A HUMAN HEALTH RISK ASSESSMENT FOR ENTEROVIRUSES AND HEPATITIS A IN RUNOFF FROM THE TIJUANA RIVER AND IN BATHING WATERS OF NEARBY IMPERIAL BEACH

PROJECT NUMBER: W-02-03

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NARRATIVE SUMMARY

Primary recreational water contact (wading, swimming, surfing, snorkeling, 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, et al. 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 sources of pollution (storm water runoff from urban and rural areas) (Cross, et al. 1992). Therefore, the recreational waters from the Tijuana River mouth to the Imperial Beach pier represent a 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 to protect public health.

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 the 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 research objective was to develop a real-time reverse transcriptase (RT)-PCR method for HAV detection in ocean water. This was successfully accomplished by cloning HAV into a plasmid vector and designing/optimizing primers based on its sequence. HAV cDNA amplified by RT-PCR using RNA from an ocean water sample contaminated with Mexican sewage was sequenced, identity confirmed, and cloned into a plasmid vector. Then four

primer sets were designed based on this sequenced HAV plasmid DNA. The primer sets were tested for amplification efficiency using the plasmid DNA as template, and the best primer set was chosen for subsequent work. This HAV plasmid DNA was also used to generate standard curves and to serve as a positive control in sample assays.

The second objective was to compare the sensitivity of HAV detection by conventional RT-PCR to the sensitivity of HAV detection by real-time RT-PCR. The limit of detection for conventional RT-PCR was determined to be 24 copies of HAV and for real-time RT-PCR the limit of detection was found to be one copy of HAV. Therefore, the data indicate that real-time RT-PCR is eight-fold more sensitive than conventional RT-PCR when plasmid DNA was used as a template.

There appeared to be a sufficient HAV load in the ocean waters surrounding the Tijuana River mouth and Imperial Beach pier following rain events to fall within the sensitivity range of conventional RT-PCR in most of the samples. However, the two samples that were negative by conventional RT-PCR either had lower HAV loads or greater concentrations of inhibitors, both of which required the increased sensitivity of real-time RT-PCR to amplify HAV. Amplification of HAV by real-time RT-PCR proved to require dilutions in order to minimize the effect of inhibitors, and therefore required the increased sensitivity to detect HAV at very low concentrations. Therefore, this real-time RT-PCR method has greater utility in determining more accurately the health risk associated with recreational waters.

In order to evaluate the data in terms of a human health risk assessment for swimming or surfing, the PCR-quantified densities must be related to infectivity. For example, it is well known that many more environmental samples are often positive by PCR than by cell culture. While infectivity data is not available for HAV, Donaldson, et al. (2002) concluded for enteroviruses, from the side-by-side comparison of cell culture and real-time PCR done by Monpoeho, et al. (2000), that a real-time PCR value of 55 particles in a sample may equate to one infectious particle. Extrapolating this infectivity relationship for HAV, if swimmers ingest an estimated 50 ml of seawater during recreation, the densities of HAV (1,303 copies/L to 21,392 copies/L) the researchers found at Imperial Beach would suggest that between 1.18 and 19.44 “viable” particles would be ingested, leading to a very high probability of infection. Such a conclusion suggests that the posting of Imperial Beach automatically after any rain event is instrumental in reducing risks to swimmers and surfers in this area.

The positive association found between the fecal coliform indicator bacteria concentrations and HAV concentrations in the ocean should be interpreted very cautiously because the observed correlation is based on only a few samples and it remains to be seen whether this association would hold when the sample number is increased. While assessing the human health risk associated with the coastal waters from the Tijuana River mouth to the Imperial Beach pier it should

be noted that the incidence of disease such as HAV in Mexico, specifically Tijuana, is greater than that of the United States and San Diego. Therefore, pathogens such as HAV, which increase in severity with decreasing incidence in a population because of a lack of acquired immunity, present a heightened risk to U.S. bathers in these coastal waters.

In summary, this investigation focused primarily on the development and application of a rapid and sensitive method to concentrate and detect HAV by SYBR Green real-time RT-PCR in environmental ocean water samples. This study was the first to not only detect, but also to quantify, HAV load in the Tijuana River mouth and Imperial Beach pier coastal waters. The method is rapid with respect to current water quality monitoring procedures, with an entire processing time of less than 24 hours. In addition, this method has the potential to offer greater sensitivity and quantitative ability that no single method currently offers. With further optimization of viral concentration procedures, the applicability of this method to high throughput reproducible assays could be developed for routine detection of human pathogens in marine recreational waters.

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INTRODUCTION

Land-based sources of pollution, such as dry and wet weather urban runoff, are responsible for beach contamination and closures throughout the region of Bight of the Californias. 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. For example, in Mexico the level of sanitation is much lower (Ganster 1999) and the incidence of the endemic diarrheal diseases are correspondingly higher than in the United States (Feachem, et al. 1983). 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 underpredict the actual risk of human disease

RESEARCH OBJECTIVES

The major objective of this proposal is to measure the level of selected enteric viruses (including hepatitis A virus) and to determine the ratio of viruses to coliforms in runoff from the Tijuana River as well as the nearshore marine bathing waters of Imperial Beach. Specifically, this project will:

1. Apply PCR methodology for the measurement of hepatitis A virus (HAV) and enterovirus levels at the mouth of the Tijuana River and in the surfzone at Imperial Beach.
2. Measure the conventional bacterial indicator levels (e.g. fecal coliform densities) at the same time as virus levels, and correlate these viral densities with fecal coliforms to better understand the relationship between indicator densities and actual pathogen levels in waters impacted by contaminated runoff from Mexico.

3. Perform a risk assessment for human health effects using the new information on measured viral densities.

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 allow 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 transcriptase (RT)-PCR in river water samples (Morace, Pisani, Divizia, and Pana 1993; Pina, et al. 2001), ground water samples (Abbaszadegan, et al. 1999), urban canal samples (Kittigul, et al. 2000), and sewage samples (Divizia, Ruscio, Degener, and Pana, 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 detection using conventional RT-PCR has also been attempted in ocean water samples with degrees of detection varying between 0% (Gilgen, et al. 1997; Katayama, et al. 2001; Miossec, Le Guyader, Haugarreau, and Pommepuy 2000; Schvoerer, et al. 2000; Tsai, Tran, Sangermano, and Palmer 1994) and 63% (Griffin, et al. 1999). In southern California, researchers failed to detect HAV in surf zone coastal water, however they detected HAV in treated sewage samples while using 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 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, it is less labor intensive, thus allowing for higher throughput as well as extremely accurate and reproducible quantification (Heid, Stevens, Livak, and Williams 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 15 samples tested positive, and a viral concentration of 9.3 viruses per milliliter (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)

Virus Recovery from Seawater

PCR is very sensitive and is especially susceptible to inhibitory substances when used for environmental samples. Humic acids, often found in environmental samples, have been shown to interfere with PCR efficiency (Ijzerman, et al. 1997; Schwab, et al. 1995; Tsai, Palmer, and Sangermano, 1993) as have fulmic acids (Burgener, Candrian, and Gilgen 2003). Many research efforts have been made to reduce the inhibitory effects of environmental inhibitors (Ijzerman, et al. 1997; Jothikumar, et al. 1998; Schwab, et al. 1995, 1996). One such study conducted by Katayama, et al. (2001) was an investigation to determine the best method to concentrate virus from seawater (and freshwater). The following methods of virus concentration were tested in all possible combinations: positive or negatively charged membranes, acid rinse step or not, and beef extract or NaOH as the elution medium. Poliovirus concentration from natural ocean water was shown to be most efficient (up to 73% recovery when 1 liter [L] filtered) when using a negatively charged membrane, with an acid rinse step and an inorganic eluate as compared to a positively charged membrane, no acid rinse step, and beef extract as the elution medium (Katayama, et al. 2001). Due to the high recovery efficiency, this recovery method was chosen for the present study of HAV in ocean water effluent of the Tijuana River.

Water Sampling

Ocean water samples were collected from two beach locations: 200 yards north of the Tijuana River mouth and the south side of the Imperial Beach Pier. A map of these sampling sites is shown in Figure 1. All samples were collected following a rain event, which was defined as precipitation of 0.2 inches (in) or more. This definition is based on the Department of Environmental Health general advisory, which is issued after 0.2 in or more of rainfall, and warns the public of possible water contamination by urban runoff. Samples were collected only after it had rained sufficiently to cause the Tijuana River to significantly increase its flow. In the beginning of the wet season the amount of rain to cause increased flow in the river was greater because the ground was very dry and soaked up much of the water before it could reach the river. Samples were collected within six hours following the peak flow of the Tijuana River so that the increased flow from the river theoretically had enough time to reach the Imperial Beach pier, 0.85 mile (1,500 yards) north of the river mouth. Peak flow was measured by an automatic sampler (ISCO automatic sampler with flow gauge) in the river. Some rain events had greater precipitation in the distant areas of the Tijuana River watershed and very little locally. This type of rain event would still cause the river to rise, although since the water was coming from far away, the peak flow would often

occur up to 24 hours after the rain event. In this case, samples would not be collected because the rain water was passing through a relatively unpopulated area of the Tijuana River watershed and therefore were not representative of the desired sampling for this study. The sampling objective was to collect water that had passed through the local urban areas and that had a greater risk of being contaminated by human waste.

Determining Fecal Coliform Bacterial Density in Water Samples
One hundred milliliters of each sample was taken for analysis of fecal coliforms. Samples were held on ice and processed within three hours of collection. The most probable number method (MPN) was used to enumerate fecal coliforms. Multiple tube fermentation was employed with five replicates of three serial dilutions (American Public Health Association 1992), and was performed by the Graduate School of Public Health at San Diego State University. Total coliform, fecal coliform, and enterococci were also tested for by the County of San Diego Department of Environmental Health, and the results used when testing times corresponded with the sampling times of this study.

Processing of Water Samples for Hepatitis A Virus Detection
Four rain events were sampled for a total of eight samples collected. Each 4 L sample was collected in a two-gallon bucket. All samples were processed within one or two days of collection. Each sample was spiked with 250 microliters (µl) of the Taura syndrome virus (TSV) homogenate. TSV is a member of the picorna-like virus family, and therefore shares physicochemical characteristics with HAV (which is a picornavirus) that will allow both viruses to act similarly through the filtration process. This viral spike will allow for assessment of the viral recovery from the filter method used to concentrate the sample. Following addition of the spike, the sample was stirred to facilitate sediment-TSV binding. Viruses tend to absorb to sediments, and have been estimated to harbor between 10 times and 10,000 times the amount of virus found in water (Metcalf, Melnick, and Estes 1995; Melnick, et al. 1978). Therefore, by stirring the spike into the sample, both the HAV-sediment binding and the HAV filtration recovery were assessed. The sample was then filtered via a vacuum pump system through a type HA 0.45-micrometer (µm) pore size, negatively charged membrane filter (Millipore, Burlington, MA) where the virus was captured. The filter was washed with 200 ml of 0.5 mM H_2SO_4 to rinse out the cations. Subsequently, the virus was eluted from the filter with 10 ml of 1 mM NaOH, into a tube containing 0.1 ml of 50 mM H_2SO_4 and 0.1 ml of 100x TE buffer to neutralize the recovery, reducing the volume of the sample to 10 ml. This sample processing was based on a virus filter/concentration method developed at the University of Tokyo (Katayama, et al. 2001).

Based on the design of the sample collection and filtration process used in this investigation (negatively charged filter and NaOH elution), positive PCR results most likely indicate intact virus particles rather than naked RNA (Abbaszadegan, Stewart, LeChevallier, and Gerba 1998; Katayama, et al. 2001). This instability of

RNA without the protection of a viral protein coat is due to the extreme susceptibility to degradation by RNases, which are abundant in the marine environment (Tsai, et al. 1995).

This eluate was then added to a Centriprep Concentrator (Millipore) and filtered at 1,500 x g for 10 minutes, reducing it further to a volume of approximately 450 µl. In order to reduce filter clogging, water samples that were high in particulate matter were filtered through a series of Whatman filters (of 11µm and 2.5 µm pore size) before applying the method described above.

RNA Extraction
The total volume of filtrate (450 µl) for each sample was transferred to one tube and RNA was extracted using TRI Reagent™ and the manufacturer's protocol (Molecular Research Center Inc., Ohio). The RNA pellet from each sample was dissolved in 20 µl of RNAse, DNAse free water (Invitrogen Corporation, Carlsbad, CA).

Conventional RT-PCR Amplification of HAV and cDNA Synthesis
HAV was amplified by conventional RT-PCR, which included cDNA synthesis, conventional RT-PCR, and gel electrophoresis. The cDNA was synthesized using random hexamer primers and the GeneAmp® Gold RNA PCR Core kit (Applied Biosystems, Foster City, CA). The reaction mix for the cDNA synthesis contained 20 µl of RNA, 1x GeneAmp RT-PCR Buffer, 2 mM of $MgCl_2$, 1.25 mM of deoxynucleoside triphosphates, 1.25 µM of random hexamers, 6.25 mM of dithiothreitol (DTT), 25 U of RNase inhibitor (RNasin), and 62.5 U of reverse transcriptase in a total reaction volume of 40 µl. All cDNA synthesis reactions were carried out at 42ºC for one hour.

Conventional RT-PCR for HAV
The HAV detection was carried out using conventional RT-PCR. The conventional RT-PCR reaction mixture contained 2 µl of cDNA, 1 x PCR buffer II, 2 mM of $MgCl_2$, 0.8 mM of deoxynucleoside triphosphates, 0.8 mM each of forward and reverse primer, and 5 U of AmpliTaq Gold® DNA Polymerase in a reaction volume of 25 µl. The primer sequences are given in Table 1. The thermal profile for conventional RT-PCR was: 10 minutes at 94ºC, following 40 cycles of 94ºC for 30 seconds, 50ºC for 30 seconds, and 72ºC for 1 minute. HAV primers have been designed specifically to target a region of the hepatitis A viral protein coding region, the VP3/VP1 junction. This region is a conserved sequence in the 5' end of the HAV genome. In comparing different strains of HAV to wild-type HAV, as well as to other picornaviruses, sequence conservation was found to be high between HAV strains and low between HAV and other picornaviruses (Cohen, Rosenblum, et al. 1987; Cohen, Ticehurst, et al. 1987; Deardorff 2001). These Hepa 1 and Hepa 2 primers have successfully amplified HAV (Deardorff 2001; Cohen, et al. 1987a; Cohen, et al. 1987b; Jothikumar, et al. 1998). The specificity of the primers were verified (Deardorff 2001).

Gel Electrophoresis
Amplified cDNA products were separated on a 2% agarose gel containing 2.5 µl of 10 mg/ml ethidium bromide. Each gel was electrophoresed for three hours at 80 V in a 1X TBE buffer (Sambrook, Fritsch, and Maniatis 1989). A 50 bp DNA ladder (Promega, Madison, WI) was used to aid in determining product band size. The DNA products were visualized with exposure to UV light using a Fisher Biotech 312 nm Variable Intensity Transilluminator (Fisher Scientific, Pittsburgh, PA). Each gel was photographed using a Photo-Documentation Hood (Fisher Scientific).

Cloning and Sequencing of HAV cDNA
HAV was isolated from an ocean water sample taken in Punta Bandera, Baja California, using the method described above. Total RNA was isolated from this sample and RT-PCR was performed as described above. The amplified cDNA was run on a 2% agarose gel and a cDNA band around the 250 base pair region was cut out. The cDNA was then purified from this band, sequenced, and the sequence analyzed in the NCBI BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST). Sequence analysis confirmed the identity of the band to be Hepatitis A virus. Once confirmed, the HAV cDNA was cloned into a TOPO cloning vector (Invitrogen, CA) following the manufacturer's protocol. The recombinant plasmid was isolated using an alkali lysis method (Sambrook, et al. 1989). The plasmid DNA was then used for generating a standard curve in real-time RT-PCR (see below).

SYBR Green Real-Time RT-PCR for HAV
The primers used for SYBR Green real-time RT-PCR are listed in Table 2. The primers for HAV were designed based on the sequence of the cloned HAV plasmids, using the Primer Express Software version 1.0 (Applied Biosystems). Four primer sets were tested for HAV amplification efficiency (Table 3), and the best primer set was chosen for real-time RT-PCR. The chosen HAV primers, HAV1FWD and HAV3RVS, amplify a region of 76 base pairs within the VP1 and VP3 region.

SYBR Green real-time RT-PCR was conducted using an iCycler iQ™ real-time PCR detection system (Bio-Rad). For real-time RT-PCR, cDNA was synthesized using random hexamers as described above. cDNA samples were diluted 1:10, 1:100, and 1:1000 with DNase, RNase free water containing herring sperm DNA (5 ng/ml) and 5 µl were taken for each amplification reaction. Real-time RT-PCR was carried out in a 25 µl reaction volume that contained 7.1 µl of iQ SYBR® Green Supermix (Bio-Rad), 0.8 mM each of the forward and reverse primer, and 1 µl of stock cDNA/ 5 µl of diluted cDNA. Each sample had three replicates and was repeated twice to ensure reproducibility of results. All reactions were carried out in 96 well plates. The thermal cycle profile for SYBR real-time PCR was 95ºC for 10 minutes, followed by 40 cycles of 95ºC for 10 seconds and 60ºC for 1 minute.

Plasmid Standard
For each SYBR Green real-time RT-PCR assay, a dilution series of HAV plasmid was run to generate a standard curve and serve as a positive control. Plasmid DNA serial dilutions were made in sonicated herring sperm DNA (5 ng/ml). This diluent serves to capture small quantities of sample DNA for PCR more efficiently. The dilution of plasmid DNA ranged from 1.1 x 10^5 copies of HAV plasmid down to a single copy of HAV plasmid.

Data Analysis for SYBR Green Real-Time RT-PCR for HAV
SYBR Green real-time RT-PCR data analyses were performed by the iCylcer iQ™ real-time PCR detection system software (Version 3.0A). In the iCylcer iQ™ detection system, the individual wells are calibrated dynamically (against the fluorescence of SYBR Green) using an internal passive reference fluorophore, fluorescein, which is in the iQ™ SYBR® Green Supermix (Bio-Rad). A sample is considered positive when ΔR_n exceeds the threshold value. The threshold value is the midpoint of ΔR_n and the cycle number plot. The threshold value of all amplifications was chosen to be 0.25. The threshold cycle (C_T) is the cycle at which a statistically significant increase is detected in R_n. This threshold cycle corresponds inversely to copy number. In other words, the higher the copy number in a sample, the lower the threshold cycle.

HAV Viral Recovery Determination and Quantification
TSV served as an internal control in assessing viral recovery during the filtration process. As stated previously, a known amount of TSV was spiked into each sample before filtering. Serial dilutions of cloned TSV plasmid were then used to generate a standard curve from which the final concentration of TSV was extrapolated. The amount of TSV remaining in the sample after filtration was compared to the amount of TSV initially seeded into the sample for determination of percent recovery. The percent recovery of TSV was assumed to be similar for HAV. Quantification of HAV was based on a standard curve generated from serial dilutions of cloned HAV plasmid, the percentage of the sample volume used in real-time PCR, and the dilution factor of the reaction. The C_T value of HAV in the unknown sample was determined and the corresponding log concentration number relative to that sample identified. The log concentration (which represents the concentration of copies) was then multiplied by 2.38 x 10^^{5} (the known number of copies per picogram of plasmid standard) to convert from concentration of copies to copy number. This copy number was then multiplied by 40 (and the dilution factor, if applicable) in order to back calculate from the portion of the sample analyzed to the original sample volume. If a dilution was used for the reaction, the calculation was also divided by five in order to account for a 5 µl volume of sample that was added to the reaction verses the 1 µl volume added for the stock. Finally, it is necessary to divide by the number of liters of ocean water filtered to obtain HAV copies per liter. This can be summarized in the following equation: the copies of HAV in 1 L = 10^ $^{[C_T - 18.088 \div -3.784]}$ (2.38 x 10^^{5} copies) x 40 x dilution factor ÷ # of liters of ocean water filtered

(÷ 5 for all dilutions, with the exception of the stock). This viral load is not adjusted for loss during the filtration process or RNA extraction.

MGB Eclipse Probe Real Time PCR for Enterovirus:
Real-time PCR was accomplished using an MGB Eclipse Probe System Kit (Amersham Biosciences). The MGB Eclipse Probe System mechanism of action involves three stages for each PCR cycle. During the initial phase, MGB Eclipse Probes assume a random coil at denaturing temperatures due to the hydrophobicity of the MGB. Fluorescence from the reporter dye is quenched by the proximity of Eclipse Dark Quencher. During the annealing phase, the MGB folds back into the minor groove of the resulting DNA duplex formed by the probe and target to stabilize hybridization. Additionally, Eclipse Dark Quencher is spatially separated from the reporter, allowing fluorescence. Following annealing, the MGB Eclipse Probe is melted from its target during the primer extension phase, which allows more efficient primer extension (Amersham Biosciences Website).

Samples were run in triplicate on a BioRad iCycler real-time PCR system according to the specifications recommended by the manufacturer of the MGB Eclipse system kit. The total volume of the real-time PCR reaction mixture was 50 µl per well and consisted of the following: 17 µl of RNAse-free water, 10 µl of 5X Qiagen One-Step RT-PCR Buffer, 10 µl of 5X Qiagen Q-Solution, 2.0 µl of dNTP Mix (10 mM of each dNTP), 2.5 µl of 20X Primer Mix, 2.5 µl of 20X MGB Eclipse Probe, 2.0 µl of Qiagen One-Step RT-PCR Enzyme Mix, and 4.0 µl of template RNA. The real-time PCR reaction conditions was as follows: reverse transcription for 30 minutes at 50^{o}C, polymerase activation for 15 minutes at 95^{o}C, and 50 cycles of denaturation for 10 seconds at 95^{o}C followed by annealing/detection for 30 seconds at 56^{o}C and finally extension for 30 seconds at 76^{o}C.

Quantification of enterovirus through real-time PCR was achieved by comparing C_T values, the number of DNA replication cycles required before a logarithmic increase in fluorescence is observed. Therefore, smaller C_T values corresponded to higher enterovirus copy numbers. In this way, C_T values could be plotted versus copy number of virus, and a regression line that best fits the curve then generated, which allowed quantitation of virus levels in any ocean water sample.

RESEARCH FINDINGS
Four sets of primer combinations were evaluated to determine their efficiency in amplifying the HAV amplicon using HAV plasmid as the template. Two dilutions of plasmid DNA (2.38×10^5 and 2.38×10^4 copies) were used for the assay and there were control reactions that contained no template DNA for each primer set. Optimal primer combination is demonstrated by the lowest C_T value and a dissociation curve with a single peak. A summary of results of the four primer combinations is given in Table 3. The amplification profiles and dissociation curves for all primer combinations are shown in Figure 2. Although all primer

combinations (with the exception of HAV2FWD and HAV3RVS) provided comparable C_T values (Table 3), the HAV1FWD and HAV3RVS primer combination (Figure 2a) was the only combination to provide a dissociation curve with a single peak at $T_M = 81°C$ and a no template control with no peak. All of the other three primer combinations had some non-specific amplification that was found even in the control reaction. For example, primers HAV1FWD and HAV4RVS (Figure 2b) had a dissociation curve with a peak at $T_M = 81°C$ for the viral product and two peaks for the no template control at $T_M = 77°C$ and 78°C. This was also seen in the HAV2FWD and HAV4RVS primer combination (Figure 2d) with two no template control dissociation curve peaks at $T_M = 76°C$ and 77°C for the control reaction. HAV2FWD and HAV3RVS (Figure 2c) was the only primer combination that did not provide any dissociation peak expected for the HAV amplicon ($T_M = 81°C$), and instead had peaks at $T_M = 76°C$ and 77°C, with no template control amplification equal to that of the viral product. Based on these findings, primers HAV1FWD and HAV3RVS were selected for subsequent work.

Detection of HAV in Tijuana River Mouth and Imperial Beach Pier Samples by Conventional RT-PCR

HAV was detected in six out of the eight samples (6/8, 75%) by conventional RT-PCR. Four of these positive samples were from the Tijuana River mouth and two were from the Imperial Beach pier (Table 4). A representative figure of the amplification of a 247 bp HAV amplicon by conventional RT-PCR is shown in Figure 3. The TSV spike was detected in five out of the eight samples (5/8, 62.5%).

For the third rain event, the sample from the mouth of the Tijuana River was concentrated using two different procedures, once with Whatman filter pretreatment and once without. The application of Whatman filter pretreatment produced an HAV positive result with conventional RT-PCR, while in the absence of this pretreatment HAV was not detected (Figure 4).

Detection of HAV in Tijuana River Mouth and Imperial Beach Pier Samples by Real-Time RT-PCR

Real-time RT-PCR detected HAV in all four Tijuana River mouth samples (4/4, 100%) and in all four Imperial Beach pier samples (4/4, 100%). Therefore, all samples that had tested positive with conventional RT-PCR also tested positive with real-time RT-PCR. In addition, two samples that had tested negative with conventional RT-PCR, tested positive with real-time RT-PCR (Table 4). It was observed that some stock cDNA samples did not successfully amplify until they were diluted between 1:10 and 1:1000 (Table 5). An amplification profile and corresponding dissociation curve for an HAV positive sample are shown in Figure 5. The TSV spike was detected in seven out of the eight samples run with real-time RT-PCR (7/8, 87.5%). An amplification profile and corresponding dissociation curve for TSV are also displayed in Figure 5.

Comparison of Sensitivity of Conventional RT-PCR Versus SYBR Green Real-Time RT-PCR

The sensitivity of conventional RT-PCR and SYBR Green real-time RT-PCR were compared by using a serial dilution of HAV plasmid DNA as template for amplification. The limit of detection for conventional RT-PCR was determined to be 24 copies of HAV (Figure 6). For SYBR Green real-time RT-PCR the limit of detection was 1 copy of HAV, shown in Figure 7. In this figure, a linear relationship was observed in real-time RT-PCR between the input plasmid DNA and the C_T values with correlation coefficients (r^2) greater than 0.99. The mean C_T values of replicate assays ranged from 19.57 ± 0.28 (1.1×10^5 copies) to 38.31 (1.1 copies) for the HAV standard curve. Therefore, the data indicated that SYBR Green real-time RT-PCR is eight-fold more sensitive than conventional RT-PCR when plasmid DNA was used as template.

Concentration in Ocean Water Samples by SYBR Green Real-Time RT-PCR

The HAV viral concentrations in Tijuana River mouth ocean water samples and Imperial Beach pier ocean water samples were determined by extrapolating the C_T values of the samples from the standard curve and applying the following equation: the amount of virus in 1 L = $10^{\wedge [C_T - 18.088 \div -3.784]}$ ($2.38 \times 10^{\wedge 5}$ copies) x 40 x dilution factor ÷ # of liters of ocean water filtered (÷ 5 for all dilutions, with the exception of the stock)(Figure 7). To more accurately assess the recovery of the virus concentration method and to strengthen the interpretation of negative results, a known amount of TSV was seeded into each sample prior to processing. Both HAV and TSV values were extrapolated from their respective standard curves and then the percent of TSV recovered was established. The percent recovery of TSV was found to be between 5.7 and <1, indicating that TSV is not a good water sample spike and therefore it was not used to assess extraction efficiency. Quantification of HAV load by SYBR Green real-time RT-PCR found 12,104, 1,349, 2,583, and 9,092 copies/L of HAV near the mouth of the Tijuana River and 9,743, 769, 1,298, and 1,311 copies/L of HAV near the Imperial Beach pier for the first, second, third, and fourth rain events, respectively (uncorrected for extraction efficiency) (Table 5).

Quantitation of Enterovirus in Ocean Water Samples by Real-Time RT-PCR

Quantification of enterovirus through real-time PCR was achieved by comparing C_T values, the number of DNA replication cycles required before a logarithmic increase in fluorescence is observed. Therefore, smaller C_T values correspond to higher enterovirus copy numbers and vice versa.

Real-time PCR was performed on a dilution series of known copy number of an enterovirus inserted plasmid provided with the MGB Eclipse kit in order to create a standard curve. C_T values of 36.0, 31.8, 28.2, and 25.8 were obtained from copy numbers of 10, 100, 1000, and 10,000 enterovirus copies, respectively (Figure 8). A standard curve was then created by plotting the log of those concentrations versus their corresponding C_T values and creating a best-fit line through these points. A standard curve with the equation C_T value = -5 Log

(Enterovirus copies) + 43.3 was developed (Figure 9). Therefore to obtain a copy number from a C_T value, the equation, Enterovirus copies = 1 x 10 ^[(C_T value-43.3)/-5] was used.

Recovery of HAV in Spiked Ocean Water Samples

Two 1 L samples from the La Jolla Shores area were each spiked with 20 µl of stock poliovirus, filtered, and the viral RNA was extracted. RNA was also directly extracted from 20 µl of unfiltered stock poliovirus. Real-time PCR was performed on both filtered and unfiltered RNA and poliovirus copy number was determined using the standard curve equation. The C_T value for a 1:10 dilution of the unfiltered sample was 29.7 (Figure 10), which corresponds to an original concentration of 5.25 x 10^3 (1 x 10 ^[(29.7-43.3)/-5] x 10) viral copies per 20 µl of stock poliovirus. C_T values for 1:10 dilutions of the two filtrates were 29.7 and 30.3 (Figure 10), which correspond to 5.25 x 10^3 (1 x 10 ^[(29.7-43.3)/-5] x 10) copies and 3.98 x 10^3 copies (1 x 10 ^[(30.3-43.3)/-5] x 10) per 20 µl of stock poliovirus, respectively. A recovery percentage of 87.93% was obtained by dividing the average copy number of the two filtrates ((5.25 x 10^3 copies + 3.98 x 10^3 copies) / 2), which equals 4.62 x 10^3 copies by the unfiltered number of copies, 5.25 x 10^3 copies.

Bacterial Concentrations of Samples

The concentrations of total coliforms, fecal coliforms, and enterococcus were determined for ocean water samples (Table 4) as described in the Research Methodology section. Analysis across rain events showed considerable variation. In general, the Tijuana River mouth samples had higher concentrations of bacteria than the Imperial Beach pier samples. The fecal coliform count in the Tijuana River mouth ocean water samples varied from 220,000 MPN/100 ml to 500,000 MPN/100 ml, whereas the bacterial count in the corresponding pier samples varied from 400 MPN/100ml to 90,000 MPN/100 ml. Within a single rain event, the reduction of fecal coliform counts between the river mouth and pier sample varied from 3.3% to 550%. The three standards used in California to indicate coastal recreational water quality, as stipulated in State Assembly Bill 411, are as follows: 10,000 MPN/100 ml (total coliforms), 400 MPN/100 ml (fecal coliforms), and 104 MPN/100 ml (enterococcus). Therefore these results indicate that six out of six samples exceeded the fecal coliform indicator threshold (as measured by the Graduate School of Public Health at San Diego State University). Total coliforms exceeded the threshold in two out of three samples, fecal coliforms exceeded the threshold in two out of three samples, and the enterococcus threshold was exceeded in three out of three samples (as measured by County of San Diego's Department of Environmental Health). In summary, two indicators in the second rain event pier sample were the only bacterial counts (out of the fifteen measured) that did not exceed a standard. At least one indicator exceeded the threshold in every sample measured.

Relationship of Fecal Coliform to HAV Concentrations in Ocean Water Samples
The relationship between indicator bacteria and HAV concentrations in ocean water was assessed using fecal coliforms. A positive association was found between the fecal coliform indicator bacteria concentrations and HAV concentrations in ocean water samples. This association is based on a sample size of six with a correlation coefficient of $r = 0.94$ (Figure 11). Using the critical values of the correlation coefficient from Table B.17 of Zar's Biostatistical Analysis (Zar 1999), the correlation coefficient is significant between $0.01 < p < 0.005$.

Risk Assessment
In order to evaluate this data in terms of a human health risk assessment for swimming or surfing, the PCR-quantified densities must be related to infectivity. For example, it is well known that many more environmental samples are often positive by PCR than by cell culture. While infectivity data is not available for HAV, Donaldson, et al. (2002) concluded for enteroviruses, from the side-by-side comparison of cell culture and real-time PCR done by Monpoeho, et al. (2000) that a real-time PCR value of 55 particles in a sample may equate to one infectious particle. Extrapolating this infectivity relationship for HAV, if swimmers ingest an estimated 50 ml of seawater during recreation, the densities of HAV (1,303 copies/L to 21,392 copies/L) the researchers found at Imperial Beach would suggest that between 1.18 and 19.44 "viable" particles would be ingested, leading to a very high probability of infection. Such a conclusion suggests that the posting of Imperial Beach automatically after any rain event is instrumental in reducing risks to swimmers and surfers in this area.

CONCLUSIONS
Primary recreational water contact (wading, swimming, surfing, snorkeling, 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, et al. 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 sources of pollution (storm water runoff from urban and rural areas) (Cross, et al. 1992). Therefore, the recreational waters from the Tijuana River mouth to the Imperial Beach pier represent a 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 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 (PCR) for monitoring. Real-time PCR is one of the recent developments in the 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 research objective was to develop a real-time reverse transcriptase (RT)-PCR method for HAV detection in ocean water. This was successfully accomplished by cloning HAV into a plasmid vector and designing/optimizing primers based on its sequence. HAV cDNA amplified by RT-PCR using RNA from an ocean water sample contaminated with Mexican sewage was sequenced, identity confirmed, and cloned into a plasmid vector. Then four primer sets were designed based on this sequenced HAV plasmid DNA. The primer sets were tested for amplification efficiency using the plasmid DNA as template, and the best primer set was chosen for subsequent work. This HAV plasmid DNA was also used to generate standard curves and to serve as a positive control in sample assays.

The second objective was to compare the sensitivity of HAV detection by conventional RT-PCR to the sensitivity of HAV detection by real-time RT-PCR. The limit of detection for conventional RT-PCR was determined to be 24 copies of HAV and for real-time RT-PCR the limit of detection was found to be 1 copy of HAV. Therefore, the data indicate that real-time RT-PCR is eight-fold more sensitive than conventional RT-PCR when plasmid DNA was used as a template.

There appeared to be a sufficient HAV load in the ocean waters surrounding the Tijuana River mouth and Imperial Beach pier following rain events to fall within the sensitivity range of conventional RT-PCR in most of the samples. However, the two samples that were negative by conventional RT-PCR either had lower HAV loads or greater concentrations of inhibitors, both of which required the increased sensitivity of real-time RT-PCR to amplify HAV. Amplification of HAV by real-time RT-PCR proved to require dilutions in order to minimize the effect of inhibitors, and therefore required the increased sensitivity to detect HAV at very low concentrations. Therefore, this real-time RT-PCR method has greater utility in determining more accurately the health risk associated with recreational waters.

In order to evaluate the data in terms of a human health risk assessment for swimming or surfing, the PCR-quantified densities must be related to infectivity. For example, it is well known that many more environmental samples are often positive by PCR than by cell culture. While infectivity data is not available for HAV, Donaldson, et al. (2002) concluded for enteroviruses, from the side-by-side comparison of cell culture and real-time PCR done by Monpoeho, et al. (2000), that a real-time PCR value of 55 particles in a sample may equate to one infectious particle. Extrapolating this infectivity relationship for HAV, if swimmers

ingest an estimated 50 ml of seawater during recreation, the densities of HAV (1,303 copies/L to 21,392 copies/L) the researchers found at Imperial Beach would suggest that between 1.18 and 19.44 “viable” particles would be ingested, leading to a very high probability of infection. Such a conclusion suggests that the posting of Imperial Beach automatically after any rain event is instrumental in reducing risks to swimmers and surfers in this area.

The positive association found between the fecal coliform indicator bacteria concentrations and HAV concentrations in the ocean should be interpreted very cautiously because the observed correlation is based on only a few samples and it remains to be seen whether this association would hold when the sample number is increased. While assessing the human health risk associated with the coastal waters from the Tijuana River mouth to the Imperial Beach pier it should be noted that the incidence of disease such as HAV in Mexico, specifically Tijuana, is greater than that of the United States and San Diego. Therefore, pathogens such as HAV, which increase in severity with decreasing incidence in a population because of a lack of acquired immunity, present a heightened risk to U.S. bathers in these coastal waters.

In summary, this investigation focused primarily on the development and application of a rapid and sensitive method to concentrate and detect HAV by SYBR Green real-time RT-PCR in environmental ocean water samples. This study was the first to not only detect, but also to quantify, HAV load in the Tijuana River mouth and Imperial Beach pier coastal waters. The method is rapid with respect to current water quality monitoring procedures, with an entire processing time of less than 24 hours. In addition, this method has the potential to offer greater sensitivity and quantitative ability that no single method currently offers. With further optimization of viral concentration procedures, the applicability of this method to high throughput reproducible assays could be developed for routine detection of human pathogens in marine recreational waters.

RECOMMENDATIONS FOR FURTHER RESEARCH

For this research on the PCR detection of viruses, further investigation is necessary to extend real-time RT-PCR methods to detection and quantitation of a whole range of viruses in ocean water. The exquisite 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.

Problems/Issues Encountered

There were no unforeseen problems or issues in this project that were beyond the scope of those normal in the course of doing applied research.

Research Benefits

The PCR research on the detection of human-specific viruses, such as HAV, resulted in the development of a new real-time PCR method to detect HAV in ocean waters, which is a novel addition to the scientific field of environmental microbiology. This method, which is sensitive, relatively rapid (six hours), and highly specific for selected viral pathogens will in the future allow a much more accurate human health risk assessment for bathing in contaminated ocean waters such as Imperial Beach.

Publications that have been published or submitted as a result of this project include Brooks, H. A., R. M. Gersberg, and A. K. Dhar. “Quantification of Hepatitis A virus in seawater using real-time RT-PCR.” Submitted to *Applied Environmental Microbiology.*

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

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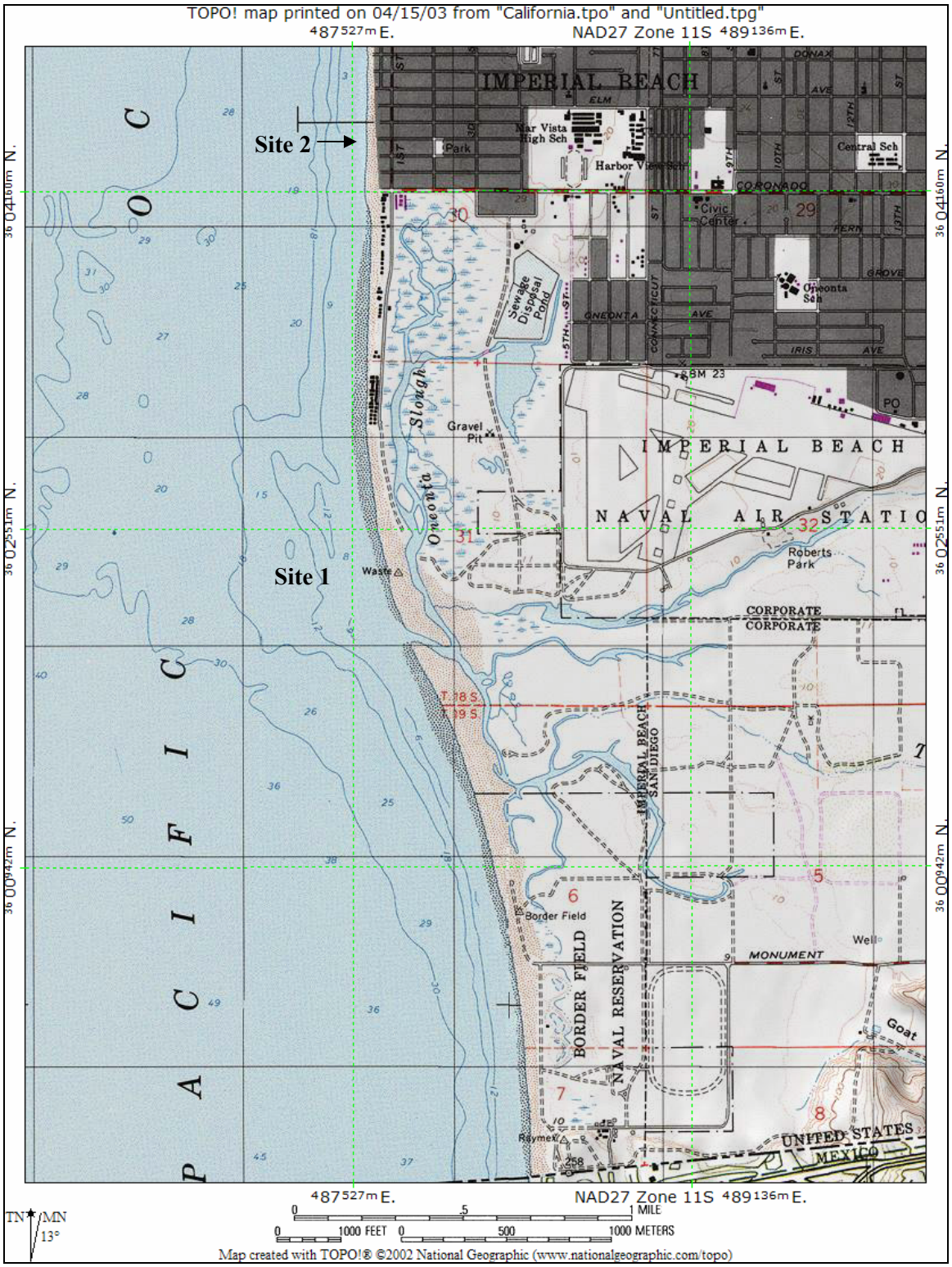
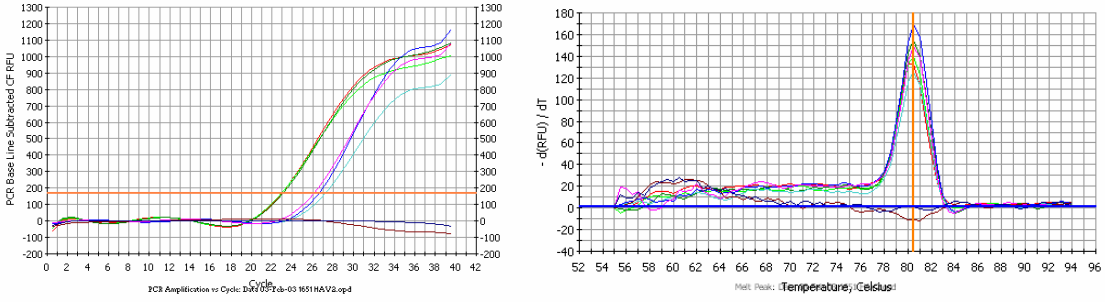
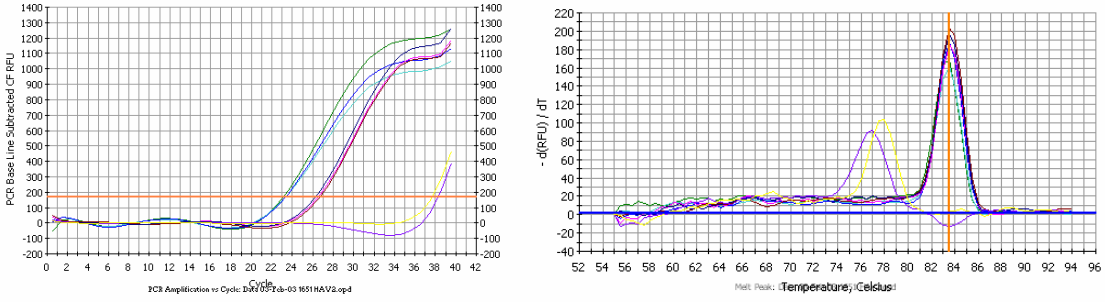


Figure 1. Aerial view of Tijuana River mouth and Imperial Beach pier sampling sites.

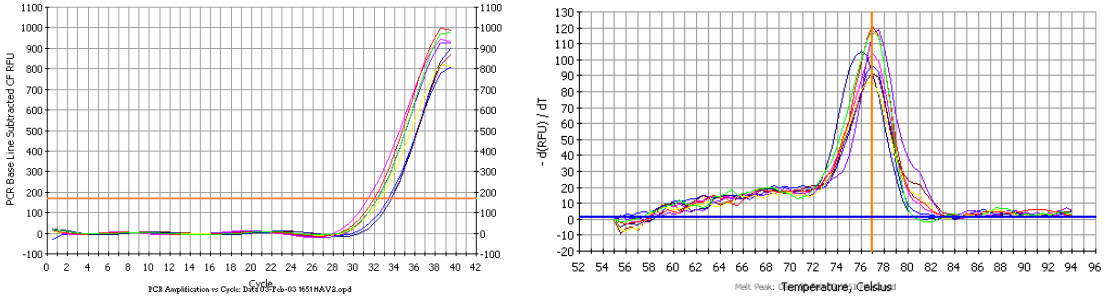
A. Primers HAV 1 & HAV 3



B. Primers HAV 1 & HAV 4



C. Primers HAV 2 & HAV 3



D. Primers HAV 2 & HAV 4

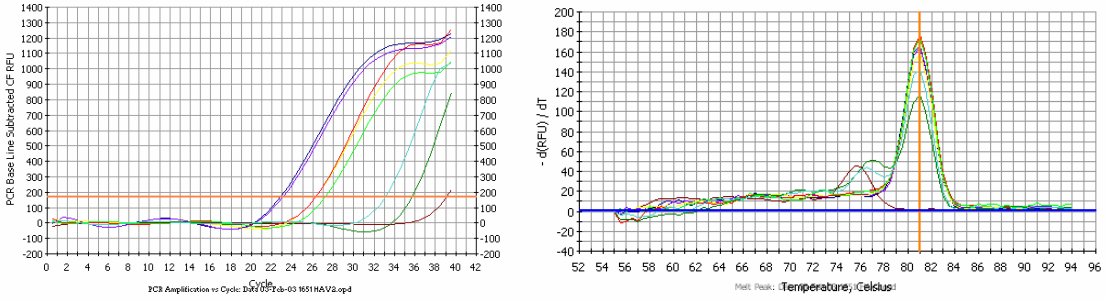


Figure 2. The amplification plots and dissociation curves for four different primer combinations used for the detection of HAV by SYBR Green real-time RT-PCR.

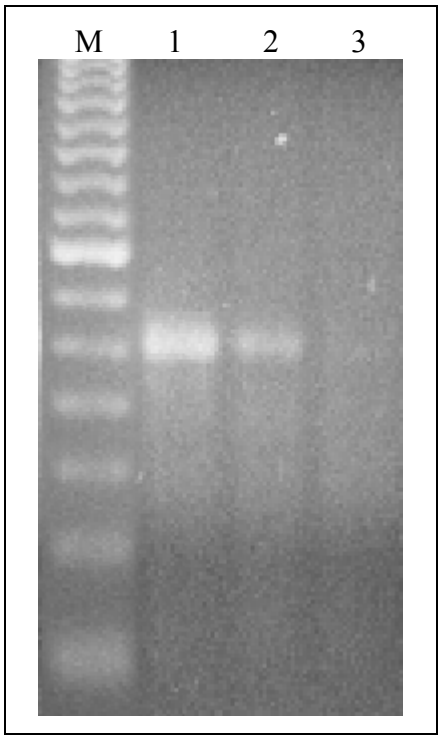


Figure 3. HAV positive Tijuana River mouth and Imperial Beach pier samples from the first rain event by conventional RT-PCR. Lanes 1 and 2 are the Tijuana River mouth and Imperial Beach pier ocean water samples, respectively. Lane 3 is a negative control. Lane M contained a 50 bp molecular weight marker and the HAV amplicon is a 247 bp product.

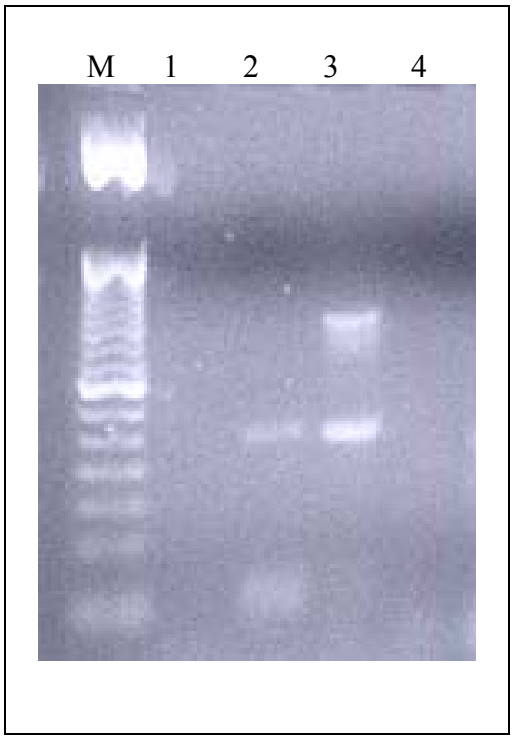


Figure 4. Tijuana River mouth sample from the third rain that was positive (by conventional RT-PCR) only after it was filtered through two different Whatman filters. Lane 1 is the Tijuana River mouth sample that was not filtered through Whatman filters, while lane 2 is the same sample after application of Whatman filters. Lane 3 is the HAV positive pier sample from this same rain event after Whatman filter treatment. Lane 4 is a negative control. Lane M is a 50 bp marker and the HAV amplicon is 247 bp.

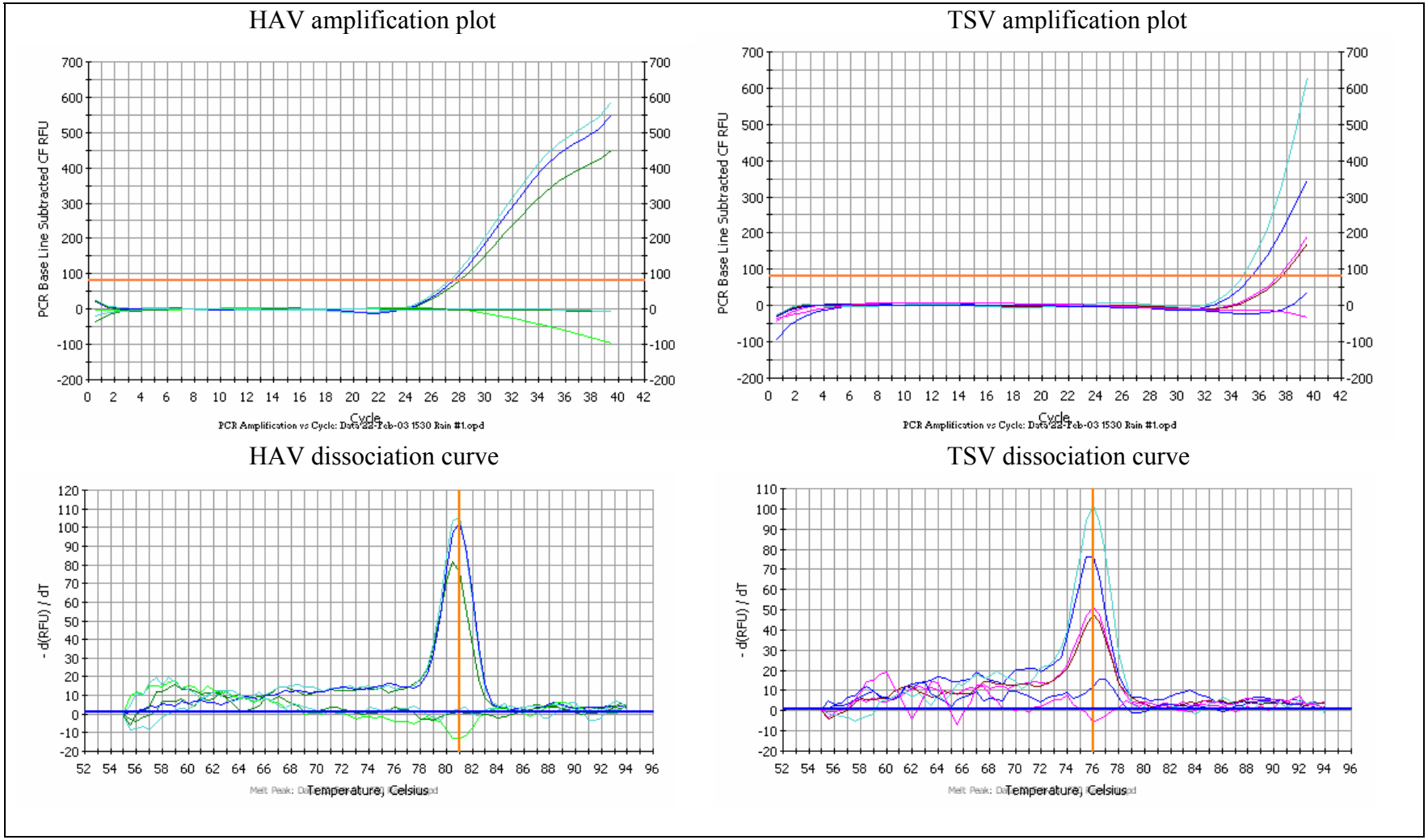


Figure 5. The amplification plots and dissociation curves of HAV and TSV in the Tijuana River mouth ocean water sample from the first rain event, by SYBR Green real-time RT-PCR.

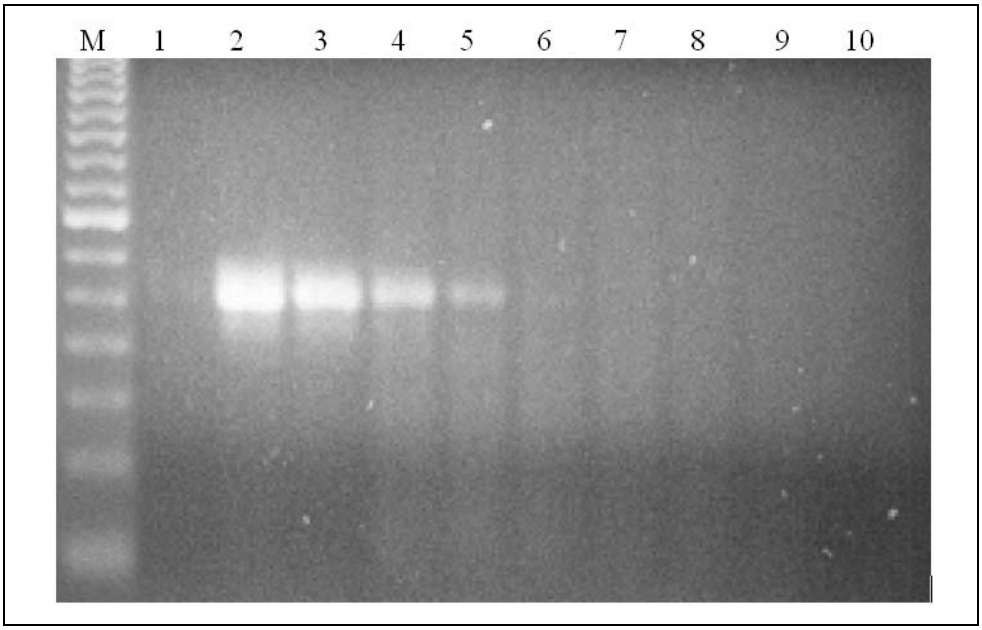


Figure 6. Determination of conventional RT-PCR sensitivity to detection of HAV plasmid standard. Visualization by agarose gel. Serial dilutions of HAV plasmid standard in lanes 2-10 contained 2.38×10^5, 2.38×10^4, 2.38×10^3, 2.38×10^2, 2.38×10^1, 12, 6, 3, 1.5 copies of HAV plasmid respectively. Lane 1 contained everything but the HAV plasmid and served as the negative control. Lane M contained a 50 bp molecular weight marker with extra bright reference band at 350 bp. Hepa 1 and Hepa 2 primers were used for HAV ampification of this 247 bp product.

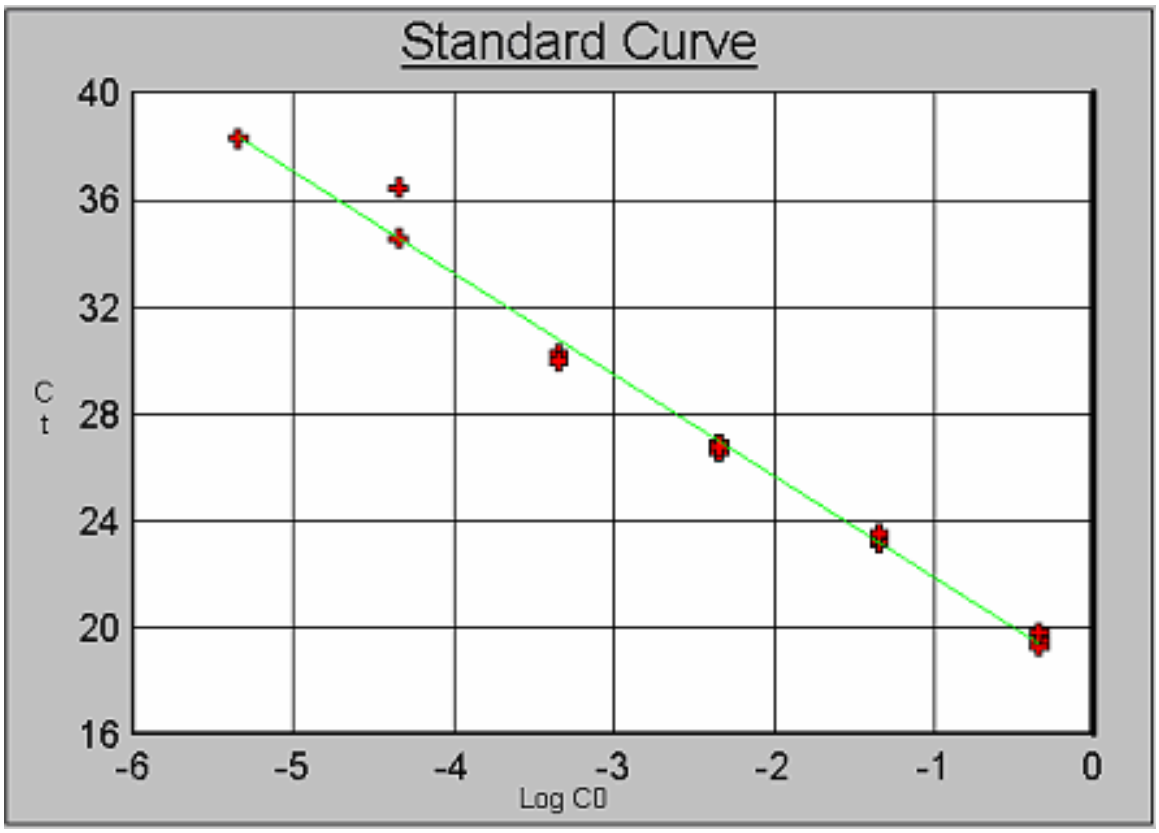


Figure 7. **Hepatitis A virus Standard Curve using SYBR Green real-time RT-PCR. $y = -3.784 (\log_{10}x) + 18.088$; $r^2 = 0.996$. Ct represents the Cycle Threshold and Log CO represents the Log_{10} Concentration. The number of copies of HAV plasmid DNA added to each reaction mixture (corresponding to each of eight groups of red points on the linear curve) from right to left were as follows: 1.1×10^5, 1.1×10^4, 1.1×10^3, 1.1×10^2, 1.1×10^1, and 1.1.**

Figure 8

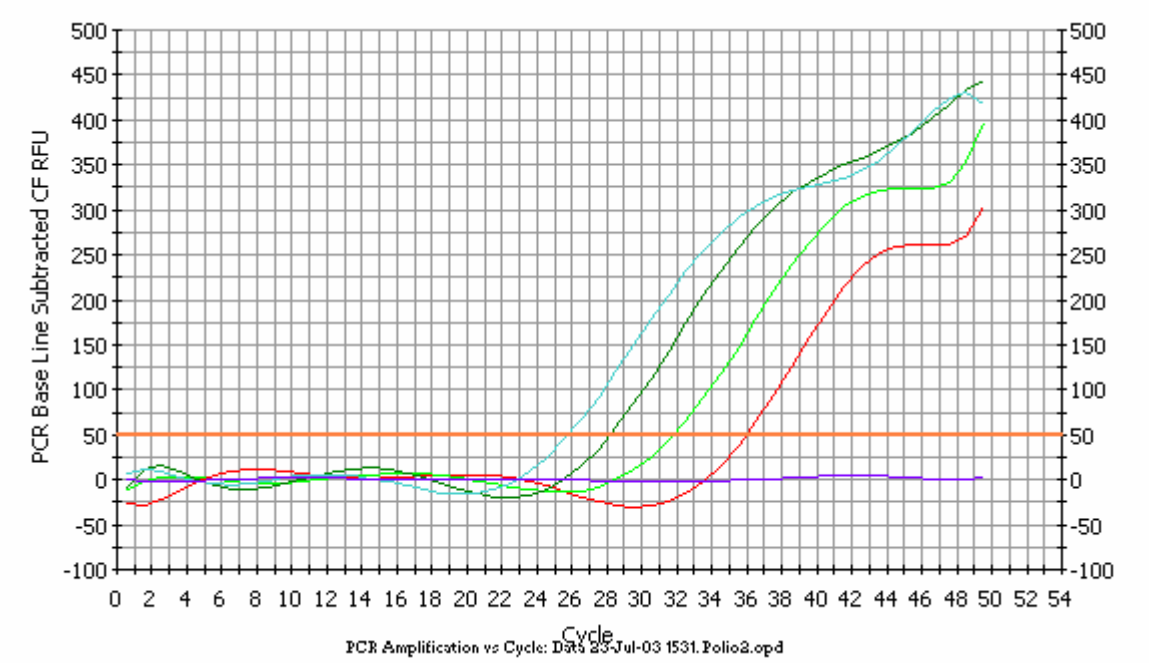

PCR Base Line Subtracted CF RFU
500
450
400
350
300
250
200
150
100
50
0
-50
-100
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54
Cycle
PCR Amplification vs Cycle: Data 23-Jul-03 1531.Polio2.opd

Figure 9

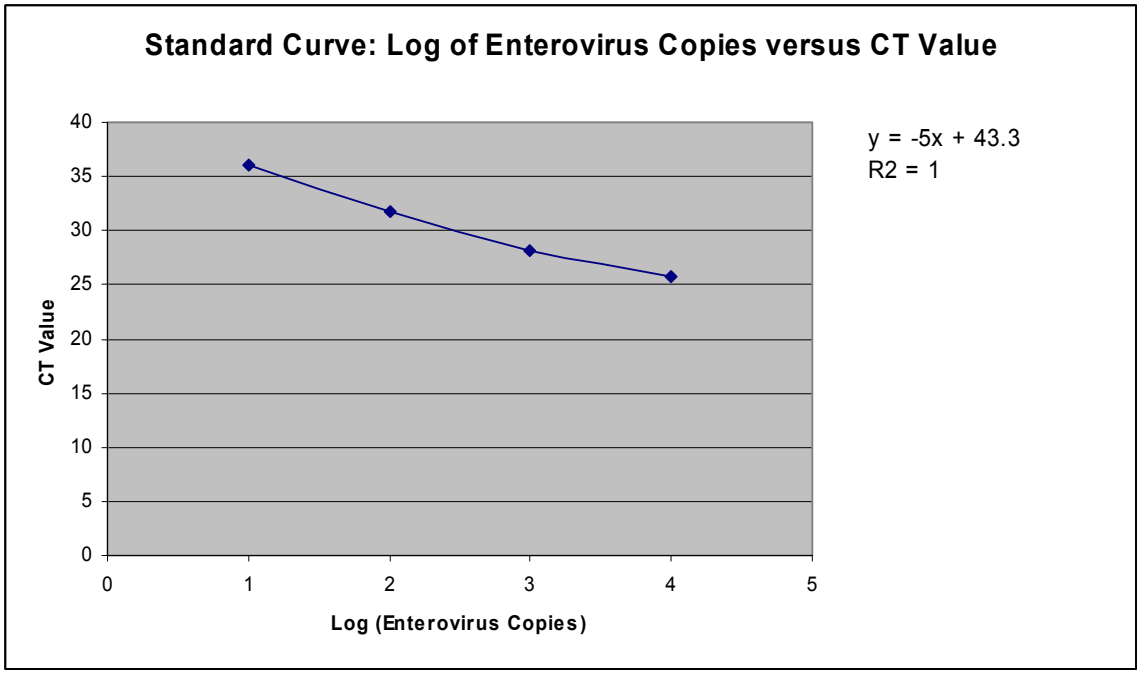
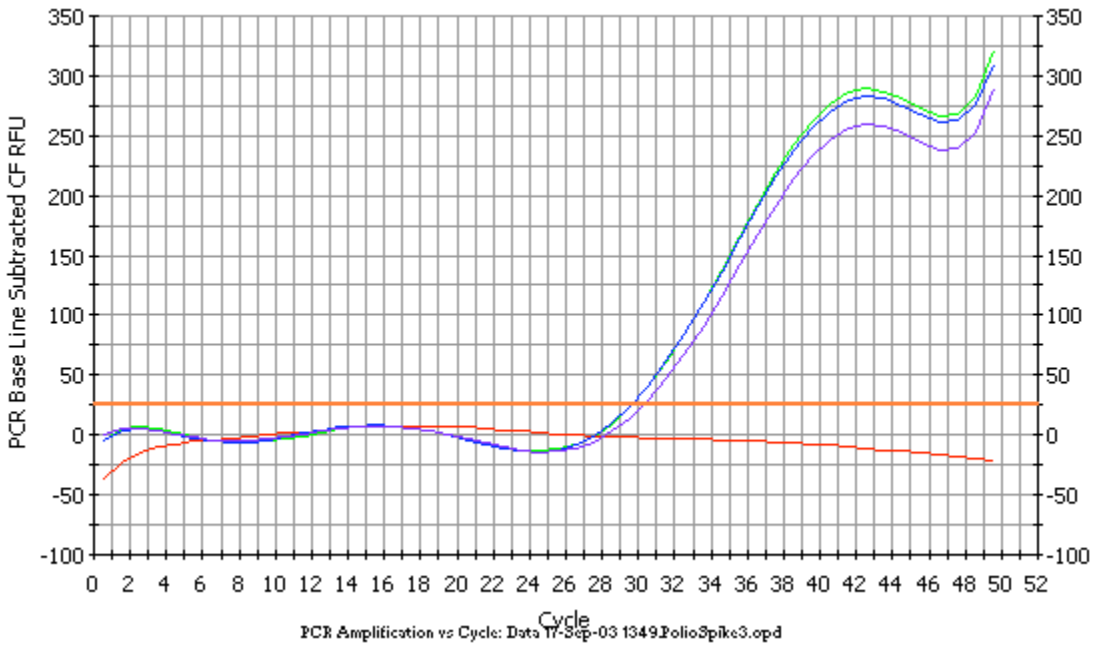

Standard Curve: Log of Enterovirus Copies versus CT Value
CT Value
40
35
30
25
20
15
10
5
0
0
1
2
3
4
5
Log (Enterovirus Copies)
y = -5x + 43.3
R2 = 1

Figure 10


PCR Base Line Subtracted CF RFU
350
300
250
200
150
100
50
0
-50
-100
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52
Cycle
PCR Amplification vs Cycle: Data 17-Sep-03 1349 Polio3spike3.opd

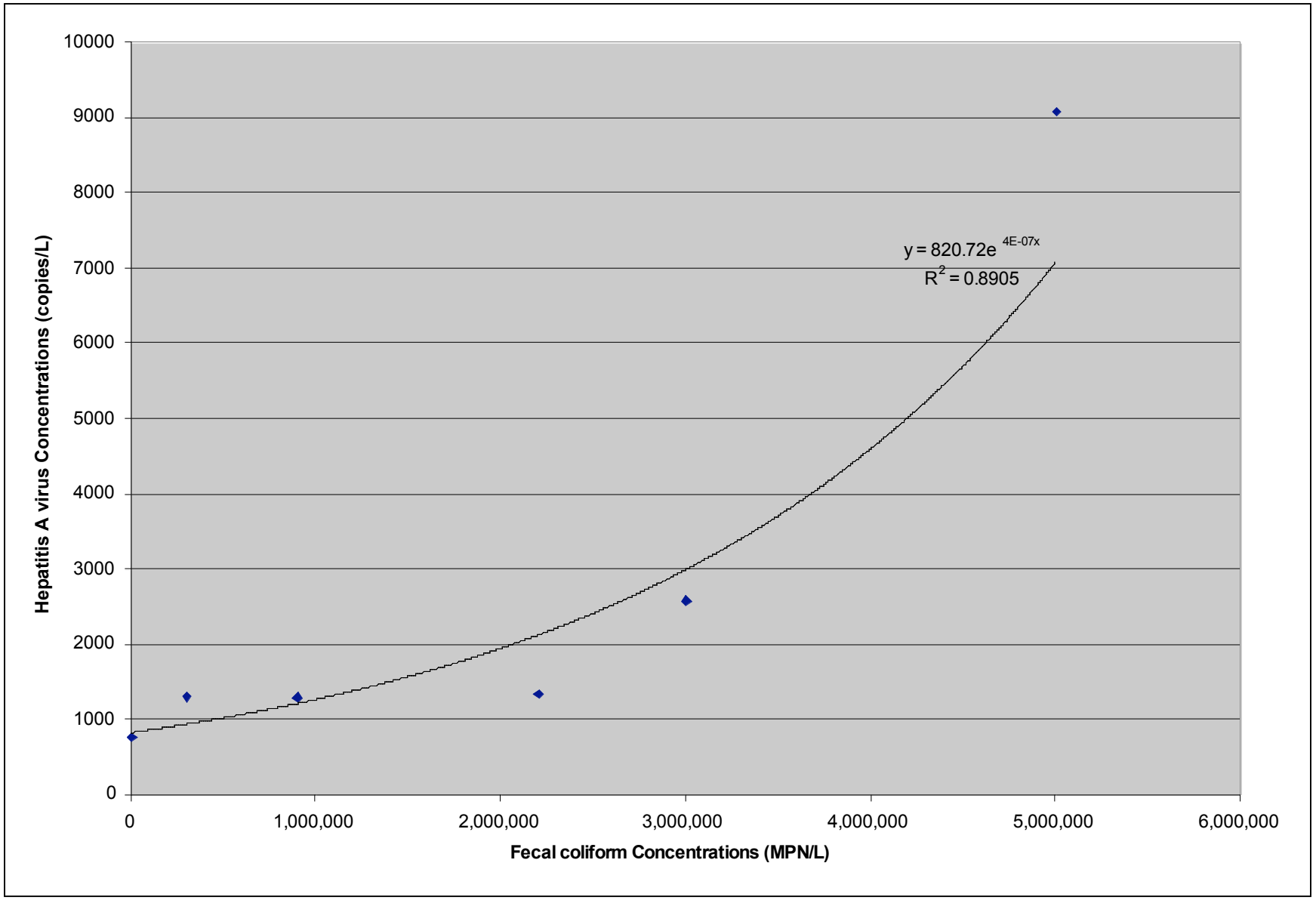


Figure 11. Correlation between fecal coliform concentration and hepatitis A virus level in coastal waters near U.S.-Mexico border

Table 1

The Sequence of HAV and TSV Primers Used for Conventional PCR

Viral Target	Primer Name	Primer Sequence (5'-3')	Product Size (bp)	Reference
Hepatitis A virus	Hepa 1	Forward: GTT TTG CTC CTC TTT ATC ATG CTA TG	247	Jothikumar et al., 1998 Deng et al., 1994 Cohen, Ticehurst et al., 1987
	Hepa 2	Reverse: GGA AAT GTC TCA GGT ACT TTC TTT G		
Taura Syndrome virus	TSVF1	Forward: TCA ATG AGA GCT TGG TCC	220	Nunan, Poulos & Lightner, 1998
	TSVR1	Reverse: AGT AGA CAG CCG CGC TTG		

Table 2

List of Primers Used for Primer Optimization, Detection, and Quantification of HAV by SYBR Green Real-Time PCR Assay

Viral Target	Primer Name	Primer Sequence (5'-3')	%GC	T_m*	Reference
Hepatitis A virus	HAV1FWD	Forward: TAC AGA GCA GAA TGT TCC TGA TCC	46	51	This study
	HAV2FWD	Forward: AGA TGG ATG TTT CAG GAG TCC AA	44	48	
	HAV3RVS	Reverse: TCC CCT ATT GGC TTT CCC TT	50	47	
	HAV4RVS	Reverse: ACT TTC TTT GCT AAA ACT GGA TCC TC	39	50	
Taura Syndrome virus	112F	Forward: CTG TTT GTA ACA CTA CCT CCT GGA ATT	40	52	Dhar et al., 2002
	162R	Reverse: TGA TAC AAC AAC CAG TGG AGG ACT AA	42	51	

Note. The primer sequence of the TSV internal control is also provided.
*At 50 mM Na^{+}.

Table 3

C_T Values, Dissociation Curve Temperatures, and No Template Controls, for All Four SYBR Green Real-Time PCR Primer Combinations for HAV

Primer combination	T_m	Expected product size (bp)	C_T value* with 2.4×10^5 plasmid copies	C_T value* with 2.4×10^4 plasmid copies	Dissociation curve (°C)	No template control
HAV1FWD/ HAV3RVS	78	76	23.0 ± 0.1	26.6 ± 0.6	Single peak at 80.5	Negative
HAV1FWD/ HAV4RVS	79	152	23.2 ± 0.1	26.5 ± 0.3	Three peaks at 83.5, 77, & 78	Two peaks at 77 & 78
HAV2FWD/HAV3RVS	68	44	32.8 ± 1.43	32.9 ± 0.8	Three peaks at 77.5, 77 & 76	Two peaks at 77
HAV2FWD/HAV4RVS	77	76	22.9 ± 0.1	26.6 ± 0.6	Single peak at 81	Two peaks at 81 each with a smaller peak at 76.5 & 77

*Average of 3 replicates.

Table 4

Determining the Bacterial Count, and the Detection and of HAV in Water Samples by Conventional and SYBR Green Real-Time RT-PCR

Rain event #/ Date	Location	Precipitation (inches)	Bacterial count	Bacterial threshold exceeded[†]	HAV conventional RT-PCR results	HAV real-time RT-PCR results
#1/ 11-10-02	Tijuana River Mouth	0.2	N/A	N/A	Positive	Positive
	Imperial Beach Pier		N/A	N/A	Positive	Positive
#2/ 12-17-02	Tijuana River Mouth	0.45	16,000 MPN/100ml (TC & FC) 2005 MPN/100ml (enterococcus) 220,000 MPN/100ml (FC)*	YES	Positive	Positive
	Imperial Beach Pier		170 MPN/100ml (TC & FC) 164 MPN/100ml (enterococcus) 400 MPN/100ml (FC)*	YES	Negative	Positive
#3/ 2-13-03	Tijuana River Mouth	2.65	300,000 MPN/100ml (FC)*	YES	Positive	Positive
	Imperial Beach Pier		90,000 MPN/100ml (FC)*	YES	Positive	Positive
#4/ 2-26-03	Tijuana River Mouth	1.59	>16,000 MPN/100ml (TC) >12,000 MPN/100ml (FC) >12,000 MPN/100ml (enterococcus) 500,000 MPN/100ml (FC)*	YES	Positive	Positive
	Imperial Beach Pier		30,000 MPN/100ml (FC)*	YES	Negative	Positive

[†]Bacterial standards for San Diego County/California recreational marine waters for a single sample are as follow; 10,000 total coliforms (TC)/100 ml, 1,000 TC/100 ml if the ratio of FC/TC exceeds 0.1, 400 fecal coliforms (FC)/100 ml, and 104 enterococci/100 ml.

*Bacterial tests done by the Graduate School of Public Health, San Diego State University. All other tests done by County of San Diego, Department of Environmental Health.

TABLE 5. *Determination of HAV Load in Ocean Water by SYBR Green Real-Time RT-PCR*

Rain event #/ Date	Location	Liters filtered	Dilution	C_T value		HAV copies per Liter*		Mean† (copies/L) ± SD	Standard Deviation (SD)
				1st Experiment	2nd Experiment	1st Experiment	2nd Experiment		
#1/ 11-10-02	Tijuana River Mouth	2	Stock	40	40	-	-	12,104	3,807
			1:10	33.25	39.41	937	22		
			1:100	32.38	33.45	15,910	8,297		
			1:1000	ND	35.54	-	23,259		
	Imperial Beach Pier	4	Stock	40	40	-	-	9,743	1,788
			1:10	34.67	40	197	-		
			1:100	32.38	31.77	7,955	11,531		
			1:1000	ND	40	-	-		
#2/ 12-17-02	Tijuana River Mouth	4	Stock	40	40	-	-	1,349	307
			1:10	32.72	32.69	647	659		
			1:100	34.96	35.72	1,655	1,042		
			1:1000	ND	36.36	-	7,061		
	Imperial Beach Pier	4	Stock	40	40	-	-	769	-
			1:10	40	40	-	-		
			1:100	36.22	40	769	-		
			1:1000	ND	40	-	-		
#3/ 2-13-03	Tijuana River Mouth	1	Stock	38.37	38.22	42	46	2,583	1,985
			1:10	37.32	36.86	157	208		
			1:100	38.91	35.57	598	4,568		
			1:1000	ND	38.87	-	6,132		
	Imperial Beach Pier	4	Stock	38.14	38.27	12	11	1,298	665
			1:10	36.46	35.8	66	99		
			1:100	36.54	34.68	633	1,963		
			1:1000	ND	40	-	-		

Table 5 (continued)

Rain event #/ Date	Location	Liters filtered	Dilution	C_T value		HAV copies per Liter*		Mean† (copies/L) ± SD	Standard Deviation (SD)
#4/ 2-26-03	Tijuana River Mouth	2.5	Stock	40	40	-	-	9,092	1,908
			1:10	36.30	33.20	117	773		
			1:100	33.32	32.62	7,184	10,999		
			1:1000	ND	34.87	-	27,973		
	Imperial Beach Pier	4	Stock	36.78	40	27	-	1,311	536
			1:10	39.20	38.34	13	21		
			1:100	34.78	36.25	1,847	775		
			1:1000	ND	39.81	-	865		

* HAV copy numbers were calculated using the equation, HAV copies per 1 L = $10^{\wedge [C_T - 18.088 \div -3.784]}$ ($2.38 \times 10^{\wedge 5}$ copies) x 40 x dilution factor ÷ # of liters of ocean water filtered (÷ 5 for all dilutions, with the exception of the stock).

† HAV mean copy number per L was calculated for each sample by taking the average of the HAV copy numbers of the 1:100 dilution from the 1st and 2nd experiments.