TC) <10 (E.Coli) <10 (enterococcus)	No No No	ND	363
Mex7	Playas Tijuana	8/9/2005	0.00	3.0	121 (TC) 52 (E.Coli) <10 (enterococcus)	No No No	ND	ND