MAMALA BAY STUDY

OVERALL IMPACT OF SAND ISLAND OUTFALL ON THE INCIDENCE OF PATHOGENS IN MAMALA BAY

PROJECT MB-7

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1 EXECUTIVE SUMMARY

The incidence of bacterial, viral, and protozoan human pathogens was monitored in sewage that was disposed of via outfalls into Mamala Bay as well as impacted marine waters and freshwater discharges. State-of-the-art methodologies were used including cultural, molecular, and immunofluorescence techniques. An evaluation of point sources of pollution indicated that Sand Island Outfall, the Ala Wai Canal, and the Manoa Stream were all sources of pathogens that can enter Mamala Bay. Non-point sources of pathogens could also arise from houseboats and bathers. Pathogens were always detected in the sewage discharged into Mamala Bay. Specific pathogens detected from point sources were *Salmonella, Escherichia coli*, enteroviruses, adenoviruses, *Giardia*, and *Cryptosporidium*. Bacterial, viral, and protozoan pathogens were detected in marine waters and Ala Wai canal. Of all the sites studied the greatest numbers and frequency of isolation of pathogens occurred from samples collected at the mouth of the Ala Wai canal. Manoa stream was not found to contain virus, but did contain bacterial and protozoan pathogens. Some bacterial pathogens appeared to be viable but nonculturable. The most commonly detected pathogens were the protozoan parasites. Based on these state-of-the-art methodologies, there is no evidence to date that the Sand Island Sewage Outfall is contributing more pathogens to bathing beaches than other sources such as the Ala Wai Canal or the Manoa Stream. Overall, the incidence of pathogens at beaches and close inshore marine waters within Mamala Bay is low. The significance of these risks are currently being determined by a formal microbial risk assessment. The Ala Wai Canal and Manoa Stream appear to be more microbially contaminated than the marine waters of Mamala Bay.

Summaries of the specific studies are presented below. Detailed objectives, methods, results, and discussion of each study are subsequently included as appendices.

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2 INTRODUCTION

2.1 Scope of Work

This study formed part of the MB-7 Project that investigated microbiological aspects of Mamala Bay. Five different teams composed the MB-7 Project. The team from the University of Arizona was to assess the occurrence and concentration of pathogens from discharges and on bathing areas, the survival of enteric viruses and protozoan parasites, and individual risks from exposure. The goal was to apply state-of-the-art methodology for pathogen detection.

2.2 Study Objectives

A major goal of this project was to demonstrate the potential application of recent advances in molecular biology to the detection of enteric pathogens in marine waters. To this end, the application and optimization of the polymerase chain reaction (PCR) to *Salmonella* and enteric virus detection was performed as part of this study. These and traditional methods were used to determine concentration of pathogens originating from the Sand Island sewage outfall, other potential land based sources of pathogens, and at bathing beaches. Another objective was to assess the survival of enteric viruses and *Giardia* in the waters of Mamala Bay. Finally, this information was used to assess the individual risk of infection from the pathogens present in the bathing waters.

2.3 Project Organization and Project Reports

This project formed a part of the MB-7 Project under the overall leadership of Dr. Charles Gerba, who was responsible for the virus and parasite sampling and analysis. Dr. Ian Pepper was responsible for the *Salmonella* and PCR analyses. Dr. Gerba, Dr. Joan B. Rose (University of South Florida), and Dr. Charles Haas were responsible for the risk analyses. Kimberly Roll served as coordinator for the entire MB-7 team sampling efforts in Hawaii and was under the direction of Charles Gerba and Roger Fujioka. She was also responsible for collecting and sending all samples to the Arizona team on a monthly basis. She was responsible for supervising the experiments and personnel on the Hawaii team on a daily basis.

Dana Johnson, a graduate research assistant, was responsible for analyses of protozoan parasites. Kelly Reynolds and Carlos Enriquez, graduate research assistants, were responsible for enteric virus analyses. Karen Josephson, a senior research specialist, and Mark Burr, a graduate research assistant, were responsible for the *Salmonella* analyses.

3 METHODS

3.1 Task Summary

The tasks for our team included developed of scientifically acceptable PCR methodology for detection of *Salmonella* and enteric viruses, determine efficiency of methods for pathogen detection, selection of methodology for detection of pathogens and methods for their assay.

3.2 Task Methodology

Methods for the concentration and detection of *Salmonella* are described in detail in Appendices A and B. Details for the methods for enteric virus concentration and enterovirus detection are detailed in Appendix C through E. Methods for the concentration and detection of adenoviruses used are described in Enriquez and Gerba, 1995. *Giardia* and *Cryptosporidium* were concentrated from water by the procedures described in Standard Methods for the Examination of Water and Wastewater, 19th Ed. (APHA, 1995).

Several experiments were conducted in Hawaii and Arizona on the survival of poliovirus, adenovirus, *Salmonella*, and *Giardia* in the marine waters of Mamala Bay. The details of the material and methods are described in Appendix E.

4 RESULTS

4.1 Occurrence of Enteric Pathogens in Mamala Bay

The occurrence and concentration of pathogens studied at each site from October 1993 through November 1994 are shown in Tables 1 through 12. As expected pathogens were always detected in the sewage leaving the Sand Island facility (Table 1). The concentrations of pathogens were in the range previously observed by other investigators. No *Salmonella* were detected at the outfall. However, enteroviruses and adenoviruses were detected on two occasions, *Cryptosporidium* on three and *Giardia* was detected on all four sampling periods (Table 2). Only *Cryptosporidium* and *Giardia* were detected at the Pearl Harbor site (Table 3). All groups of pathogens studied were detected at the entrance of the Ala Wai Canal (Table 4). *Cryptosporidium* and *Giardia* were the most commonly detected followed by *Salmonella*. The presence of enteroviruses suggest that human sewage was the source. In contrast to the entrance of the Ala Wai Canal, only *Salmonella* and *Giardia* were detected offshore of the Ala Wai Canal (Table 5). *Salmonella* and the protozoan parasites were detected in Manoa Stream, but no enteric viruses were isolated (Table 6).

Pathogens were detected at all of the study beaches except the Sand Island Beach (Table 11). Enteroviruses were isolated on one occasion on Waikiki Beach and the protozoan parasites four times each (Table 7). The protozoan parasites were also isolated at Queen's Surf Beach (Waikiki), as well as, adenoviruses on two occasions (Table 8). All of the study pathogens were isolated at Ala Moana Beach, except *Salmonella*, on at least one occasion (Table 9). Enteroviruses were isolated on two occasions at Hanauma Bay and *Giardia* once (Table 11). No pathogens were isolated at the offshore site near Diamond Head.

4.2 Bacterial Pathogens

4.2.1 Detection of *Salmonella* in Mamala Bay by Cultural Methods

The objective of this study was to use traditional cultural techniques to determine the occurrence of *Salmonella spp.* in sewage, marine water, and fresh water samples collected from

Honolulu, Hawaii. Based on a Standard Methods most probable number technique, the steps included sample concentration and enrichment followed by plating on selective media. Confirmation of presumptive isolates was accomplished by the use of biochemical, serological, and PCR techniques. All of the sewage samples were contaminated with *Salmonella* with values ranging from 10^2 to 10^4 CFU's per liter. Manoa stream samples for all four quarterly sampling periods were positive for *Salmonella* with around 1 CFU per liter. In the Ala Wai Canal, seven of the thirteen samples indicated the presence of *Salmonella* with a range of 0.4 to 4.3 CFU's per liter. Ala Wai Offshore sample date 2/15/94 had 0.4 CFU's per liter, while both other dates for this sample were negative. In all other marine samples, no contamination with *Salmonella spp.* was found. Our results indicate

a) *Salmonella* inputs into Mamala Bay include the outfall, the Ala Wai Canal, and fresh water stream inputs.

b) Despite these inputs, contamination of beaches and marine water within Mamala Bay appears to be minimal, at least based on cultural methodologies.

4.2.2 Direct PCR Detection of Naturally Occurring *E.coli* and *Salmonella* in Mamala Bay

We have developed methodologies for direct PCR detection of naturally occurring *E.coli* and *Salmonella* in environmental samples. The efficacy of the protocol was demonstrated on sewage, marine water, and freshwater samples collected from Honolulu, Hawaii. Vortex flow filtration, along with centrifugation, was used to concentrate bacteria from aquatic samples. Cell lysis was accomplished by sonication and thermal shock treatments. Extracted DNA was purified by passage through Elutip d minicolumns. Purified DNA was analyzed by PCR amplification using *E.coli* and *Salmonella* specific primers. Use of this PCR based methodology allowed detection of *E.coli* in sewage, marine, and fresh waters, whereas *Salmonella* was found only in sewage. Simultaneous analysis of the environmental samples by traditional cultural assay allowed for evaluation of the PCR protocol to detect specific bacterial DNA sequences in aquatic environments. A comparison of culturable and PCR methodologies suggests that each approach has its limitations. Culturable counts will not detect viable but nonculturable organisms, whereas

PCR will. However, the PCR process can be compromised, especially when environmental samples with high levels of inhibitors such as metals or humic substances are concentrated along with low levels of target DNA. Qualification of PCR amplified DNA by HPLC allowed for estimates of the amount of pathogens within the environmental samples to be made. Such estimates would include all viable organism including viable but non-culturable cells. A comparison of theses counts with culturable counts suggests that the environmental samples do contain viable but non-culturable organisms.

4.3 Viruses

4.3.1 Development of PCR Methodology for Virus Detection

Virus adsorption/elution (Viradel) methods of filtration were examined to evaluate recovery efficiency and suitability for enterovirus detection in marine waters using RT-PCR and culture in a BGM cell line. Both natural and Poliovirus (type 1 strain LSc-2ab) inoculated marine water samples were concentrated using either electropositive (1 MDS) or electronegative (Filterite and K27) 10-inch cartridge filters which were eluted using 1.5% Beef Extract V. Double PCR was utilized, in addition to cell culture, to evaluate the presence of RT-PCR inhibitory factors and the sensitivity of enterovirus detection in BEV eluants and reconcentrates. Column chromatography resins (Sephadex G-25, -50, -200 and Chelex-100) were tested for their effective removal of inhibitory factors and their efficiency of virus recovery. Filterite electronegative filters were the most efficient, with an enterovirus recovery efficiency of 66% and also produced the most consistent PCR results. Enterovirus primed double RT-PCR was capable of detecting 0.1 PFU of Poliovirus (type 1 strain LSc-2ab) in distilled water; however, detection sensitivity decreased by 2 to 4 orders of magnitude in reconcentrated eluants. Sephadex/Chelex columns, used to remove RT-PCR inhibitory factors, improved sensitivity by 2 orders of magnitude in sewage outfall samples. Sephadex G-25 was the least effective at removing inhibitory factors; however, 100% of the virus in the sample was recovered. In contrast, while Sephadex G-200 was most effective for removing inhibitory factors, 97% of the virus was lost in the column. Therefore, the trade-off between inhibition removal and virus recovery efficiencies must be considered for each individual sample site.

4.3.2 Detection of Viruses in Mamala Bay by PCR and Cell Culture Methodologies

Point and nonpoint pollution sources discharged into marine waters were evaluated for their impact on public recreational beaches in Mamala Bay, Oahu. Twelve sites were sampled in Mamala Bay, either quarterly or monthly, from October, 1993, to November, 1994. Marine and canal waters were concentrated from 400 L to 30 mL using electronegative cartridge filters (Filterite) and 1.5%, glycine buffered Beef Extract (BEV) eluent, pH 9.5. All samples were analyzed for enteroviruses using a BGM cell line. Equivalent samples were further evaluated by direct RT-PCR, using enterovirus-specific primers. Levels of RT-PCR inhibition varied with each concentrated sample; however, column purification increased RT-PCR sensitivity by at least one order of magnitude in sewage outfall and recreational beach water samples. Using tissue culture methodology, viable enteroviruses were found in 50% and 17% of all outfall and canal samples, respectively, but samples were positive at potentially impacted beaches only 8% of the time. Results from this study indicate that frequent bather use may also be contributing to Oahu's recreational water quality with respect to enteroviruses.

4.3.3 Development of an Integrated Cell Culture/PCR Methodology to Detect Viruses

Reverse transcriptase-polymerase chain reaction (RT-PCR) is a rapid, sensitive detection technique that has been used effectively for direct detection of enteroviruses in a variety of environmental samples. The implication of positive results, however, has been difficult to evaluate due to amplification of nonviable particles. Traditional cell culture detects viable virions, but may take 14-21 days before cytopathic effects (CPE) are confirmed. In this study we have combined the advantages of both methodologies for more rapid detection of cell culture infective enteroviruses using RT-PCR. Buffalo green monkey kidney (BGM) cells were inoculated with poliovirus type 1 (strain LSc-2ab) or primary sewage, and observed up to 28 days for cytopathic effect. Replicate flasks were frozen and thawed 3 times, Freon extracted, and saved for RT-PCR analysis. Poliovirus inoculums of <1 PFU/flask demonstrated CPE after 3 days; however, RT-PCR was positive after only one day of incubation on BGM cells. The integrated cell culture/PCR methodology, therefore, allowed for more rapid detection of enteroviruses than cell culture alone. Furthermore, in a comparison of primary cell culture assay, RT-PCR and the integrated cell culture/RT-PCR, the

combined methodology was at least ten fold more sensitive for detecting infectious poliovirus type 1 (strain LSc-2ab) than either method alone. Similarly, enteroviruses were detected by primary cell culture in dilutions of primary sewage samples after 10-14 days of incubation. Using the combined method of cell culture inoculation followed by RT-PCR, enteroviruses were detected after only 1-5 days. Therefore, the integrated cell culture/PCR method was nearly 5 times more rapid for the detection of cell culture infectious enteroviruses than currently used methodologies.

4.4 Survival of *Giardia*

Giardia muris and *G.lamblia* were found to die-off fairly rapidly in marine waters with a 99.9% reduction in viability in three hours in the presence of sunlight. The same degree of inactivation occurred in the dark within 77 hours. Poliovirus type 1 survived longer in marine waters than did *Salmonella typhimurium* and *G. muris*.

5 CONCLUSIONS

1. Sources of enteric pathogenic viruses, protozoan parasites, and *Salmonella* identified were the Sand Island Outfall, and the Ala Wai Canal. *Salmonella* and the protozoan parasites were also detected in Manoa Stream.

2. Studies at Hanauma Bay, with no known point sources suggested that bathers were a potential source of enteroviruses and *Giardia*.

3. Of all the sites studied the greatest numbers and frequency of isolation of pathogens occurred at the mouth of the Ala Wai Canal.

4. Enteric viruses and protozoan parasites were detected in low at Waikiki Beach, Queen's Surf Beach, Manoa Beach., and Hanauma Beach.

5 .Overall, the incidence of pathogens at beaches and close inshore marine was within Mamala Bay is low.

6. *Giardia* cysts are unlikely to survive more than three days in the marine waters of Mamala Bay.

6 RECOMMENDATIONS

1. Since enteric viruses survive the longest in marine waters information on the concentration of enteric viruses in the wastewater being discharged from the Sand Island Plant such be done on a regular basis.

2. Any treatment of the wastewater before discharge should be tested to assess the degree of removal of enteric viruses and protozoan parasites. This would allow for an estimate of the risk reduction from pathogens discharges into Mamala Bay.

3. Since the Ala Wai Canal was a significant source of pathogens the source of these pathogens should be determined so that appropriate action can be taken to reduce the threat of pathogens from this source.

7 REFERENCES

Enriquez, C.E. and C.P. Gerba. 1995. Concentration of enteric adenovirus 40 from tap, sea and wastewater. Water Res. 29:2554-2560.

American Public Health Association (APHA). 1995. Standard Methods for the Examination of Water and Wastewater. 19th Edition. Washington, D.C.

Table 1. Occurrence of pathogens in the sewage at Sand Island before discharge (SI).

Date	*Salmonella* MPN/L	Enterovirus MPN/L	Adenovirus MPN/L	Crypto oocysts/L	*Giardia* cysts/L
10/25/93	+*	1.3E+04	4.4E+02	2.6E+01	2.5E+02
12/06/93	+	3.7E+03	4.0E+01	2.1E+02	1.7E+03
02/01/94	+	6.8E+03	3.7E+02	1.0E+01	3.7E+02
02/14/94	1.0E+04	2.8E+03	4.5E+02	2.4E+01	8.0E+02
03/09/94	2.0E+04	5.6E+03	ND	7.5E+01	3.0E+02
04/21/94	2.3E+02	5.0E+03	ND	9.0E+01	1.5E+03
05/02/94	2.4E+03	1.1E+03	ND	3.8E+02	4.6E+03
06/20/94	1.5E+03	3.6E+02	4.0E+02	1.3E+01	1.3E+03
07/12/94	2.4E+03	1.8E+02	ND	3.2E+02	8.0E+02
08/16/94	2.3E+02	2.3E+01	ND	8.0E+02	2.1E+03
09/20/94	4.3E+02	8.6E+02	ND	5.0E+02	5.5E+03
10/31/94	4.3E+02	ND**	ND	1.6E+02	3.7E+03
11/14/94	7.5E+02	8.2E+01	ND	2.0E+02	7.4E+03
maximum value	2.0E+04	1.3E+04	4.5E+02	8.0E+02	7.4E+03
mean	3.8E+03	3.3E+03	3.4E+02	2.2E+02	2.3E+03
minimum value	2.30E+02	2.28E+01	4.0E+01	1.0E+01	2.5E+02

*+ = detected by, not quantified
**ND = not done or not assayed

Table 2. Occurrence of pathogens at the Sand Island Sewage Outfall (D2B).

Date	*Salmonella* MPN/L	Enterovirus MPN/L	Adenovirus MPN/L	Crypto oocysts/L	*Giardia* cysts/L
10/28/93	-*	**4.4E-02**	**4.8E-02**	**1.1E-01**	**1.0E-01**
02/16/94	<0.3	**3.6E-02**	**3.4E-01**	**7.5E-02**	**1.5E-01**
06/23/94	<0.3	<1.2E-02	ND	**1.6E-01**	**4.6E-01**
11/16/94	<0.3	<9.6E-03	ND	<5.0E-03	**1.0E-02**
maximum value	<0.3	4.4E-02	3.4E-01	1.6E-01	4.6E-01
mean	<0.3	4.0E-02	1.9E-01	1.2E-01	1.9E-01
minimum value	<0.3	<9.6E-03	4.8E-02	<5.0E-03	4.8E-02

*Samples were not quantified (presence/absence only)
ND = not done

Table 3. Occurrence of pathogens at the Pearl Harbor Outfall (C2).

Date	*Salmonella* MPN/L	Enterovirus MPN/L	Adenovirus MPN/L	Crypto oocysts/L	*Giardia* cysts/L
10/29/93	-*	<1.3E-02	<1.5E-02	<1.1E-02	<1.1E-02
02/17/94	<0.3	<1.1E-02	<1.5E-02	<5.1E-03	**1.0E-02**
06/22/94	<0.3	<1.0E-02	<1.5E-02	**1.0E-02**	<5.2E-03
11/17/94	<0.3	<1.0E-02	ND	<5.0E-03	**5.0E-03**
maximum value	<0.3	<1.3E-02	<1.5E-02	1.0E-02	1.0E-02
mean	<0.3	<1.1E-02	<1.5E-02	1.0E-02	7.5E-03
minimum value	<0.3	<1.0E-02	<1.5E-02	<5.0E-03	5.0E-03

*Samples were not quantified (presence/absence only)
ND = not done

Table 4. Occurrence of pathogens at the entrance of the Ala Wai Canal (AW1).

Date	*Salmonella* MPN/L	Enterovirus MPN/L	Adenovirus MPN/L	Crypto oocysts/L	*Giardia* cysts/L
10/25/93	+*	**2.5E-02**	**2.0E-02**	<1.0E-02	<1.0E-02
12/07/93	<0.3	<2.5E-02	ND	<4.6E-02	<4.6E-02
01/20/94	<0.3	ND	ND	**2.5E-02**	**3.0E-02**
02/07/94	ND	<1.1E-02	<1.5E-02	ND	ND
02/14/94	**4.3**	**4.5E-02**	**2.2E-02**	**1.0E-02**	**3.5E-02**
03/21/94	<0.3	ND	ND	**1.5E-02**	**5.0E-03**
04/18/94	**0.9**	<1.0E-02	ND	**1.0E-02**	**1.0E-02**
06/01/94	<0.3	<1.1E-02	ND	**5.0E-03**	**1.0E-02**
06/20/94	**0.4**	<1.0E-02	**5.6E-02**	**1.0E-02**	**1.0E-02**
07/05/94	**0.9**	<1.2E-02	ND	**2.0E-02**	**2.0E-02**
08/09/94	<0.3	<9.0E-03	ND	**5.0E-03**	<5.0E-03
09/12/94	<0.3	<1.0E-02	ND	**1.0E-02**	**1.0E-02**
10/17/94	**0.9**	<1.1E-02	ND	**1.0E-02**	**7.0E-02**
11/14/94	**0.9**	<1.7E-02	ND	**1.0E-02**	**1.0E-02**
maximum value	4.3E+00	4.5E-02	5.6E-02	2.5E-02	7.0E-02
mean	1.4E+00	3.5E-02	2.4E-02	1.2E-02	2.1E-02
minimum value	<0.3	<9.0E-03	<1.5E-02	5.0E-03	<5.0E-03

*Sample was not quantified (presence/absence only)
ND = not done

Table 5. Occurrence of pathogens offshore of the Ala Wai Canal (AW2).

Date	*Salmonella* MPN/L	Enterovirus MPN/L	Adenovirus MPN/L	Crypto oocysts/L	*Giardia* cysts/L
02/15/94	**0.4**	<9.6E-03	<1.5E-02	<5.4E-03	**5.0E-03**
06/21/94	<0.3	<9.4E-03	<1.5E-02	<4.9E-03	**1.0E-02**
11/15/94	<0.3	<1.6E-02	ND	<5.0E-03	**1.0E-02**
maximum value	4.0E-01	<1.6E-02	<1.5E-02	<5.4E-03	1.0E-02
mean	<0.33	<1.2E-02	<1.5E-02	<5.1E-03	8.3E-03
minimum value	<0.3	<9.4E-03	<1.5E-02	<4.9E-03	5.0E-03

ND = not done

Table 6. Occurrence of pathogens in Manoa Stream (MS). *

Date	*Salmonella* MPN/L	Enterovirus MPN/L	Adenovirus MPN/L	Crypto oocysts/L	*Giardia* cysts/L
10/25/93	+*	<2.0E-02	ND	<8.1E-03	<8.1E-03
02/14/94	**1.5**	<7.4E-02	<1.6E-02	<1.0E-02	<1.0E-02
06/24/94	+	<1.3E-02	<1.6E-02	**5.5E-02**	**4.5E-02**
11/18/94	**0.9**	<1.6E-02	ND	**1.0E-02**	**5.0E-02**
maximum value	1.5E+00	<7.4E-02	<1.6E-02	5.5E-02	5.0E-02
mean	1.2E+00	<3.1E-02	<1.6E-02	3.3E-02	4.8E-02
minimum value	9.0E-01	<1.3E-02	<1.6E-02	<8.1E-03	<8.1E-03

*Samples were not quantified (presence/absence only)
ND = not done

Table 7. Occurrence of pathogens at Waikiki Beach (W1).

Date	*Salmonella* MPN/L	Enterovirus MPN/L	Adenovirus MPN/L	Crypto oocysts/L	*Giardia* cysts/L
10/26/93	-*	<1.1E-02	ND	<4.4E-03	<4.4E-03
12/01/93	-*	ND	ND	<4.6E-03	<4.6E-03
12/15/93	ND	<1.0E-02	ND	ND	ND
01/27/94	-*	<9.6E-03	<1.6E-02	**1.0E-02**	**1.0E-02**
02/18/94	<0.3	<1.4E-02	<1.5E-02	<5.0E-03	<5.0E-03
03/16/94	<0.3	<1.0E-02	ND	<3.7E-03	<3.7E-03
04/25/94	<0.3	<2.5E-02	<1.5E-02	**5.0E-03**	<4.9E-03
05/11/94	<0.3	ND	ND	ND	ND
06/07/94	ND	<1.1E-02	<1.5E-02	<5.0E-03	**5.0E-03**
06/20/94	<0.3	<1.2E-02	ND	**1.0E-02**	<5.0E-03
07/11/94	<0.3	**>2.1E-01****	ND	<4.8E-03	<4.8E-03
08/15/94	<0.3	<2.1E-02	<1.5E-02	**5.0E-03**	**5.0E-03**
09/13/94	<0.3	<1.0E-02	<1.6E-02	**<5.0E-03**	**5.0E-03**
10/24/94	<0.3	<1.2E-02	<1.7E-02	<5.0E-03	<5.0E-03
11/14/94	<0.3	<1.0E-02	<1.5E-02	<5.0E-03	<5.0E-03
maximum value	<0.3	>2.1E-01	<1.7E-02	1.0E-02	1.0E-02
mean	<0.3	>2.1E-01	<1.5E-02	7.5E-03	6.3E-03
minimum value	<0.3	<9.6E-03	<1.5E-02	5.0E-03	5.0E-03

*Samples were not quantified (presence/absence only)
**Sample was lost so total concentration of virus could not be determined
ND = not done

Table 8. Occurrence of pathogens at Queen's Surf Beach (Waikiki Beach) (Q1).

Date	*Salmonella* MPN/L	Enterovirus MPN/L	Adenovirus MPN/L	Crypto oocysts/L	*Giardia* cysts/L
12/01/93	-*	<1.1E-02	ND	<4.3E-03	<4.3E-03
12/15/93	-*	ND	ND	ND	ND
01/27/94	-*	<9.7E-03	ND	<4.9E-03	**5.0E-03**
03/16/94	<0.3	<9.0E-03	**4.0E-02**	<4.1E-03	<4.1E-03
04/25/94	<0.3	<1.9E-02	ND	<3.6E-03	<3.6E-03
05/11/94	<0.3	ND	ND	ND	ND
06/07/94	ND	<1.0E-02	ND	**1.0E-02**	**5.0E-03**
07/11/94	<0.3	<1.1E-02	**1.7E-02**	<4.9E-03	<4.9E-03
08/15/94	<0.3	<1.1E-02	ND	<5.1E-03	<5.1E-03
09/13/94	<0.3	<9.6E-03	<1.6E-02	<5.0E-03	<5.0E-03
10/24/94	<0.3	<1.1E-02	<1.6E-02	<5.0E-03	<5.0E-03
maximum value	<0.3	<1.9E-02	4.0E-02	1.0E-02	5.0E-03
mean	<0.3	<1.1E-02	2.8E-02	1.1E-04	5.0E-03
minimum value	<0.3	<2.2E-02	<1.6E-02	<3.6E-03	<3.6E-03

*Samples were not quantified (presence/absence only)
ND = not done

Table 9. Occurrence of pathogens at Ala Moana Beach (AM1).

Date	*Salmonella* MPN/L	Enterovirus MPN/L	Adenovirus MPN/L	Crypto oocysts/L	*Giardia* cysts/L
10/25/93	-*	<1.4E-02	<1.9E-02	<9.8E-03	<9.8E-03
12/07/93	-*	<1.4E-02	<1.9E-02	<3.3E-03	<3.3E-03
01/20/94	-*	ND	ND	<4.0E-03	<4.0E-03
01/31/94	ND	<1.3E-02	<1.9E-02	ND	ND
02/14/94	<0.3	<9.1E-03	<1.9E-02	<4.9E-032	<4.9E-032
03/21/94	<0.3	ND	ND	<4.9E-032	<4.9E-032
04/18/94	<0.3	<1.4E-02	ND	**5.0E-03**	**8.0E-02**
06/01/94	<0.3	<1.2E-02	ND	<5.0E-03	<5.0E-03
06/20/94	<0.3	<1.2E-02	**5.6E-02**	**2.5E-02**	**5.0E-03**
07/05/94	<0.3	**3.7E-02**	ND	<5.0E-03	<5.0E-03
08/09/94	<0.3	<9.9E-03	ND	<5.0E-03	<5.0E-03
09/12/94	<0.3	<1.1E-02	<1.6E-02	**1.5E-02**	**1.0E-02**
10/17/94	<0.3	<1.1E-02	<1.7E-02	<1.4E-03	<1.4E-03
11/14/94	<0.3	<1.3E-02	<2.0E-02	<5.0E-03	**1.5E-02**
maximum value	<0.3	3.7E-02	5.6E-02	2.5E-02	8.0E-02
mean	<0.3	3.7E-02	5.6E-02	1.5E-02	2.8E-02
minimum value	<0.3	<1.1E-02	<1.6E-02	<1.4E-03	<1.4E-03

*Samples were not quantified (presence/absence only)
ND = not done

Table 10. Occurrence of pathogens at Sand Island Beach (SBI).

Date	*Salmonella* MPN/L	Enterovirus MPN/L	Adenovirus MPN/L	Crypto oocysts/L	*Giardia* cysts/L
12/01/94	<0.3	ND	ND	ND	ND
02/17/94	<0.3	<1.1E-02	ND	<5.0E-03	<5.0E-03
06/22/94	<0.3	<9.2E-03	ND	<5.0E-03	<5.0E-03
11/17/94	<0.3	<1.2E-02	<1.5E-02	<5.0E-03	<5.0E-03
maximum value	<0.3	<1.2E-02	<1.5E-02	<5.0E-03	<5.0E-03
mean	<0.3	<1.1E-02	<1.5E-02	<5.0E-03	<5.0E-03
minimum value	<0.3	<9.2E-03	<1.5E-02	<5.0E-03	<5.0E-03

ND = not done

Table 11. Occurrence of pathogens at Hanauma Bay (HB1).

Date	*Salmonella* MPN/L	Enterovirus MPN/L	Adenovirus MPN/L	Crypto oocysts/L	*Giardia* cysts/L
10/26/93	<0.3	<1.1E-02	<1.5E-02	<1.0E-04	<1.0E-04
12/15/93	<0.3	<2.1E-02	ND	<4.7E-03	<4.7E-03
02/07/94	<0.3	<1.3E-02	<1.6E-02	<4.7E-03	<4.7E-03
02/18/94	<0.3	<1.4E-02	<1.9E-02	<5.0E-03	<5.0E-03
03/09/94	<0.3	<1.0E-02	<1.9E-02	<5.0E-03	<5.0E-03
04/21/94	<0.3	<1.6E-02	ND	<5.0E-03	**5.0E-03**
05/02/94	<0.3	**4.4E-02**	ND	<4.7E-03	<4.7E-03
06/24/94	<0.3	<1.3E-02	<1.7E-02	<5.1E-03	<5.1E-03
07/12/94	<0.3	<1.2E-02	<1.7E-02	<5.1E-03	<5.1E-03
08/16/94	<0.3	**1.2E-02**	<1.6E-02	<5.0E-03	<5.0E-03
09/20/94	<0.3	<9.5E-03	<1.6E-02	<5.0E-03	<5.0E-03
10/31/94	<0.3	<1.0E-02	ND	<5.0-E-03	<5.0E-03
11/18/94	<0.3	<1.2E-02	<1.6E-02	<4.8E-03	<4.8E-03
maximum value	<0.3	4.4E-02	<1.7E-02	<5.1E-03	5.0E-03
mean	<0.3	2.8E-02	<1.6E-02	<4.6E-03	5.0E-03
minimum value	<0.3	<9.5E-03	<1.5E-02	<1.0E-04	<1.0E-04

*Hanauma Bay is not impacted by the sewage outfall and Ala Wai Canal.
ND = not done

Table 12. Occurrence of pathogens offshore of Diamond Head (E4S).

Date	*Salmonella* MPN/L	Enterovirus MPN/L	Adenovirus MPN/L	Crypto oocysts/L	*Giardia* cysts/L
10/27/93	-*	<1.7E-02	<1.7E-02	<1.0E-02	<1.0E-02
02/15/94	<0.3	<1.4E-02	ND	<5.0E-03	<5.0E-03
06/21/94	<0.3	<1.5E-02	<2.0E-02	<4.9E-03	<4.9E-03
11/15/94	<0.3	<1.5E-02	ND	<5.0E-03	<5.0E-03
maximum value	<0.3	<1.5E-02	<2.0E-02	<1.0E-02	<1.0E-02
mean	<0.3	<1.5E-02	<1.8E-02	<6.2E-03	<6.2E-03
minimum value	<0.3	<1.4E-02	<1.7E-02	<4.9E-03	<4.9E-03

*Samples were not quantified (presence/absence only)
ND = not done

APPENDICES

A. ISOLATION OF SALMONELLA FROM ENVIRONMENTAL SAMPLES BY CULTURE METHODS

OVERVIEW

Salmonellas are the pathogenic microorganisms most frequently found in polluted waters. In addition to surviving in the animal intestinal tract, they may also exist as free living forms which proliferate under environmental conditions. (Hendricks and Morrison, 1967). Since the presence of Salmonellae in marine recreational waters creates a public health hazard, detection of these pathogens is important in prevention of salmonellosis outbreaks.

For the detection of *Salmonella* in marine water as well as stream, canal, and sewage samples, a traditional culture methodology was used (Alonso, et al. 1992). Based on a Standard Methods Most Probable Number technique, the steps included sample concentration and enrichment followed by plating on selective media. Confirmation of presumptive isolates was accomplished by the use of biochemical, serological and/or PCR techniques. The sample sites are located on the map in Fig 1. The sample processing scheme is shown in Figure 2.

METHODS

SAMPLE CONCENTRATION

For the sewage sample, volumes of 10ml, 1ml and 0.1ml was used to directly inoculate the enrichment broth. However, for all other sites, including the canal, stream, beach and ocean waters, the samples were concentrated prior to enrichment. Volumes of 1 liter, 100ml, and 1ml of water were concentrated by filtration using a sterile 0.45um pore size membrane (Millipore). The membrane containing the trapped bacteria was directly immersed into the enrichment broth for subsequent culturing. For MPN analysis 3 replicates of three volumes were examined in triplicate.

ENRICHMENT

For each sample, three replicates of the three volumes were inoculated into a bottle of NR10 enrichment broth. The formulation per liter is Tryptone 4.54g, Sodium Chloride 7.2g, Potassium

Dihydrogen Phosphate 1.45g, Magnesium Chloride, Anhydrous, 13.4g, and Malachite Green Oxalate, 0.036g. Final ph 5.1. For Sewage samples, enrichment broth was supplemented with 40mg/ml of sodium novobiocin. For all other samples, 10mg/ml of sodium novobiocin was added. Samples were incubated for 48hrs at 43C. This step allowed for the growth of salmonella while inhibiting the growth of competing organisms which may produce *Salmonella*-like colonies.

ISOLATION ON SELECTIVE AGAR PLATES

A subsample from each enrichment bottle is streaked onto agar plates containing media which selects for growth and distinct colony appearance of *Salmonella*. For this study, Hectoen Enteric agar (Difco) was used. After incubation for 24-48 hours at 35C, plates were examined for the presence of colonies indicative of salmonella. Individual isolates that had the appearance of salmonella were selected and streaked for single colony isolation onto XLT (FMTI Inc, Riviera Beach, Florida) agar plates, another media selective for salmonella. Colonies on XLT with the morphology of *Salmonella* were considered presumptive and were subjected to confirmation.

CONFIRMATION OF SELECTED ISOLATES

Biochemical and serological confirmation of presumptive isolates was performed using the Biolog Identification system (Biolog, In, Hayward, CA), and Oxiod Salmonella Rapid Latex Kit (Unipath, Ltd., Hampshire England) using the manufacturers' recommended protocol. In addition, the Polymerase Chain Reaction using a multiplex system developed in our lab (Way et. al. 1993) was performed on individual environmental isolates for further confirmation of the presence of *Salmonella* in the water or sewage samples.

ENUMERATION

Isolates from every tube in which *Salmonellae* were confirmed were regarded as positive. The MPN of *Salmonella* was obtained from probability tables (Standard Methods for the Examination of Water and Wastewater.)

RESULTS

Sample locations and dates positive for *salmonella* are listed in Table 1. Table 2 lists the sample locations and dates that did not have culturable *salmonella* present. All thirteen sewage samples were positive for salmonella. Values ranged from 10^2 to 10^4 CFU's per liter. In addition, stream samples for all four sampling periods were positive for salmonella, with values around 1 *salmonella* with a range of 0.4 to 4.3 CFU's per liter. Ala Wai offshore (AW2) sample date 2/15/94 had 0.4 CFU's per liter, while both other dates for this sample were negative. Samples positive for salmonella are listed in Table 1.

In all other samples, no contamination with Salmonella was found. Beaches tested included Sand Island, Ewa Park, Queen's Surf, Hanauma Bay, Waikiki, and Ala Moana. Ocean samples include Diamond Head (surface), Sand Island (Bottom) and Pearl Harbor. A list of the samples negative for salmonella by culture methods , as well as the sampling dates, are found in Table 2.

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Table 1: SAMPLES POSITIVE FOR SALMONELLA BY CULTURE METHODS

SEWAGE (S1) 13 positive samples out of 13 tested

DATE	MPN PER LITER
10/25/93	+*
12/06/93	+*
02/01/94	+*
02/14/94	1.0×10^4
03/09/94	2.0×10^4
04/21/94	2.3×10^2
05/02/94	2.4×10^3
06/20/94	1.5×10^3
07/12/94	2.4×10^3
08/16/94	2.3×10^2
09/20/94	4.3×10^2
10/31/94	4.3×10^2
11/14/94	7.5×10^2

ALA WAI CANAL (AW1) 7 positive samples out of 13 tested

DATE	CFU PER LITER
10/25/93	+*
02/14/94	4.3
04/18/94	0.9
06/20/94	0.4
07/05/94	0.9
10/17/94	0.9
11/14/94	0.9

ALA WAI OFFSHORE (AW2) 1 positive sample out of 3 tested

DATE	CFU PER LITER
02/15/94	0.4

MANOA STREAM (MS) 2 positive samples out of 4 tested

DATE	CFU PER LITER
10/25/93	+*
02/14/94	1.5
06/24/94	+
11/18/94	0.9

*Samples not quantified (presence/absence only)

Table 2: SAMPLES NEGATIVE FOR SALMONELLA BY CULTURE METHODS

ALA WAI OFFSHORE (AW2)	06/21/94	11/15/94	
2 negative samples out of 3 tested			
EWA BEACH PARK (EW1)	12/01/93		
1 negative sample out of 1 tested			
DIAMOND HEAD - SURFACE (E4S)	10/27/93	06/21/94	
4 negative samples out of 4 tested	02/15/94	11/15/94	
SAND ISLAND - BOTTOM (D2B)	10/28/93	06/23/94	
4 negative samples out of 4 tested	02/16/94	11/16/94	
PEARL HARBOR (C2)	10/29/93	06/22/94	
4 negative samples out of 4 tested	02/17/94	11/17/94	
SAND ISLAND BEACH (IS1)	12/01/93	06/22/94	
4 negative samples out of 4 tested	02/17/94	11/17/94	
ALA WAI CANAL (AW1)	12/07/93	06/01/94	
6 positive samples out of 13 tested	01/20/94	08/09/94	
	03/21/94	09/12/94	
QUEEN'S SURF BEACH (Q1)	12/01/93	05/11/94	
10 negative samples out of 10 tested	12/15/93	07/11/94	
	03/16/94	09/13/94	
	04/25/94	10/24/94	
HANAUMA BAY (HB1)	10/26/93	04/21/94	09/20/94
13 negative samples out of 13 tested	12/15/93	05/02/94	10/31/94
	02/07/94	06/24/94	11/18/94
	02/18/94	07/12/94	
	03/09/94	08/16/94	
WAIKIKI BEACH (W1)	10/26/93	04/25/94	09/13/94
13 negative samples out of 13 tested	12/01/93	05/11/94	10/24/94
	01/27/94	06/20/94	11/14/94
	02/18/94	07/11/94	
	03/16/94	08/15/94	
ALA MOANA BEACH (AM1)	10/25/93	04/18/94	09/12/94
13 negative samples out of 13 tested	12/07/93	06/01/94	10/17/94
	01/20/94	06/20/94	11/14/94
	02/14/94	07/05/94	
	03/21/94	08/09/94	

*Samples were not quantified (presence/absence only)

**Negative result for quantified samples (after 02/07/94) indicates less than 0.3 CFU per liter

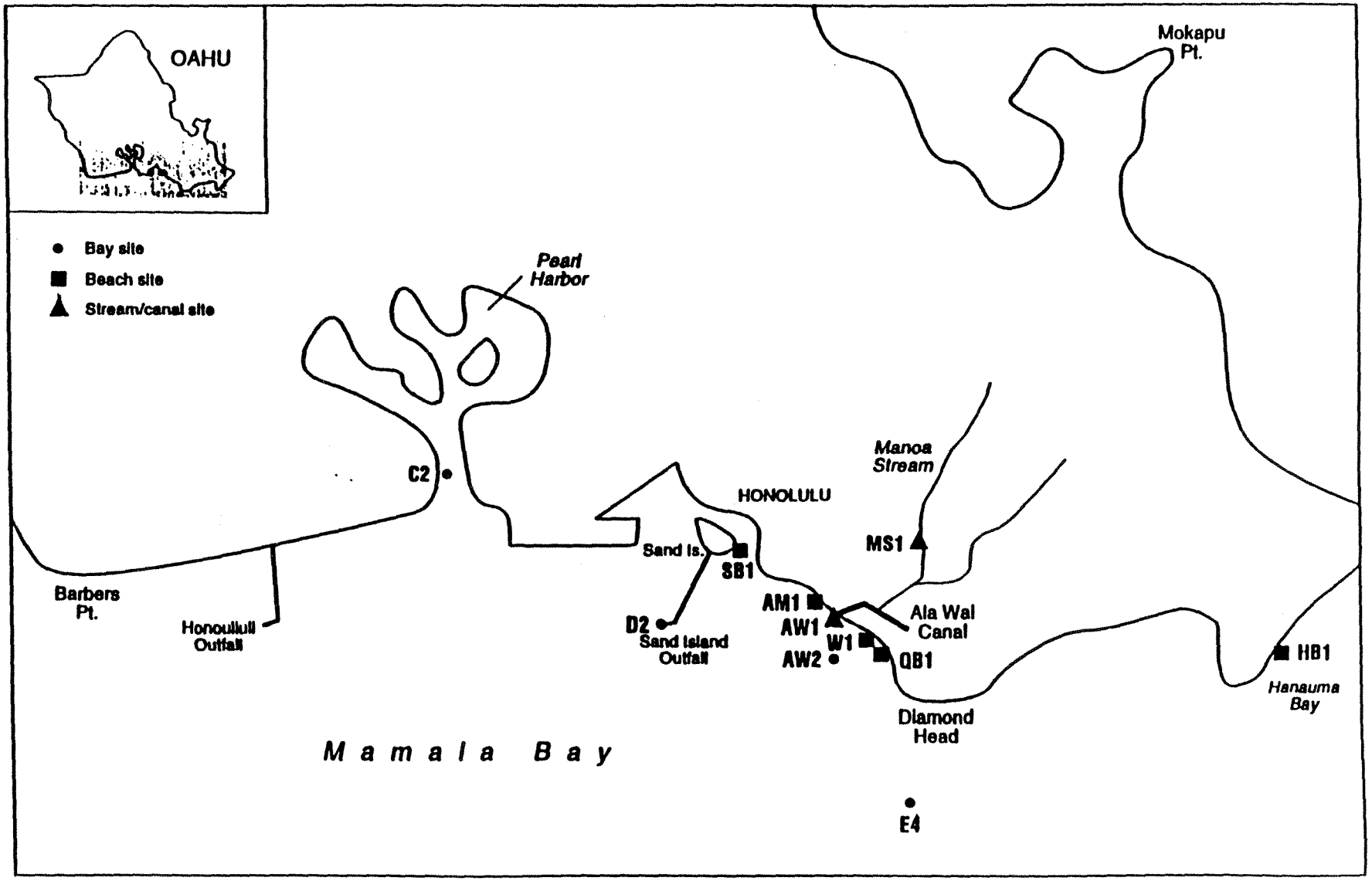

Figure 1. Mamala Bay Study Sites

Fig 2: SAMPLE PROCESSING SCHEME FOR SALMONELLA

1. SAMPLES

Filtration/Concentration	Volume	Direct Inoculation	Volume
Stream	1000mls	Sewage	10mls
Canal	100mls	1ml	
Marine Water	10mls	0.1ml	
Three replicates of each volume		Three replicates of each volume	

↓ Inoculation

2. ENRICHMENT

Bottles of Selective Broth

Incubation at 43C for 48hrs

↓

A loopful from each tube

3. ISOLATION

Inoculation of Selective agar plates

Incubation 35C 24-48hrs

↓

Colonies with typical Salmonella morphology

4. SECONDARY ISOLATION

Inoculation of Selective agat plates

Incubation 35C 24hrs

Colonies with typical Salmonella morphology

↓

5. CONFIRMATION

Biochemical (Biolog)	Serological (Agglutination)	Molecular (PCR)

↓

6. ENUMERATION

Quantification by MPN Tables

B. DIRECT PCR DETECTION OF NATURALLY OCCURRING *E.COLI* AND *SALMONELLA* FROM DIVERSE ENVIRONMENTAL SAMPLES

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This study describes methodologies for direct PCR detection of naturally occurring *E.coli* and *Salmonella* in environmental samples. The efficacy of the protocol is demonstrated on sewage, marine water and fresh water samples collected from Honolulu, Hawaii. Vortex flow filtration, along with centrifugation, was used to concentrate bacteria from aquatic samples. Cell lysis was accomplished by sonication and thermal shock treatments. Extracted DNA was purified by passage through Elutip d minicolumns. Purified DNA was analyzed by PCR amplification using *E.coli* and *Salmonella* specific primers. Use of this methodology allowed detection of *E.coli* in sewage, marine and fresh waters. *Salmonella* was found in sewage. Simultaneous analysis of the environmental samples by traditional cultural assay allowed for evaluation of the PCR protocol to detect specific bacterial DNA sequences in aquatic environments. A comparison of culturable and PCR methodologies suggests that each approach has its limitations. Culturable counts will not detect viable but nonculturable organisms, whereas PCR will. However, the PCR process can be compromised, especially when environmental samples with high levels of inhibitors such as metals or humic substances are concentrated along with low levels of target DNA.

In clinical studies, pathogenic bacteria are often routinely cultured using dilution and plating techniques. However, these techniques are not always successful for the detection of pathogens in complex environmental samples such as marine and fresh waters, or sewage that can contain high concentrations of colloids, humic substances, inorganic salts and/or metals (Tsai and Olson, 1992b). These compounds can cause abiotic stress on introduced pathogens resulting in organisms that are sublethally injured, and subsequently viable but nonculturable (Roszak and Colwell). Xu et al., (1982) found that viable *E. coli* persist in natural aquatic ecosystems even when the organism was no longer recoverable by conventional culture methods. In addition, concentrations of pathogens in environmental samples may be extremely low, particularly in comparison to higher levels of non-target community bacteria, making selective enrichment a requirement prior to dilution and plating. Consequently, pathogens in the environment capable of impairing human health and welfare may go undetected by cultural assay.

Polymerase chain reaction (PCR) has been used to detect a variety of pathogenic or indicator organisms including *E.coli* in sewage (Tsai et al., 1993) and *Salmonella* in groundwaters (Way et al.). Unlike the cultural assays, PCR will detect nonviable as well as viable but

nonculturable bacteria (Josephson et. al. 1993). However, analysis of environmental samples by PCR can also be problematic.

Total cell lysis within the environmental sample is necessary for efficient DNA extraction and amplification. Picard et al., (1992) successfully used sonication to lyse and release bacterial cells from soil samples. In addition, DNA purification is a critical yet difficult step for successful DNA amplification, since inhibitory substances can interfere with Taq enzyme activity resulting in false negatives (Tsai and Olson, 1992b). Ancillary to this, the PCR detection of organisms in marine or fresh waters often requires large sample volumes to be processed due to their naturally low bacterial biomass. Concentration of large sample volumes to the small amounts used as template in PCR reactions also results in the concentration of PCR inhibitory substances (Kelly et al., 1995).

The vortex flow filtration (VFF) Benchmark system has been shown to be an efficient method for the concentration of microbial populations from aquatic environments (Paul et al., 1991; Jiang et al., 1992). In this system, Taylor vortices are established by rotation of a cylindrical filter inside a second filter. The sample is fed under pressure between the two cylindrical surfaces forcing fluid across the filter, and into the inner cylinder for collection or out to waste. Recently VFF has been used to collect enteroviruses and hepatitis A virus from ocean water with subsequent detection by PCR (Tsai et al., 1993).

In this study, we have developed a protocol that allows direct PCR detection of naturally occurring *E.coli* and *Salmonella* in environmental samples. VFF was used to concentrate bacteria from environmental waters. Following lysis of bacteria by sonication, the DNA was harvested, purified and analyzed by PCR. In addition, environmental samples were assayed for *E. coli* and *Salmonella* using standard cultural techniques. The efficacy of the methodology was evaluated on sewage as well as marine, stream and canal water sampled around Honolulu, Hawaii. These samples potentially contained pathogens from point sources including sewage outfalls, streams and canals, or from humans in direct recreational contact with the ocean water.

MATERIALS AND METHODS

Sample collection. Water samples were collected during a period starting October 1993 and ending September 1994. Samples for cultural assay were stored on ice during collection and

transport, and were processed immediately upon return to the laboratory. Larger volumes of 20 to 30 liters were needed for DNA extraction and PCR analysis. Concentration of this larger volume was performed by Membrex filtration. Water samples were filtered immediately upon arrival in the laboratory. Alternatively, a few samples were held at 4C and filtered within 24 hours. The location of all sampling sites is shown in Fig. 1.

Sample concentration for DNA extraction. A Membrex Benchmark VFF device fitted with a 400 cm^2 100 kDa filter was used to concentrate marine, canal and stream samples. Initial volume of the environmental sample was 20-30 liters. Filtration was performed at 7-9 lb/in^2 with a rotation speed of 1500 rpm. Final volume of the sample after membrexing was approximately 50 mls. Samples were further concentrated to 4-8 mls by centrifugation at 10,000 x g for 30 min. A cleaning protocol based on manufacturers recommendations was used to assure that there was no contamination between sample runs. For sewage, a 25 ml sample was concentrated by centrifugation at 10,000 x g for 10 min to a final volume of 2.5 ml. Samples were stored at -20C until subjected to purification and PCR analysis.

Efficiency membrex filtration for recovery of bacteria. To check the performance of our Membrex unit, recovery studies were performed prior to its use on environmental samples. Eight liters of sterile deionized water was seeded with pure cultures of *E.coli* ATCC 15225 or *Salmonella* ATCC 6539 at a rate of 10^4 to 10^6 CFU per ml. The sample was concentrated using the manufacturers protocol as discussed above, to a final volume of 30-40 ml. The resulting concentrate was subject to dilution and plating to obtain culturable counts of bacteria recovered during filtration. Comparison of introduced CFU's to those recovered by filtration was used to calculate percent recovery. In addition, 2 liters of sterile deionized water was subjected to the concentration protocol after undergoing the disinfection procedure to assure the proper cleaning of the unit between samples.

Cell lysis and DNA extraction. The DNA extraction protocol was based on a method developed by Picard et al., 1992. To the previously concentrated sample, 5x buffer (1M NaCl, 100 mM Tris HCl, 5 mM EDTA, 5% polyvinylpolypyrrolidone, pH 7.4) was added to a final concentration of 1x. The sample was quickly thawed in a water bath and held on ice while processing. The sample, contained in a 15 ml polypropylene centrifuge tube, was immersed in a microcup horn attachment of a high intensity ultrasonic processor (Cole Palmer Instrument

Company, Chicago, Ill). Cooling was accomplished by continuous water circulation through the system. Sonication was performed for 10 min at 20 watts. After sonication, the sample was centrifuged for 2 min at 8000 x g to pellet cell debris. The supernatant containing the free DNA was saved.

To extract DNA remaining in association with the pelleted debris, one ml of 1 x buffer was added to the pellet, and it was subjected to 3 cycles of thermal shock: 10 min in dry ice ethanol, 10 min at 65C. The sample was again centrifuged for 2 min at 8000 x g and the supernatant saved. Two more cycles of washing/centrifugation were performed to recover DNA from the environmental sample. All the supernatants from a sample were pooled and subjected to purification. To evaluate the efficiency of extraction of DNA from the environmental sample, a portion of the remaining pelleted cellular debris was resuspended in water and, visualized by electrophoresis using a 1.6% agarose gel.

DNA purification. Using the manufacturers instructions, Elutip d purification columns were prepared (Schleicher and Schuell, Keene, N.H.). The pooled supernatant from each sample was passed through the Elutip d prefilter and column. The sample was recovered from the column with two 400 ul washes with high salt buffer. The DNA was precipitated in 2.5 volumes of ethanol at -20C overnight and pelleted by centrifugation in the cold for 20 min at 14,000. The pellet was washed in 70% ethanol, air dried, and resuspended in TE buffer with (1 mg per ml) RNAse. The DNA was stored at 4C. Canal and stream water samples were subjected to 2 Elutip d purifications.

Quantification and amplification of DNA. Fluorometer model TKO 100 (Hoefer Scientific Instruments, San Francisco, CA) was used to quantify the extracted DNA. In addition, some DNA samples were visualized on a 1.6% agarose gel stained with EtBr, and viewed under a transilluminator. A portion of the resulting DNA was subjected to PCR analysis using previously published protocols (Josephson et al.,1991; Way et al., 1993). PCR reaction volume was 50ul. The scheme for the extraction/purification protocol is shown in Fig. 2.

Efficiency of extraction/purification protocol. The efficiency of the extraction/purification protocol was tested using pure culture isolates of *E.coli* and *Salmonella*. Late log phase broth cultures were frozen at -20C overnight to simulate the storage conditions of the environmental samples. A volume of 2.5 ml was lysed by sonication and extracted by thermal

shock treatments and successive washes of the pellet as described above. After Elutip d purification and EtOH precipitation, the purified DNA was quantified using the fluorometer. Direct counts of the broth culture were also made using acridine orange staining (Hobbie et al., 1977). Using the assumption that one cell is equivalent to 9 fg of DNA, a theoretical number of cells recovered after processing the DNA was obtained. Results for *E.coli* and *Salmonella* are listed in Table 1A and 1B. Serial dilutions of the resulting *E.coli* or *Salmonella* DNA were subjected to PCR amplification using *lamB* primers and Multiplex primers respectively (Josephson et al., 1991; Way et al., 1993).

Cultural counts of *E.coli.* Enumeration of *E. coli* in the water samples was performed using a membrane filtration technique as outlined in Standard Methods for the Examination of Water and Wastewater 17th edition. (APHA, 1989). For the marine samples, 100mls of water was filtered, whereas the sewage, canal and stream samples were diluted prior to filtration. As a resuscitation step, membranes were placed on Difco mTEC media, and incubated for 2 hrs at 30C. This was followed by incubation in a water bath for 22 hr at 44.5C. The membrane was transferred to a filter pad saturated with urease. After 15min yellow or yellow-brown colonies were considered presumptive *E. coli*

Cultural counts of *Salmonella.* Modification of a standard methods MPN technique was used for the isolation of *Salmonella* from the environmental samples (Alonso, et al., 1992). Samples were subjected to selective enrichment for 48hrs at 42C in RV broth supplemented with 40 mg/ml of sodium novobiocin for sewage, and 10mg/ml of sodium novobiocin for all other samples. For the sewage sample, volumes of 10ml, 1ml, and 0.1ml were assayed. For all other sites, volumes of 1 liter, 100ml and 1ml were concentrated by filtration using a sterile 0.45 um membrane (Millipore). The membrane containing the trapped bacteria was directly immersed into the enrichment broth with subsequent culturing onto Hectoen Enteric Agar plates (Difco). After incubation at 35C for 24-48hrs, plates were examined for the presence of colonies indicative of *Salmonella*. Three replicates of three volumes were examined.

Presumptive isolates were subjected to confirmation using the Biolog identification system (Biolog, Inc., Hayward, CA) and Oxoid Salmonella Rapid Latex Kit (Unipath, Ltd., Hampshire England) using the manufacturers recommended protocol. In addition, PCR was performed on individual isolates using the multiplex system developed in our lab (Way et. al. 1993) Every tube in

which isolates were confirmed, was regarded as positive. The MPN of *Salmonella* was obtained from probability tables.

RESULTS AND DISCUSSION

Efficiency of recovery of viable bacteria after membrex concentration. Overall recovery rates were 79.6% and 57.0% for *E.coli* and *Salmonella* respectively. A reduction in the number of culturable bacteria after filtration was likely due to the death of some cells during processing. The fact that recovery rates were lower for *Salmonella* than *E.coli* is consistent with the fact that *Salmonella* are not as hardy as *E.coli*, and are more prone to be lethally injured and/or viable but non culturable. Since detection of bacteria by PCR relies on amplification of gene sequences rather than on culturable bacteria, loss of culturability of pathogens originally present in the environmental sample is inconsequential. Jiang et al. (1992) used DAPI epifluorescent microscopy to evaluate the efficiency of the Benchmark membrex system for concentrating bacterial cells. In their study, direct counts of bacteria yielded an overall recovery of 80.5%. These data indicate that membrex filtration is a suitable technology for the recovery of bacteria from water samples.

To prevent contamination between samples, the membrex unit was disinfected and rinsed with 2 liters of sterile distilled water. As a negative control, 2 liters of sterile deionized water was subjected to membrex concentration. No culturable organisms were detected in the control retentate. Furthermore, the control membrex sample was further concentrated by centrifugation and a portion was used as template for PCR amplification of the *lam b* sequence. Lack of amplification of the control sample after double PCR negated the possibility of cross contamination of samples.

Efficiency of recovery of DNA after extraction and purification. The yield of DNA extracted from pure cultures of *E.coli* and *Salmonella* is shown in Table 1. The yields are based on a theoretical input of DNA from bacterial cells assuming 9 fg of DNA per cell. These calculated DNA values were then compared to the total amount of purified DNA obtained after the extraction and purification procedure. The % of DNA recovered from *E.coli* was 19.0, 23.7, and 21.0 for samples a, b, and c, respectively. Equivalent recovery rates for *Salmonella* were a: 10.2%, b: 12.0% ,and c: 14.6%. These values are based on the input of AODC direct counts.

For all 3 samples *E. coli* recovery rates reflected an approximate 0.5 log reduction in number of cells recovered (theoretical) compared with the original number of total cells (measured). Salmonella samples showed a log reduction for all three trials.

To access the ability of the protocol to lyse and extract total DNA in environmental samples, a portion of the resulting purified community DNA was visualized using gel electrophoresis. The sonication step resulted in the release of DNA ranging in size from 4000 to 100 bp. In addition, a portion of the pelleted debris remaining after DNA extraction was subjected to agarose gel visualization. No DNA was observed on the gel, suggesting efficient recovery of the DNA from the sample debris.

Successful PCR amplification of a specific gene sequence from an environmental sample necessitates complete cell lysis and efficient extraction of pure DNA. To date, many bacterial DNA extraction/PCR amplification procedures have been performed on sediments or soils. Methods have included bead beating plus sodium dodecyl sulfate (SDS) to lyse the cells, followed by phenol-chloroform extraction of DNA and purification via CsCl-ethidium bromide density gradient ultracentrifugation (Ogram et al., 1987). Tsai and Olson (1991) treated sediments with lysozyme and rapid freezing and thawing (-70 to 65°C) to lyse cells, followed by phenol-chloroform extraction of DNA, precipitation with isopropanol and purification by Sephadex 100 (Pharmacia). More recently Leff et al. (1995) compared several methods of DNA extraction from soil. They concluded that the method of choice depended on which analysis was to be performed subsequent to DNA extraction.

More et al. (1994) compared several methods of cell lysis procedures including bead-mill homogenization and freeze/thaw methodologies. They concluded that physical disruption of cells was critical to harvest maximal amounts of DNA. Picard et al., (1993) took advantage of the fact that intact DNA was not required for the PCR process, and used sonication to lyse and release bacteria from soil samples. This is the foundation for our approach, in which sonication was a critical component of the cell lysis procedure.

Purification of extracted DNA can involve CsCl density ultracentrifugation (Ogram), Sephadex spun columns (Tsai and Olson, 1992b; Tsai et al., 1993) or commercial DNA purification columns such as Spin Bind cartridges (FMC Bioproducts, Rockland Maine). Problems with these procedures include length of time for analysis as well as dilution effects and reproducibility of spun

columns. In this study, we found that commercial Elutip columns were easy to use, relatively inexpensive, reproducible and efficient at DNA purification. Such purification is vital to remove PCR inhibitory substances such as humic substances. Tsai and Olson (1992a) showed that PCR detection of 16S rDNA sequences had a limit of sensitivity of 1 x 10^5 CFUs/g of soil without DNA purification, compared to 70 CFUs/g of soil with purification (1992b). Purification has also been demonstrated to be effective on sewage and sludge (Tsai et al., 1993).

Sensitivity of detection of extracted *E.coli* and *Salmonella* DNA by PCR analysis. Successful PCR detection of extracted *E.coli* and *Salmonella* DNA is shown in Table 2. Aliquots of extracted DNA were diluted and subjected to PCR amplification using *lamB* and multiplex primers for *E.coli* and *Salmonella* respectively. Table 2 shows the PCR results for each theoretical total number of cells used as template. Sensitivity after single PCR was 10^3 - 10^4 cells for *E.coli* compared to 10^2 - 10^3 cells for *Salmonella*. This data is similar with our previous work using both the lam b primers (Josephson et al.,1991) and the multiplex primers (Way et al., 1993), where crude cell lysates of pure cultures were used as template for the amplification of *E. coli* and *Salmonella* respectively. It also confirms the work of Picard et. al. (1992), who found that fragmentation of DNA did not affect the yield of PCR product when short sequences are targeted for amplification. These data show that for purified DNA, PCR sensitivity was excellent and unaffected by extraction/purification procedure.

PCR detection of *E.coli* and *Salmonella* in environmental samples. Marine samples were collected from various sites within Mamala Bay Hawaii, outside of Honolulu. Between 10 and 30 liters of sample was used for extraction, with an equivalent volume of 1 to 3 liters used as template in the PCR reaction for *E. coli* amplification, and 8 liters for detection of *Salmonella*.. This equivalent volume for PCR can be further equated to the number of CFU's present in the PCR reaction based on cultural data. Table 3 shows results of *E.coli* via *lamB* amplification after single (30 cycles) and double (60 cycles) of PCR for these marine samples. Double PCR was performed only when single PCR was negative. Counts obtained by culture methods are also shown. Six out of nine sites were PCR positive for *E.coli* on one or more occasions, compared to 5 out of 9 sites based on CFU's. In all but one instance, when culturable counts of *E.coli* were obtained, PCR results were positive. Sometimes marine samples contained no culturable counts of *E.coli* and were similarly PCR negative. However on three other occasions samples were PCR positive but plate count negative. In seven instances, PCR detection was below the expected sensitivity of

10^3-10^4 cells per 30 cycles of PCR, or 10^2-10^3 cells per 60 cycles. Thus some marine waters did not appear to contain any *E.coli*, whereas other samples either contained dead cells or viable but no culturable cells. Josephson et al. (1993) demonstrated PCR detection of non viable *E.coli* cells. However, they also showed that in non sterile natural waters, cell and DNA degradation was rapid indicating that a positive PCR result implied the presence of viable or recently viable *E.coli* in the environmental sample. Roszak and Colwell (1987) have described the latter phenomenon of viable but non culturable cells. Finally note that one marine sample contained low culturable counts but was PCR negative. In this instance amplified DNA concentrations were likely too low to be visible on the stained gel.

Detection of *E.coli* in other environmental samples is presented in Table 4. For sewage, which sustains a large population of *E. coli* (10^8 CFU/liter), PCR results were consistently positive with 30 cycles (equivalent of 10^5 CFU's in PCR). However, stream and canal water samples were more problematic for PCR. After membrex concentration these samples resembled sediment, although samples were highly variable in appearance. This unpredictability in sample quality, along with lower CFU values than sewage (10^2-10^5 CFU/L), made PCR assay difficult. Seeded studies in which *E coli* cells were introduced into the PCR reaction along with the environmental sample showed that two column purifications were necessary to remove or reduce PCR inhibitory substances. Reduction of the equivalent volume of canal or stream water in the PCR reaction was another effective approach for obtaining positive PCR. Inhibition may be a function of imperfect purification of the DNA extract, but may also be due to an excess amount of total community DNA which decreases the detection threshold of the sample. (Nesme, et. al. 1995) Since 2 column purifications were necessary to remove PCR inhibition, the minicolumn purification method may not be the method of choice for samples low in target DNA and high in inhibitory substances.

Data in Table 5 shows comparable data for *Salmonella*. For marine samples, no *Salmonella* was detected by cultural or PCR analysis. Presumably these marine samples truly did not contain either culturable *Salmonella* or PCR amplifiable sequences, or were below the detection limits of our assay. Fresh water canal and stream samples were culturally positive but PCR negative. These samples contain PCR inhibitory substances which prevented DNA amplification. Similar PCR inhibition from these samples was reported by Reynolds (1995). Sewage samples were always positive for *Salmonella* regardless of whether analysis was by PCR or cultural methodologies.

Overall, these data demonstrate that the methodologies employed in this study can be used in ecological studies. Little data is available in the literature on direct PCR detection of real pathogens in environmental samples. Most studies have involved sterile systems and/or detection of seeded organisms (Tsai and Olson, 1992a, 1992b). Tsai et al. (1993) did utilize PCR to directly detect *E.coli* in sewage and sludge. Palmer et al., (1993) reported the detection of *Legionella* species in sewage and ocean water. However, their approach was perform PCR on diluted samples, thus diluting out PCR inhibitory substances. To our knowledge no one has reported direct PCR detection of bacterial pathogens in marine and fresh waters using our combined methodologies.

Evaluation of the data suggest that direct PCR detection is only useful on certain kinds of environmental samples. PCR analysis of marine samples which contained relatively low amounts of PCR inhibitory substances, and low numbers of target organisms was successful. Similarly in sewage samples which contained high numbers of target organisms but large amounts of humic like substances, PCR was also successful. However in the canal and stream waters which contained PCR inhibitory substances, and where target organism concentrations were moderate, PCR was not always successful and required larger equivalent sample volumes and/or double purification through the Elutip columns.

In summary, for environmental samples use of cultural analyses or PCR analyses alone can result in false negatives due to the presence of viable but non culturable organisms and PCR inhibitory substances respectively. Both techniques should be conducted to evaluate the real potential health hazard of the pathogens. The methodologies presented in this paper allow for successful direct PCR detection of such pathogens.

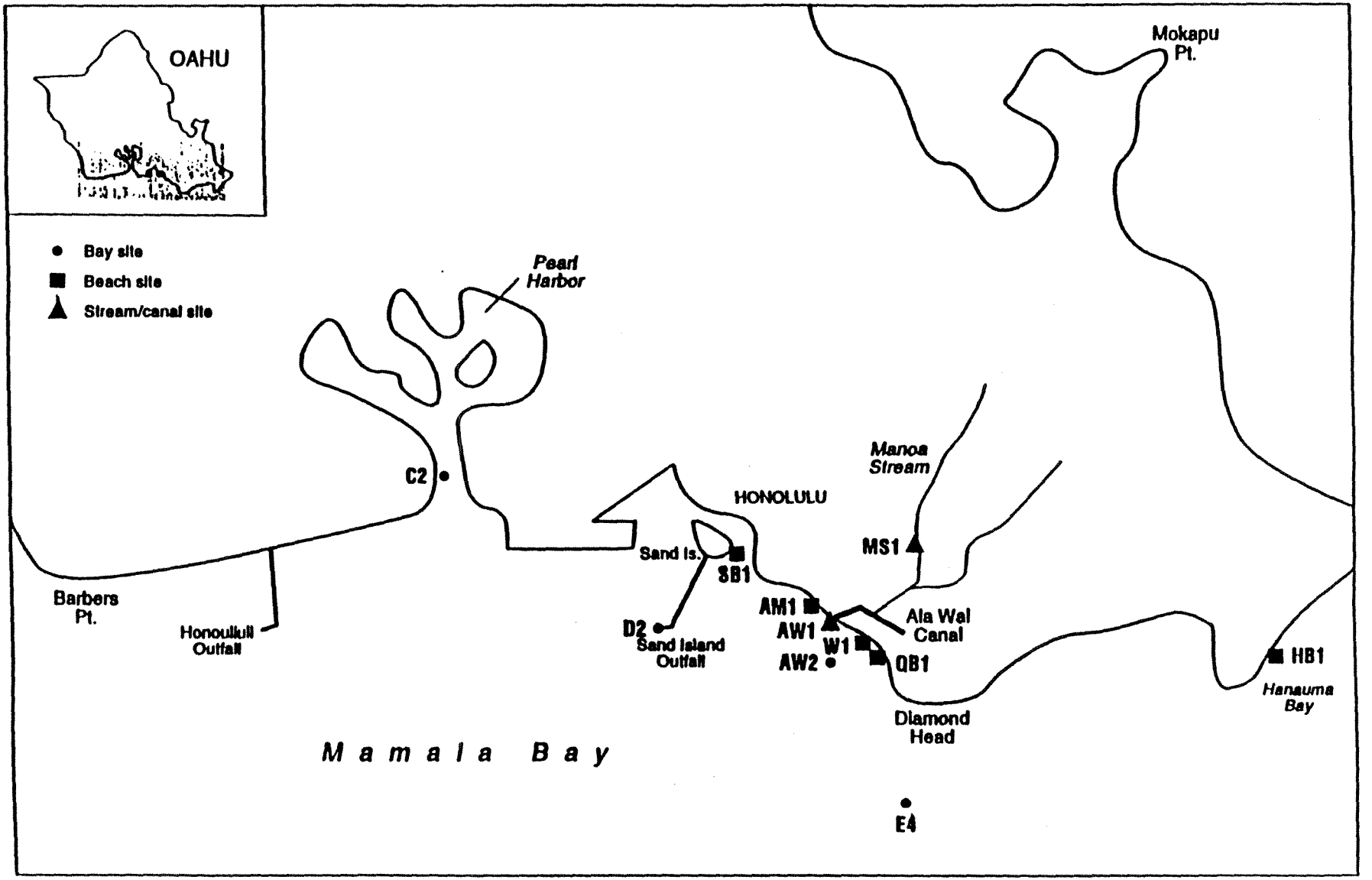

Figure 1. Location of Sampling sites within Mamala Bay, Hawaii

Figure 2. DNA extraction and purification protocol.

1. SAMPLE CONCENTRATION

Concentration by Membrex Filtration and Centrifugation		**Concentration by Centrifugation only**	
Initial Volume		Initial Volume	
Stream	20 - 30 Liters	Sewage	25 mls
Canal	20 - 30 Liters		
Marine Water	20 - 30 Liters		

2. EXTRACTION

Sonication of sample plus buffer
Thermal Shock
Washing and Centrifugation of Pellet
Pooling of Supernatants

3. PURIFICATION

Elutip D Column Purification
EtOH Precipitation
Resuspension of DNA in TE

4. PATHOGEN DETECTION

PCR amplification for detection of *E.coli* or *Salmonella*

Quantification of community DNA via fluorometer

Visualization of community DNA via electrophoresis

5. PCR PRODUCT ASSAY

Visualization of amplification product by gel electrophoresis

Table 1. Yield of DNA from pure culture after extraction and purification.

A. *E.coli*

Rep	Amount of Sample Processed			Yield After Processing	
	AODC Total Counts	CFU's (Actual)	µg equivalent DNA* (Theoretical)	Total µg DNA (Actual)	Total Equivalent Cells* (Theoretical)
A.	1.5×10^9	2×10^8	13.5	2.6	2.9×10^8
B.	1.5×10^9	2×10^8	13.5	3.2	7.2×10^8
C.	7.8×10^9	1×10^9	70.2	14.8	1.6×10^9

B. *Salmonella*

Rep	Amount of Sample Processed			Yield After Processing	
	AODC Total Counts	CFU's (Actual)	µg equivalent DNA* (Theoretical)	Total µg DNA (Actual)	Total Equivalent Cells* (Theoretical)
A.	2.4×10^9	5×10^8	21.6	2.2	2.4×10^8
B.	2.4×10^9	5×10^8	21.6	2.6	2.9×10^8
C.	1.2×10^{10}	2.5×10^9	108	15.8	1.8×10^9

*Calculated based on assumption of 9 fg of DNA per cell for AODC total counts.

Table 2. Sensitivity of PCR amplification of extracted DNA.

E.COLI

Rep A		Rep B	
Cells Per PCR Template* (Theoretical)	30 cycles PCR	Cells Per PCR Template*	30 cycles PCR
5.8 x 10^7	+	2.9 x 10^8	+
5.8 x 10^6	+	2.9 x 10^7	+
5.8 x 10^5	+	2.9 x 10^6	+
5.8 x 10^4	+	2.9 x 10^5	+
5.8 x 10^3	+	2.9 x 10^4	+
5.8 x 10^2	–	2.9 x 10^3	–
5.8 x 10^1	–	2.9 x 10^2	–
5.8 x 10^0	–	2.9 x 10^1	–

SALMONELLA

Rep A		Rep B	
CFU's Per PCR Template* (Theoretical)	30 cycles Multiplex PCR	CFU's Per PCR Template*	30 cycles Multiplex PCR
1.8 x 10^7	+	1.1 x 10^8	+
1.8 x 10^6	+	1.1 x 10^7	+
1.8 x 10^5	+	1.1 x 10^6	+
1.8 x 10^4	+	1.1 x 10^5	+
1.8 x 10^3	+	1.1 x 10^4	+
1.8 x 10^2	–	1.1 x 10^3	+
1.8 x 10^1	–	1.1 x 10^2	–
1.8 x 10^0	–	1.1 x 10^1	–

***Based on theoretical yield after processing.**

Table 3. PCR amplification of marine samples: *E.coli*

Sampling Date	Location	Volume Processed (liters)	Equivalent Volume in PCR Reaction (liters)	CFU's in PCR Reaction	PCR Single	PCR Double	Cultural CFU'S* per liter
MARINE SAMPLES							
Diamond Head							
2/15/94	E4S	10	1	0	+	N	0
6/21/94	E4S	15	2	0	+	N	0
Hanauma Bay							
10/26/93	HB1	30	3	690	_	+	230
6/24/94	HB1	15	2	60	+	_	30
Waikiki Beach							
10/26/93	W1	30	3	180	_	+	60
7/11/94	W1	15	2	20	+	N	10
6/20/94	W1	15	2	20	_	_	10
Queen's Surf Beach							
2/15/94	Q1	11.4	1	--	_	+	ND
7/11/94	Q1	15	2	60	+	N	30
Pearl Harbor							
10/26/93	C2	30	3	30	_	+	10
6/22/94	C2	15	2	0	+	_	0
Sand Island Beach							
6/24/94	IS1	15	2	0	_	_	0
Ala Wai Offshore							
6/21/94	AW2	15	2	0	_	_	0
Ala Moana Beach							
6/20/94	AM1	15	2	0	_	_	0
Sand Island Outfall							
6/23/94	D2B	15	2	18080	+	N	9,040

*Culture Data from Univ. of Hawaii.
N = Not necessary
ND = No sample

Table 4. PCR amplification of environmental samples: *E.coli*

Sampling Date	Location	Volume Processed	Equivalent Volume in PCR Reaction	CFU's in PCR Reaction	PCR Single	PCR Double	Cultural CFU'S* per liter
SEWAGE SAMPLES							
4/21/94	S1	25 ml	2.5 ml	3.75×10^5	+	N	1.5×10^8
5/2/94	S1	25 ml	2.5 ml	4.25×10^5	+	N	1.7×10^8
9/20/94	S1	25 ml	2.5 ml	3.0×10^5	+	N	1.2×10^8
10/31/94	S1	25 ml	2.5 ml	3.25×10^5	+	N	1.3×10^8
6/20/94	S1	25 ml	2.5 ml	3.25×10^5	+	N	1.3×10^8
STREAM SAMPLES							
2/14/94	MS1	10 Liters	1 Liter	5.5×10^4	_	_	5.5×10^4
		10 Liters	100 ml	5.5×10^3	_	+	5.5×10^4
		10 Liters*	1 Liter	5.5×10^4	+	N	5.5×10^4
10/25/93	MS1	15 Liters*	750 ml	7.5×10^4	_	_	1.0×10^5
		15 Liters*	375 ml	3.75×10^4	_	+	1.0×10^5
6/20/94	MS1	15 Liters	2 Liters	6.8×10^4	_	_	3.4×10^4
CANAL SAMPLES							
2/14/94	AW1	6 Liters	600 ml	7.2×10^3	_	_	1.2×10^4
		6 Liters*	600 ml	7.2×10^3	+	N	1.2×10^4
1/20/94	AW1	8.6 Liters	860 ml	1.2×10^4	_	_	1.4×10^4
		8.6 Liters*	420 ml	6.0×10^3	+	N	1.4×10^4
10/25/93	AW1*	15 Liters	1.5 Liters	2.4×10^5	_	_	1.6×10^5
		15 Liters	750 ml	1.2×10^5	_	+	1.6×10^5
8/9/94	AW1	15 Liters	2 Liters	2.0×10^3	+	N	1.0×10^3
7/5/94	AW1	10 Liters	2 Liters	9.8×10^2	+	N	4.9×10^2
6/20/94	AW1	15 Liters	2 Liters	6.4×10^3	_	_	3.2×10^3
	*Double Column Purification						

*Culture Data from Univ. of Hawaii.
N = Not necessary

Table 5. PCR amplification of environmental samples: *Salmonella*

Sampling Date	Location	Volume of Sample Used for DNA extraction	Equivalent Volume in PCR Reaction	CFU's in PCR Reaction	PCR Single	PCR Double	CFU'S* per liter
MARINE SAMPLES							
Ala Moana Beach							
6/20/94	AM1	15 Liters	8 Liters	<2.4	_	_	<0.3
Diamond Head							
6/21/94	E4S	15 Liters	8 Liters	<2.4	_	_	<0.3
Ala Wai Offshore							
6/21/94	AW2	15 Liters	8 Liters	<2.4	_	_	<0.3
Hanauma Bay							
6/24/94	HB1	15 Liters	8 Liters	<2.4	_	_	<0.3
and Island Outfall							
6/23/94	D2B	15 Liters	8 Liters	<2.4	_	_	<0.3
Waikiki Beach							
6/20/94	W1	15 Liters	8 Liters	<2.4	_	_	<0.3
Sand Island Beach							
6/24/94	IS1	15 Liters	8 Liters	<2.4	_	_	<0.3
CANAL SAMPLES							
6/20/94	AW1	15 Liters	8 Liters	<2.4	_	_	0.4
STREAM SAMPLES							
6/20/94	MS1	15 Liters	8 Liters	+*	_	_	+*
SEWAGE SAMPLES							
9/20/94	S1	25 mls	5 mls	2.1	+	N	4.3×10^2
6/20/94	S1	25 mls	5 mls	7.5	+	N	1.5×10^3

N = Not Necessary.
*Not quantified.

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C. OPTIMIZATION OF RT-PCR DETECTION OF ENTEROVIRUSES IN MARINE WATER SAMPLES: SENSITIVITY VS INHIBITION

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ABSTRACT

Virus adsorption/elution (Viradel) methods of filtration were examined to evaluate recovery efficiency and suitability for enterovirus detection in marine waters using RT-PCR and culture in a BGM cell line. Both natural and Poliovirus (type 1 strain LSc-2ab) inoculated marine water samples were concentrated using either electropositive (1 MDS) or electronegative (Filterite and K27) 10 inch cartridge filters which were eluted using 1.5% Beef Extract V. Double PCR was utilized, in addition to cell culture, to evaluate the presence of RT-PCR inhibitory factors and the sensitivity of enterovirus detection in BEV eluants and reconcentrates. Column chromatography resins (Sephadex G-25, -50, -200 and Chelex-100) were tested for their effective removal of inhibitory factors and their efficiency of virus recovery. Filterite electronegative filters were the most efficient, with an enterovirus recovery efficiency of 66% and also produced the most consistent PCR results. Enterovirus primed double RT-PCR was capable of detecting 0.1 PFU of Poliovirus (type 1 strain LSc-2ab) in distilled water, however detection sensitivity decreased by 2 to 4 orders of magnitude in reconcentrated eluants. Sephadex/Chelex columns, used to remove RT-PCR inhibitory factors, improved sensitivity by 2 orders of magnitude in sewage outfall samples. Sephadex G-25 was the least effective at removing inhibitory factors however 100% of the virus in the sample was recovered. In contrast, while Sephadex G-200 was most effective for removing inhibitory factors, 97% of the virus was lost in the column. Therefore, the trade-off between inhibition removal and virus recovery efficiencies must be considered for each individual sample site.

INTRODUCTION

Enteroviruses are introduced into marine waters from a variety of sources including storm water runoff, sewage outfalls (Fagen et al., 1992), boat (Loh et al., 1979) and bather wastes (Cabelli, 1983). Enteroviruses in marine waters may pose a significant public health risk to bathers (Wade, 1989) as well as shellfish consumers (Desenclos et al., 1991)

Previous studies have examined a variety of filters for their efficiency at concentrating enteroviruses from water for cell culture detection (Dutka, 1981; Gerba, 1982). However, little information is currently available evaluating seawater filtration methodology for PCR detection of enteroviruses. This study focused on evaluation and optimization of current methodologies for the collection and reconcentration of enteroviruses from large volumes of marine waters, and the use of a variety of resin columns to remove RT-PCR inhibitory compounds from concentrates.

Several groups have evaluated RT-PCR for the detection of enteroviruses in marine environments (Tsai et al., 1993 and 1994; LeGuyader et al., 1994; DeLeon et al., 1990). While researchers agree that viruses are typically present in very low numbers in environmental samples and that waters must be concentrated prior to PCR analysis, methods of concentration and purification are highly variable. One of the limitations inherent to PCR reactions is that only small volumes may be examined (Ma, et al., 1995). Tsai et al., (1994), used vortex flow filtration and centrifugal microconcentration to concentrate viruses from seawater. Using these methods, they were able to detect as few as 1 PFU of poliovirus in primary influent. They also found enterovirus positives in equivalent volumes of 150 mL of marine water samples using RT-PCR. However, cell culture could not be used due to the fact that only 15 L of marine water was filtered and <1 viral PFU/15 L was present. Membrane filtration allows for the filtration of large volumes of environmental water samples and has been commonly used for enterovirus concentration (Goyal and Gerba, 1982). Membrane filtration, or Viradel filtration has the advantage of being able to filter hundreds of liters of seawater, and is limited only by the turbidity of the sample. Four hundred liters of seawater may be easily concentrated using Viradel methodology and in a fraction of the time it takes for vortex flow filtration.

OVERVIEW OF METHODOLOGIES FOR ENTEROVIRUS DETECTION

1) Viradel Filtration

Virus adsorption and elution (Viradel) methods of filtration were examined for recovery efficiency and suitability for enterovirus detection in marine waters using RT-PCR and cell culture. Viradel filters were evaluated, including an electropositively charged filter, 1-MDS Virosorb, and two electronegative filters, Filterite and K-27. In addition to varying charges, Viradel filters differ with respect to cost and medium employed to attract viruses (Table 1). Viradel filters concentrate viruses based on opposing electrostatic charges. At their isoelectric point (IEP), viruses have a net charge of zero. However, isoelectric points vary greatly with different enteroviruses, ranging from 3.8 to 8.2. Most viruses have an IEP less than the pH of seawater and are negatively charged in marine water environments. Thus electropositive filters may be used directly, but, their efficiency of recovery begins to decrease above a sample pH of 7.0. Previous studies indicate most sewage enteroviruses are positively charged at the pH of 3.5 (Gerba, 1985), so, when using electronegative filters, samples must first be adjusted to pH 3.5. In addition, trivalent cations may be added to enhance flocculation of charged precipitates and increase viral adsorption efficiency (Farrah et al., 1978.)

2) Vortex Flow Filtration

Vortex Flow Filtration (VFF) membranes have been recently used to concentrate viruses by utilizing density gradients and molecular sizes rather than surface charge interactions (Paul et al., 1991; Jiang et al., 1992; Tsai et al., 1993). VFF has the advantages of automatic volume reduction and typically higher recovery efficiencies (73% with T2 tailed viruses; Paul et al., 1991). However, it is limited by large initial overhead (Membrex Inc., Fairfield, NJ; $9,000-25,000) and maintenance costs, and slow filtration rates (4 L/hr). Therefore, maximum sample volumes are typically 15-30 L with VFF concentrations. Table 2 compares Membrex VFF methodology with Viradel filtration for the concentration of enteroviruses from environmental samples. The impracticality of filtering large volumes of seawater greatly reduces the effectiveness of Membrex VFF of environmental samples for virus recovery. Although recovery efficiencies with Viradel methods are slightly lower than VFF, this is easily compensated by increased volume filtration using Viradel cartridge filters. The rapid flow rates possible with Viradel

filtration methods allow 400 L to be filtered and eluted in <40 minutes. On the other hand, Membrex VFF of only 30 L would take nearly 8 hours to complete.

TABLE 1: Characteristics of Viradel Filters Evaluated for Enterovirus Concentration

Viradel Filter	Filter Medium	Surface Charge	Cost Per Sample[1]
Filterite	pleated epoxy fiberglass	negative	$37
K27	fiberglass depth cartridge	negative	$18
1-MDS	charge modified cellulose	positive	$100

[1]1995 estimated cost of a single filter.

Regardless of the filtration method used, all concentrates must subsequently be suitable for enterovirus analysis using cell culture or RT-PCR analysis. Membrex concentrates do not require further processing prior to purification and analysis. In contrast, Viradel filters must first be eluted with 1 L of beef extract V and reconcentrated by organic flocculation to a smaller volume prior to purification (Katzenelson et al., 1976). Organic reconcentration increases the equivalent volume of marine water per volume analyzed, however, it also results in increased concentration of RT-PCR inhibitory compounds. Thus, concentration of large volumes of water to recover low levels of virus may result in an increased concentration of inhibitors, and lower sensitivity of detection via RT-PCR.

TABLE 2: Vortex Flow Filtration Versus Viradel Filtration for Concentration of Viruses

	VFF	Viradel Filtration
Concentration Mechanism	molecular density separations	electrostatic interactions
Flow Rate	4 L/hour	12 L/minute
Practical Sample Size	<30 L	>400 L
Recovery Efficiency In Marine Waters	73%	30-66%

3) RT-PCR Sensitivity

The polymerase chain reaction provides an inexpensive, rapid and sensitive method for detection of enteroviruses in environmental samples. Cell culture sensitivity is defined as 1 plaque forming unit (PFU), but it is important to note that cell culture may not detect all infectious viruses of the same or multiple species present (Payment et al., 1985; Benton et al., 1990). The low infective dose of viruses reinforces the need for improved concentration and detection methodologies and the need for more sensitive detection techniques to evaluate environmental samples. PCR has the potential to provide increased sensitivity for enterovirus detection, because it targets viral nucleic acids and therefore detects noninfectious as well as infectious virus particles. Environmental samples are estimated to have greater than 100 virus particles present per PFU detected by tissue culture (Sharp, 1965; Heinz et al., 1986). This ratio theoretically enables PCR to detect less than 0.01 PFU per equivalent volume of environmental sample. However, PCR detection efficiency is limited by small reaction volumes and the presence of inhibitory substances. Inhibitory factors may be composed of organics, including humic acids, proteins, particulate matter, metals, salts or other unidentified compounds acting to reduce PCR amplifications. Therefore, the intrinsic sensitivity for detection of viruses by PCR is a function of several factors: i) original sample volume filtered; ii) concentration of inhibitors in the sample; iii) concentrate purification; iv) the RT-PCR volume and v) virus concentration in the sample. The influence of each of these factors ultimately affects PCR detection sensitivity. In addition, the interactive nature of these factors, and the fact that many choices are available for filtration, concentration and purification, result in variable detection sensitivities.

4) Purification

The major limitation of enterovirus detection in marine water using RT-PCR is the trade off between inhibition and the recovery efficiency of viruses from the purification procedure. Since increased concentrations of inhibitory compounds result in decreased sensitivity of virus detection by RT-PCR, Abbaszadegan et al., (1993), developed a method of groundwater purification for RT-PCR using columns of combined Sephadex (G-50, G-100, and G-200) and Chelex resins. These columns were also used to purify sewage samples for RT-PCR (Straub et al., 1994). Sephadex resins were used in this experiment to purify marine water samples from inhibitors, such as humic acids, based on principles of size exclusion chromatography. In addition, Chelex-100

removes inhibitory compounds, such as metals, based on ion exchange chromatography. These two resins, singularly and combined in columns, were evaluated for their ability to remove RT-PCR inhibitors from marine water samples without appreciable loss of target viruses.

The present study focused on the evaluation of concentration, reconcentration, and purification methodologies for conventional and molecular methods of enterovirus detection in marine waters.

MATERIALS AND METHODS

Figure 1 provides an overview of techniques used in this study for enterovirus detection in marine waters. Virus concentration was initiated with either Viradel or VFF, and resulted in final marine water volumes of approximately 30 mL. Optimization methods included a 0.1 M NaCl purge of electronegative filters, a BEV elution of all Viradel filters, and resin column purification of both VFF and Viradel concentrates.

1) Marine Water Filtration

Poliovirus type 1 (strain LSc-2ab), obtained from Dr. Charles P. Gerba, University of AZ, was inoculated into 100 L volumes of natural and artificial seawater. Artificial seawater was made by adding 3841 g of Instant Ocean (Aquarium Systems, Mentor Ohio) into 100 L of tapwater dechlorinated with sodium thiosulfate (10 g/100 L).

Viradel filtration apparatus included a Homelite gas/oil pump, a 10inch cartridge filter, and a flow meter. For recovery efficiency studies, 2.8 x 10^6 PFU of poliovirus type 1 (strain LSc-2ab) was added to 200 mL of seawater. Ten mL was saved for analysis and the remaining 190 mL was added to the 400 L of marine water from La Playa Bonita Beach, Puerto Penasco, Mexico. Samples were filtered using either electronegative Filterite (Filterite Corp., Timonium, MD) and K27 (Commercial Filter Division, Carborundum Co., Lebanon, IN), or electropositive, 1-MDS Virosorb filters (AMF, CUNO Division, Meridian, CT). Other samples, including an offshore marine water site, a sewage outfall, and a canal reservoir, were collected in Mamala Bay, HI, and concentrated using Filterite filters. These samples were used to evaluate different purification methods on a variety of marine environments.

Samples processed with electronegative filters were preconditioned by lowering the pH to 3.5 with 1 M HCl and adding Al_2Cl_3 to a final concentration of 0.0015 M. All Viradel filtration rates were approximately 12 L/min. Recovery of enteroviruses from Filterite filters was optimized with a purge of 1 L of 0.1 M NaCl (pH 3.5) using positive pressure (vacuum pressure chamber or Nitrogen gas). Two passes of a 1 L volume of glycine buffered 1.5% beef extract V (BEV), pH 9.5 was used to elute enteroviruses from the filter surface. Recovery efficiencies were compared in which electronegative filters were eluted immediately in the field and after 24 hours of storage at 20°C. Electropositive filters were likewise stored and eluted using 1.5% glycine buffered BEV (pH 7.0). All BEV filter eluants were adjusted to pH 7.2 prior to reconcentration and final storage at -80°C.

Twenty L of marine water near a sewage outfall site in Bimini, Bahamas were concentrated using a Membrex Benchmark Gx Vortex Flow Filtration (VFF) System with a 400 cm^2, 100 kDa pore size, Membrex UltraFilic membrane (Membrex Inc., Fairfield, NJ). The VFF unit was run in the recirculation configuration at 7-8 psi with a filter rotation speed of 1500 rpm. The sample was pumped via tangential flow across the filter until the feed solution vessel was empty. The small batch configuration was then used by applying positive pressure to the filter producing a final retentate volume of 33 mL.

2) Reconcentration

Following BEV elution, samples were reconcentrated by organic flocculation. Eluent pH was lowered to 3.5 with 1 M HCl and continuously stirred for 15 min. Flocculated organic precipitates were pelleted by centrifugation at 3700 x g for 30 min. The pellet was resuspended in 30 mL of Na_2HPO_4 and the pH then lowered to 7.2. The adjusted samples were stirred for 5 min. Reconcentrated samples were shaken with equal volumes of fluorocarbon (freon) for 15 min., and centrifuged at 3700 x g for a further 15 min. The less dense fraction containing virus was collected, avoiding the interface, and incubated at 37°C with penicillin/streptomycin (100 U), mycostatin (10^4 IU), kanamycin (100 ug/mL), and gentimicin (10^4 ug/mL) for 30 min. Samples were stored at -80°C until further processed.

3) Column Purification

Sample concentrates were evaluated for the level of inhibitory substances by inoculation of

the concentrates with 10-fold dilutions of poliovirus (0.1 to 1000 PFU). If RT-PCR sensitivity was lower relative to distilled water, environmental concentrates were purified by Sephadex/Chelex resin columns and subjected to repeat RT-PCR analysis.

A variety of Sephadex resin columns (G-25, G-50, G-200) were evaluated for their ability to remove inhibitory substances. One mL syringes were plugged with Silane-treated glass wool and layered with 1 mL of either Sephadex, Chelex, or with 0.5 mL combinations layered within the same syringe. Columns were autoclaved and rehydrated prior to use. Fifty mL sample volumes were applied to columns and allowed to absorb into the resin for a minimum of 10 min. Syringes were placed within 15 mL centrifuge tubes and samples were recovered by low speed centrifugation (3000 x g) in a 1.5 mL capless microfuge tube.

4) Direct RT-PCR

Reverse transcriptase (RT) was used to synthesize a complementary DNA strand (cDNA) of poliovirus RNA to be used for subsequent PCR amplifications. For the RT reaction, a master mix of 3 mL 10X buffer, 7 mL 25 mM $MgCl_2$, and 8 mL 10 mM dNTP was made for each 100 mL PCR (Perkin Elmer Cetus, Norwalk, CT). Virus samples were heated for 5 min. at 99°C to release their RNA genomes from their outer capsids. After adding 1 mL each of reverse transcriptase, random hexamers and RNase inhibitor, samples were subjected to 1 cycle of: 24°C for 10 min.; 44°C for 50 min.; 99°C for 5 min., and 5°C for 5 min. to transcribe the RNA to cDNA. For cDNA amplification, a master mix was made with 7 mL of 10X buffer, 3 mL of 25 mM $MgCl_2$, 57.5 mL of double distilled water, and 0.5 mL of *Taq* enzyme (5U/mL and 0.5 mL of each primer at 0.5 mg/mL. The downstream primer, base pairs 577-594 (5'> TGT CAC CAT AAG CAG CC <3') and an upstream primer, base pairs 445-465 (5'> TCC GGC CCC TGA ATG CGG CT >3'), (Abbaszadegan, et al., 1993) were used for enterovirus amplification with the following PCR reaction conditions: 94°C for 1 min., 55°C for 45 sec., and 72°C for 45 sec. After 25-30 cycles, the final extension cycle was 72°C, for 7 min. For double PCR, a 5 uL aliquot of sample from the single PCR was added to half reactions (50 mL) of fresh reagents, and amplification was repeated for a further 25 cycles. Twenty mL of the PCR product combined with a ficol based loading buffer was added to a 1.6% agarose gel matrix for electrophoresis at 100 V for 1.5 h. The gel was then stained with ethidium bromide for 15 min., destained in sterile distilled water for 45 min., and viewed under a UV transilluminator for a 149 base pair amplification product.

Amplifications were rated on a scale of 0-3 based on the intensity of the fluorescing product. A rating of 0 indicated no visible amplification product, a 1 was given for faint band intensity, 2 for medium band intensity, and 3 for maximum band intensity.

5) Cell Culture

Poliovirus PFU were quantitated using Buffalo Green Monkey (BGM) continuous cell lines. Monolayers were grown for four days at 37°C in a 5% CO_2 incubator using 6-well trays with a growth media of 1X minimal essential medium (MEM), 5% fetal bovine serum (FBS), 200 mM glutamine and antibiotics (penicillin/streptomycin [100 U], mycostatin [10^4 IU], kanamycin [100 ug/mL], and gentimicin [10^4 ug/mL] buffered at pH 7.2 with 7.5% sodium bicarbonate and 1 M Hepes buffer (N-2- hydroxy-ethylpiperazine-N-2-ethane sulfonic acid). Prior to sample addition, cells were rinsed with sterile Tris-buffered saline and inoculated with 100 uL of serially-diluted sample. Viruses within the sample were allowed to adsorb to cells for 1 h and 3 mL of overlay media was added to each well. Overlay media was identical to growth media except that it was made double strength prior to dilution to 1X with 1.5% agar and enhanced with 3% FBS. After 48 h incubation, the agar was removed and plaques were visualized by crystal violet staining.

Natural marine water samples were similarly assayed but in 75 cm^2 flasks with up to 3 mL of sample inoculum. Rather than agar media, cells were overlaid with maintenance medium which was identical to growth media but with 2% FBS. Inoculated cells were allowed to incubate for 14 days for total cytopathic effect (CPE). For secondary passage, sample flasks were frozen and thawed 3 times and 100 uL reinoculated onto new cell monolayers as described for primary cultures. Secondary passages were observed for another 14 days for CPE.

RESULTS AND DISCUSSION

1) Evaluation of Filtration Methodologies

Many researchers have studied Viradel filtration methods for the concentration of enteric viruses from tapwater (Farrah et al., 1977; Dizer et al., 1982; Goyal et al., 1981; Gerba et al., 1986; Sobsey et al., 1985a and b). These studies found recovery efficiencies ranging between 52-64% regardless of whether electropositive or electronegative filters were used. In seawater however, electronegative filters are the medium of choice due to the fact that recovery efficiencies decrease above pH levels of 7.0 with electropositive filters, and the pH of marine waters may be greater than

7.0. Previous studies reported that recovery efficiencies of enteric viruses from seawater, using electronegative filters, approached those of tapwater, ranging from 42-53% (Goyal et al., 1983; Sobsey et al., 1977; Farrah et al., 1977; and Payment et al., 1976; Gerba et al., 1977a). Data from this study shows comparative results of electronegative and electropositive filtration for concentration of enteroviruses from seawater analyzed using RT-PCR (Table 3). Electronegative filter concentrates were positive by RT-PCR more often than electropositive filter concentrates when inoculated with the same amount of poliovirus, prior to filtration. Furthermore, results were more consistent with Filterite concentrates. In addition, based on cell culture analysis, Filterite filters were most efficient at recovery of viruses from seawater, with an average percent recovery of 66% compared to 1-MDS and K27 filters which averaged 43 and 61%, respectively. The higher efficiency of enterovirus detection using Filterite filters may be due to either less concentration of inhibitory factors or an increased virus recovery efficiency with the electronegative filter, or a combination of both. Based on these studies, Filterite filters were chosen for subsequent optimization of purification procedures.

TABLE 3: Comparison of RT-PCR Detection of Poliovirus Concentrated By Viradel Filtration Methods[1]

Filter	Virus Recovery Efficiency[2]	PFU/ Reaction	RT-PCR[3]: Trial 1	Trial 2	Trial 3
1-MDS	43%	12	0	0	0
Filterite	66%	18	2+	2+	2+
K27	61%	16	1+	2+	2+

[1]Detection in 1 L BEV eluants. [2]Determined by cell culture. [3]Initial PFU added to 400 L of seawater=2.8 x 10^6. PCR result based on a scale of 0 (no amplification) to 3+ (maximum intensity of amplification product).

Results of initial quantitative studies of poliovirus recovery from natural marine waters and tapwater, using Filterite filters, were disappointing. Recovery efficiencies averaged below 10 and 40% respectively. The elution procedure was therefore modified to include a 0.1% NaCl rinse step followed by 2 passes of a 1 L volume of 1.5% glycine buffered BEV eluent, pH 9.5. Recovery efficiencies improved to 70% in tapwater, 77.5% in artificial seawater and 66% in natural marine waters.

To evaluate virus stability and detection at low pH levels, viruses were collected using Filterite filters that were stored on ice for 24 h prior to elution. Therefore, viruses were in contact with the filters at pH 3.5 for an extended period of time. After 24 hours, viruses were eluted from the filter, as previously described, and analyzed by cell culture and PCR. A significant decrease of infectious virus particles was evident by cell culture analysis, but RT-PCR detection results were unaffected. In fact PCR continued to be positive for enteroviruses even when cell culture analysis was negative.

Although Membrex VFF technology has been reported to be very efficient at recovering viruses, sample volume is limited to <30 liters. Therefore, detection of low levels of enteric viruses in large volumes of water would be difficult using VFF alone. Preliminary results from this study indicate that inhibition of PCR is extremely high in Membrex concentrates of marine waters. Even after inoculating the PCR reaction directly with 1,000 PFU of poliovirus, no signal could be detected. However, Membrex VFF methodology may be useful in future studies as a second step reconcentration of Viradel eluants.

2) Efficiency of Column Purification and Virus Recovery

In order to increase the equivalent volume of Viradel sample analyzed by PCR, BEV eluants were reconcentrated via organic flocculation. Equivalent volumes examined per PCR increased from 4.7 mL with beef extract eluants to 189 mL of original seawater. Once samples were reconcentrated, the intensity of the PCR positive band decreased in the majority of the samples analyzed, presumably because of the trade off between sensitivity and inhibition. Although more viruses are concentrated, inhibitory compounds are also concentrated which may have decreased the ultimate sensitivity of the PCR.

TABLE 4: Evaluation of Various Sephadex Columns for Recovery Efficiency of Enteroviruses in Marine Water Sewage Outfall Concentrates

Resin Column	Dilution Factor[1]	Percent Recovery[1]	1X RT-PCR
None	NA	NA	3+
Sephadex G-200	0.5	3%	2+
Sephadex G-50	0.4	36%	3+
Sephadex G-25	0	100%	3+

[1]Average of 3 trials.

Future studies, therefore, focused on removal of inhibitory compounds in highly concentrated marine water samples. Table 4 compares various sizes of Sephadex resin beads for their ability to recover enteroviruses from a marine water sewage outfall site. Samples were inoculated with 1 x 10^4 PFU to compare recoveries of viruses with and without resin treatment. When no resin column treatment was used, RT-PCR analysis of distilled water inoculated with Poliovirus type 1 (strain LSc-2ab) produced the maximum band intensity result of 3+. However the passage of virus inoculated marine water concentrates through the resin columns often resulted in some virus loss to the column. According to cell culture analysis, the average percent recovery from Sephadex G-200 columns was 3% compared to 36% using Sephadex G-50 and nearly 100% with Sephadex G-25 columns. Therefore the larger the bead size the greater the virus recovery efficiency, since in identical volumes, the interactive surface area is smaller with larger beads. Furthermore, larger beads may be emptied of excess residual column buffer prior to marine water sample addition. Less residual buffer means less dilution of the final sample volume. Table 4 also shows an increase in sample volume dilution with a decrease of bead size. In particular, Sephadex G-200 columns were very inconsistent with regard to final sample recovery volumes. Final sample dilutions ranged from 0-1.5 times the initial volume applied to the column. Evaluation of various column resins necessitates a trade-off between effective removal of inhibitory factors and maximal recovery of the target viruses following column purification. These results show that smaller sized resin beads, such as those in Sephadex G-200 resins are the least efficient for virus recovery. However, the greater surface area of smaller resin beads provides the most effective removal of RT-PCR inhibitory substances. In contrast, G-25 resins exhibited considerably higher virus recovery efficiencies but correlated with less efficient removal of inhibitory compounds in environmental samples (data not shown).

To evaluate the sensitivity of enterovirus primers without the interference of inhibitory factors, poliovirus type 1 (strain LSc-2ab) was inoculated at various dilutions into distilled water (Table 5). Target concentration >1000 PFU often resulted in a diffused band or smear around the expected 149 bp marker. Sensitivity of enterovirus primed PCR using a laboratory strain of poliovirus type 1 (strain LSc-2ab) was 10 and 0.1 PFU for single and double PCR respectively. Next, environmental samples were inoculated with poliovirus, prior to and after column purification, to determine the effect of inhibitory factors relative to distilled water (Table 6). Sensitivity often decreased in environmental samples compared to distilled water, presumably due

to the presence of natural inhibitors. However, RT-PCR detection sensitivity in environmental samples was greatly improved by Sephadex/Chelex column purification treatments.

TABLE 5: Sensitivity Of Enterovirus Primers

Estimated PFU	PCR[1]:	
Per PCR Reaction	**Single RT-PCR**	**Double PCR**
5000	3+	smear
1000	3+	3+
100	1+	3+
10	1+	2+
1	0	2+
0.1	0	1+
0.01	0	0
0.001	0	0
0	0	0

[1]RT-PCR volumes were 50 uL with a sample volume of 5 uL.

TABLE 6: Evaluation of RT-PCR Sensitivity in Polio Inoculated Marine Water Filterite Concentrates[1]

Sample[2]	Minimum PFU Detected	Decreased Sensitivity (log)
Distilled Water	0.1	NA
Sewage Outfall	100.0	3
Treated Sewage Outfall[2]	1.0	1
Offshore Seawater	0.1	0
Treated Offshore Seawater[3]	0.1	0
Canal Water	>1000.0	4
Treated Canal Water[4]	>1000.0	4

[1]Results of double PCR. [2]Purified using Sephadex G-200. [3]Purified using Sephadex G-25. [4]Purified using Sephadex G-50, and G-200 employing a single and double pass through the columns

Prior to treatment, environmental marine water samples were inoculated with 0.1, 1.0, 100, and 1,000 PFU of Poliovirus type 1 (strain LSc-2ab). Samples from an offshore sewage outfall site were 1000-fold more inhibitory to PCR than distilled water, while canal samples were at least 10,000-fold more inhibitory to PCR. Other environmental samples varied drastically with respect to their inhibition of RT-PCR. For example, offshore seawater concentrates did not appear to be limited by inhibitory factors. Following column purification of sewage outfall samples, PCR detection sensitivity improved such that 1 PFU could be detected. The level of inhibitory factors in canal samples, on the other hand, were not improved by column purification even when 1000 PFU of poliovirus was added.

Although PCR is a very rapid and specific technique for detection of viruses in the environment, it can be significantly affected by the presence of inhibitory substances. Samples from different environments contain varying levels of inhibition and thus must be individually evaluated to minimize the occurrence of false negative results. Samples with increased levels of viruses present may be most effectively purified using Sephadex G-50 or G-200 resin columns compared to Sephadex G-25 columns. Sewage outfall samples were purified with G-200 resins for an improvement in detection sensitivity of two logs compared to the untreated sample. However, samples with low levels of viruses should be purified using Sephadex G-25 resins since virus recovery efficiencies are highest with this size resin. For offshore marine water sites, which appear to be free of RT-PCR inhibitors, Sephadex G-25 is still recommended as a routine step since time and spatial variations in the presence of inhibitory substances may occur. Some samples, such as those from the canal, were extremely turbid and concentrated with inhibitors such that column purification was ineffective, regardless of the bead size used. Future studies will continue to focus on alternative methods for purification of large volume concentrates in order for RT-PCR analysis to be routinely applied.

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D. COMPARATIVE DETECTION OF ENTEROVIRUSES IN MARINE WATERS BY CELL CULTURE AND RT-PCR

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ABSTRACT

Point and nonpoint pollution sources discharged into marine waters were evaluated for their impact on public recreational beaches in Mamala Bay, Oahu. Twelve sites were sampled in Mamala Bay, either quarterly or monthly, from October, 1993, to November, 1994. Marine and canal waters were concentrated from 400 L to 30 mL using electronegative cartridge filters (Filterite) and 1.5%, glycine buffered Beef Extract (BEV) eluent, pH 9.5. All samples were analyzed for enteroviruses using a BGM cell line. Equivalent samples were further evaluated by direct RT-PCR, using enterovirus-specific primers. Levels of RT-PCR inhibition varied with each concentrated sample; however, column purification increased RT-PCR sensitivity by at least one order of magnitude in sewage outfall and recreational beach water samples. Viable enteroviruses were found in 50% and 17% of all outfall and canal samples respectively, but samples were positive at potentially impacted beaches only 8% of the time. Results from this study indicate that frequent bather use may also be contributing to Oahu's recreational water quality with respect to enteroviruses.

INTRODUCTION

The Federal Clean Water Act and The Pollution Prevention Act established goals to eliminate discharge of pollutants into navigable waters by 1985, and control pollution at both nonpoint and point sources (Adler et al., 1993; Kolluru, 1994). Treatment plants, such as the one serving the entire city of Honolulu, Hawaii, continue to discharge untreated municipal waste into marine waters, with unknown effects on the environment and public health. Pathogens are invariably present in untreated sewage and may survive for days to months once discharged into marine waters (Melnick et al., 1980; Bosch et al., 1987; Chung et al., 1993). Survival studies of enteric viruses, specifically in Hawaiian marine waters, have shown infectious persistence for up to four days (Loh et al., 1979; Fujioka et al., 1980), during which time they may be transmitted to humans via recreational exposure (Shaiberger et al., 1982; Cabelli, 1983; Wade, 1989; Fagan et al., 1992). In addition to point discharge of sewage by treatment plants and catchment canals, nonpoint sources of pollution, such as boat wastes, bather load, streams, and land runoff have been suspected of compromising public and environmental health (Seabloom, 1969; Cassin et al., 1971; Rose et al., 1987; Cabelli, 1989). Pathogens found in marinas with live-on boats indicate that uncontrollable sources of direct pollution are also being discharged into Hawaiian harbors (Loh et

al., 1979). Furthermore, rain events, followed by notable increases in fecal indicators in streams, are common occurrences in Hawaii, therefore suggesting the contribution of land runoff (Paul, 1994). The impact of nonpoint source wastes, however, is difficult to evaluate unless they are combined into a single, collective point source, such as canal reservoirs.

Direct bather contamination is also a suspected source of microbial pollution in recreational waters (Health and Welfare Canada, 1980; Suk et al., 1987; Sherry, 1989). Nearly 13,000 tourists visited Hawaii in 1994 (Hawaii Visitors Bureau, 1995). Waikiki, Hanauma Bay and Ala Moana beach, three popular recreational bathing areas in Oahu, accommodated an average of 7,752,051, 1,933,915, and 1,453,605 people in 1994, respectively (Hawaii Department of Business, Economic Development and Tourism, 1995).

Concern for the protection of Hawaii's beaches and marine habitat prompted the study of pollution sources and water quality of Mamala Bay. Over 100 enteric viruses have been identified in humans, producing a wide variety of clinical symptoms (Gerba, 1988). Large numbers of enteric viruses may be excreted by infected individuals resulting in raw sewage which is conservatively estimated to contain 10^3 PFU/L (Ruddy et al., 1969). Through a variety of waste disposal practices and recreational use, the possibility exists for enteric viruses to be present in environmental waters. Many researchers have reported adverse health effects from recreational exposure to polluted marine waters (Goyal 1984; Saliba et al., 1990; Rees, 1993;) and shellfish consumption (Goyal et al., 1979; Richards, 1985; Desenclos et al., 1991). As a result, quantitative risk assessment analysis has been suggested for predicting the potential impact of pathogenic viruses in coastal and shellfish-harvesting waters. However, more information is needed on virus occurrence and exposure (Rose et al., 1993).

Previous studies of the Mamala Bay area have evaluated the public health impact of sewage-borne viruses on marine waters and popular swimming beaches around Oahu (Loh et al., 1979). Viruses were detected at the Sand Island sewage outfall site 85% of the time and in areas extending in every direction from the discharge plume up to 228.5 meters. Viruses were also found near certain bathing beaches, in the Ala Wai canal, and in several boat marinas. Since the time of this study, the outfall pipe has been extended, however no information is currently available measuring the effectiveness of this extension.

In the past, viral evaluation of polluted waters was extrapolated from the numbers of bacterial indicators present. This method proved to be unreliable since enteroviruses tend to survive longer than bacterial indicators in marine environments (Goyal et al., 1979; Ellender et al., 1980; Sobsey et al., 1988). In addition, recent evidence suggests that traditional bacterial indicators, may be naturally present in pristine tropical environments (Hazen et al., 1987; Bermudez et al., 1988). Several studies have determined the presence of fecal indicators in nonpolluted areas and suggest the use of alternative indicators (Rivera et al., 1988; Toranzos, 1991).

Direct detection of viral pathogens is needed to accurately evaluate their presence in marine waters. Cultural methods are now available for direct detection of several enteric viruses, however, routine monitoring has been limited due to laborious and costly methods (Bitton, 1980). In addition, not all human viruses grow on the same cell line (Schmidt et al., 1978). For those viruses which can be detected using cell culture, confirmed results may still take up to four weeks. Development of molecular approaches, such as gene probes, for rapid, specific and more cost effective detection of enteroviruses in environmental waters have circumvented many of the disadvantages to cell culture (Margolin et al., 1985 and 1987; Jiang et al. 1987; Richardson et al., 1988). The polymerase chain reaction (PCR) has been used most recently, providing extremely rapid and sensitive methods for the detection of enteroviruses. PCR may allow for routine screening of recreational and shellfish-harvesting waters (DeLeon et al., 1991; Sobsey, 1993; Kopecka et al., 1993). Specific primers are now available for detection of enteroviruses which may be difficult or impossible to detect using cell culture, including: polio, echo, coxsackie A & B, Hepatitis A, adeno, and rotavirus (DeLeon et al. 1990; Wilde et al., 1992; Abbaszadegan et al., 1993; Girones et al., 1993). Protocols have been developed for detection of RNA viruses by reverse transcriptase-PCR (RT-PCR) in a variety of environments which may be complicated by the presence of inhibitory factors (Schwab et al., 1991; Atmar et al., 1993; Graff et al., 1993; Jothikumar et al., 1993), specifically in shellfish extracts and concentrated seawater (Tsai et al., 1993; Jaykus et al., 1993). Several investigators have used Sephadex and Chelex column resins for the reduction of RT-PCR inhibitory compounds in environmental samples (Abbaszadegan et al., 1993; Straub et al., 1994 and 1994a). However, little information is available on the effectiveness of resin column purification for seawater concentrates.

The objectives of this work were to develop a sensitive method for direct RT-PCR detection of enteroviruses in marine waters and evaluate the water quality of Mamala Bay with respect to enteroviruses.

MATERIALS AND METHODS

Monitoring of viruses off the coast of Oahu began with a baseline analysis of the Sand Island sewage effluent, Ala Wai canal, and Manoa Stream followed by analysis of the ocean discharge sites for each point source. Areas of potential human exposure, such as recreational beaches, were sampled along the coastline and offshore of Mamala Bay. Marine water samples of up to 400 L were filtered using Filterite adsorption/elution technology (Viradel; Gerba et al., 1978), followed by organic flocculation, to a final reconcentrated volume of 30 mL (Katzenelson, 1976). Approximately one-third (9 mL) of the reconcentrates were assayed on a BGM cell line. In addition, inhibitory compounds were reduced using Sephadex/Chelex resin columns and samples were analyzed by RT-PCR using enterovirus specific primers (Abbaszadegan, 1993). Original volume collection was influenced by the concentration of viruses expected, exposure potential, and turbidity of the sample. Increased turbidity limited filterable and assayable sample volume, since highly turbid samples clogged filters, were toxic to cell cultures, and inhibited RT-PCR.

Negative controls were taken from areas not impacted by point and nonpoint sources and were subsequently analyzed for background levels of enteric viruses. Poliovirus type 1 (strain LSc-2ab), obtained from Dr. Charles P. Gerba, was used as a positive control for testing RT-PCR inhibition levels, filtration efficiencies, and cell culture assay effectiveness.

Selection of Sampling Sites

Mamala Bay is defined as all ocean areas from Barber's Point to Diamond Head, south of the Hawaiian island of Oahu. Honolulu, the largest city in Hawaii, lies adjacent to the Mamala Bay Shoreline. Sampling sites were chosen based on indicated pollution sources, such as a stormwater canal and sewage outfalls, as well as sites of potential public exposure, including bathing beaches. Sewage from Sand Island treatment plant (S1), and the Ala Wai canal (AW1) were sampled directly and at their offshore discharge points (D2B and AW2, respectively). Pearl Harbor (C2) was also sampled since it is known to be impacted by a sewageoutfall serving the local Naval Base. Manoa stream (MS1), a nonpoint source site, was often sampled during storm events,

which were followed by increased runoff levels. Several public bathing beaches in Mamala Bay were chosen in close proximity to either the outfall or canal (Waikiki, W1; Ala Moana, AM1; Sand Island, SB1; Queens, QB1) to determine the presence of enteroviruses. In addition, Hanauma bay, a unique crater bay on Oahu's southeastern shore, was selected as a control beach (HB1), with respect to point source pollution, since it is thought not to be impacted by sewage or canal outfalls. Therefore if beaches of Mamala Bay are being impacted by the sewage and canal outfalls alone, one would expect to find relatively fewer viruses at Hanauma Bay. Hanauma bay is, nonetheless, subjected to natural nonpoint source contamination, such as storm water runoff and recreational bathers which may contribute to poor water quality (Fujioka et al., 1990). Finally, two offshore sites were sampled, one near Queens beach (W2) and another several miles offshore from Diamond Head (E4) (Table 1). E4 was chosen as a negative control since it is thought to be unimpacted by the outfalls, canal, bathers, or land runoff.

Filtration and Reconcentration

All samples were filtered using Filterite electronegative, 10-inch, pleated cartridge filters (Filterite Corp., Timonium, MD) after preconditioning to a pH of 3.5 with 1 N HCl and a final concentration of 0.0015 M $AlCl_3$ (Kessick et al., 1978). Flow rates were approximately 20 L/min. Filters were purged with 1L of 0.1 N NaCl, pH 3.5 (Standard Methods, 1992) and eluted with two passes of 1 L volumes of 1.5% glycine buffered beef extract virus (BEV), pH 9.5. Eluents were stored at -20°C and reconcentrated within 48 hours.

Eluents were reconcentrated by organic flocculation (Katzenelson, 1976) after adjusting the pH to 3.5 with 1 N HCl and stirring for 15 min. The flocs were pelleted by centrifugation at 3000 x g for 30 min., resuspended in 30 mL of $NaPO_4$, adjusted to pH 7.2 and stirred for 5 min. Equal volumes of reconcentrated samples were freon extracted (1,1, 2-2-trichlorotrifluorethane: Aldrich, Milwaukee, WI) by vigorous mixing for 15 min. followed by centrifugation at 3000 x g for another 15 min. The upper fraction was collected and treated with antibiotics (penicillin/ streptomycin, mycostatin, kanamycin, and gentamicin: 100 mg/mL) for 30 min., at 37°C, prior to storage at -80°C until further processing.

TABLE 1: Mamala Bay Sampling Locations.

Sample Code	Site Description	Depth (Meters)	Frequency (# Of Samples Collected)
S1	Primary Sewage Effluent	NA	monthly (13)
D2B	Honolulu Outfall	70	quarterly (4)
C2	Pearl Harbour	3	quarterly (4)
AW1	Ala Wai Canal	1	monthly (12)
AW2	Ala Wai Canal Discharge Point into Mamala Bay	3	quarterly (3)
MS1	Manoa Stream	1	quarterly (4)
SB1	Sand Island Beach	1	quarterly (3)
AM1	Ala Moana Beach	1	monthly (12)
W1	Waikiki Beach	1	monthly (13)
QB1	Queens Beach	1	monthly (9)
HB1	Hanauma Bay Beach	1	monthly (13)
E4	Diamond Head Offshore Site	3	quarterly (4)

Cell Culture Assay

Final concentrate equivalent volumes of approximately 100 L of original seawater were assayed in three 75 cm^2 flasks of 4 day old passages of Buffalo Green Monkey kidney cells (BGM). After a 45 min. incubation, monolayers were overlaid with 1X Eagle's minimal essential maintenance media (Irvine Scientific Co., Santa Ana, CA: 2% fetal bovine serum; 200 mM glutamine; penicillin/streptomycin, mycostatin, kanamycin: 100 ug/mL; sodium bicarbonate; and 1 M Hepes, N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid). Inoculated cells were observed for 14 days for cytopathic effect (CPE), which is indicative of virus infection. All cell culture samples were frozen and thawed three times in preparation for a secondary passage and assay. Samples which were negative on the first passage were assayed and observed for another 14 days on fresh BGM cells, while samples which showed a positive CPE on the first passage were reconfirmed using 25 cm^2 flasks and a 1 ml inoculum from the original assay.

Sample Purification and RT-PCR Detection

Initial RT-PCR quality control testing involved inoculation of poliovirus into marine

samples to evaluate specificity of primers, characteristic banding patterns, optimal Mg++ concentration and the sensitivity of PCR in marine waters. Samples to be evaluated with enterovirus primer RT-PCR were first purified using layered Sephadex G-25/Chelex-100 resins in Silane-treated, glass wool plugged, 1 mL syringes since marine waters contain humic acids which are common inhibitors of PCR (Tsai, et al.,1992). Fifty mL sample volumes were applied to columns and allowed to absorb into the resin for a minimum of 10 min. Columns were centrifuged at 3000 x g, and samples were collected for RT-PCR analysis (Abbaszadegan et al., 1993; Straub et al., 1994; 1994a). Reverse transcriptase (RT)-PCR was used to synthesize a complimentary DNA strand for seminested PCR amplification. To complete the RT reaction, a master mix of 3 mL of 10X buffer, 7 mL of 25 mM $MgCl_2$, and 8 mL of 10 mM dNTP was made for each 100 mL reaction. RNA was extracted from potential targets in 10 mL samples by heating at 99°C for 5 minutes. In order to transcribe the released RNA, 1 mL each of reverse transcriptase, random hexamers and RNase inhibitor were added to the reaction mix (Perkin Elmer Cetus, Norwalk, CT). Samples were subjected to 1 cycle of: 24°C for 10 min.; 44°C for 50 min.; 99°C for 5 min.; and 5°C for 5 min. The cDNA was subsequently amplified using a master mix of 7 mL of 10X buffer, 3 mL of 25 mM $MgCl_2$, 57.5 mL double distilled water, 0.5 mL of *Taq* enzyme (5 U/mL), and 0.5 mL of the downstream (base pairs 577-594: 5'> TGT CAC CAT AAG CAG CC <3') and upstream (base pairs 445-465: 5'> TCC GGC CCC TGA ATG CGG CT <3') primer at a concentration of 0.5 mg/mL. Primer sequences were selected from the vpg region of poliovirus positive sense RNA and determined specific for nearly all strains of human enteroviruses (Abbaszadegan, et al., 1993). Samples were subjected to the following PCR conditions: 94°C for 1 min., 55°C for 45 sec, and 72°C for 45 sec. After 30 cycles, the final extension cycle was 72°C for 7 min. Seminested PCR was used to increase sensitivity and confirm positive results from single PCR. Five mL of the single PCR volume was added to half reactions (50 mL total reaction volume), containing fresh reagents, with the upstream external primer, as previously described. A downstream primer internal to the two external primers used in single PCR (base pairs 531-550: 5'> CCC AAA GTA GTC GGT TCC GC <3') was also added to the reaction mix. The amplification conditions were repeated for a second set of 30 cycles.

A 20 mL portion of the amplified PCR product was combined with 3 mL of Ficoll-based loading buffer and visualized by ethidium bromide staining after electrophoresis in a 1.6% agarose gel run for 1.5 hours at 100 V. The gel was with solution (0.4 mg/mL) for 15 min.

and destained in distilled water for 45 min. Presumptive positive and confirmed seminested product bands fluoresced at the 149 and 105 standard base pair marker, respectively, when exposed to a UV- transilluminator. Amplifications were rated on a scale of 0-3 according to band intensity, with three being the greatest intensity and zero indicating no amplification.

Reconcentrates were tested for inhibition by seeding low levels of poliovirus into the PCR ($\leq$1 PFU). If inhibition was evident, samples were passed through a Sephadex G-25/Chelex 100 resin column. If inhibition remained, the sample was passed through a fresh resin column or through one with a smaller bead size, such as Sephadex (G-200).

RESULTS

Filter Concentration and Cell Culture Analysis

Volumes of 100 L of artificial seawater and natural marine waters of Mamala Bay were inoculated with a known amount of Poliovirus type 1 (strain LSc-2ab), to test the recovery efficiency of the Filterite Viradel method (APHA, 1992). Efficiency experiments were performed 3 times in artificial seawater and after every quarterly sampling of Mamala Bay, for a total of 9 trials. Recoveries averaged 78 $\pm$ 13% in initial trials with artificial seawater and 60 $\pm$ 9% in natural seawater samples (data not shown).

Enteroviruses were isolated in cell culture in 21 out of 94 samples collected from the Mamala Bay area (Table 2). Thirteen of these samples were sewage effluent which was positive 100% of the time and contained an average of 3584 PFU/L. Enteroviruses were also detected in relatively low concentrations (0.04 PFU/L) at the offshore sewage outfall discharge site 50% (2/4) of the time. Approximately the same level of enteroviruses were found in the canal (0.03 PFU/L), with 17% of the samples being positive (2/12). However, no viruses were recovered from the canal entrance to Mamala Bay (0/4). Enteroviruses were detected infrequently from bathing beaches along the shoreline of Mamala Bay. Virus was isolated from only one sample collected at Waikiki Beach (1/13) but this sample had the highest concentration of enteroviruses (0.21 PFU/L) out of all beaches tested. Viruses were also isolated once at Ala Moana Beach out of 12 samples collected. The average concentration of virus at Ala Moana Beach was 0.04 PFU/L.

Hanauma Bay, which is thought to be unimpacted by point source discharges, showed the highest frequency of enterovirus isolation (2/13) compared to all other beaches sampled;

however the average virus concentration was relatively low (0.03 PFU/mL). The Diamond Head offshore site was chosen as a negative control since it is thought to be unimpacted by point source discharge sites and high bather loads. All four samples collected from this site were negative with respect to enteroviruses.

TABLE 2: Occurrence of Enteroviruses in Mamala Bay by Cell Culture

Sample	Sample Code	# Positive/ #Sampled	Average Equivalent Volume Assayed(L)[1]	PFU/L[2]
Sewage	S1	13/13	0.61	3584
Ala Wai Canal	AW1	2/12	84	0.03
Ala Moana Beach	AM1	1/12	90	0.04
Honolulu Outfall	D2B	2/4	91	0.04
Waikiki Beach	W1	1/13	94	0.21
Hanauma Bay	HB1	2/13	90	0.03

[1]Average equivalent volume of original seawater used in cell culture assay of reconcentrated samples.

[2] Average of positive samples only.

Purification and RT-PCR

Ion exchange purification procedures were evaluated for removal of substances inhibitory to marine water analysis using RT-PCR. Sephadex/Chelex column resins increased PCR sensitivity in many marine water samples that were previously inhibitory to RT-PCR (Table 3). However, the increase in sensitivity was dependent on the overall quality of the water sample. Samples which rapidly clogged the filters or were visibly turbid, such as the Ala Wai canal, showed poor RT-PCR sensitivity, presumably due to increased inhibitors.

Poliovirus-inoculated distilled water served as the standard for evaluating the detection sensitivity of seminested RT-PCR methodologies. Double PCR was able to detect less than 0.1 PFU of poliovirus in 100 uL PCR volumes when no inhibitory substances were present. For example, sensitivity of detection in offshore marine water concentrates was 0.1 PFU, indicating no significant interferences to RT-PCR. Although the Diamond Head offshore site was not inhibitory to RT-PCR, the outfall, beach and canal samples were inhibitory to various degrees. Table 3 shows a comparison of sensitivities obtained from untreated and treated marine water samples inoculated with poliovirus. Waikiki Beach samples were found to be inhibitory to PCR relative to distilled

water samples. For example, PCR detection sensitivity in the Waikiki Beach samples were 1.0 PFU of inoculated poliovirus compared to 0.1 PFU detection sensitivity in distilled water. Following column purification, however, the detection sensitivity of the PCR reaction increased to 0.1 PFU, indicating removal of PCR inhibitory compounds. Similarly the sewage outfall sample was inhibitory to the RT-PCR, with a thousand fold reduction in potential sensitivity prior to treatment. Following treatment, sensitivity was still slightly inhibited, but improved 100-fold relative to the untreated sample. Ala Wai Canal samples did not amplify even with multiple passes through several resin columns of varying bead size, or when seeded with as many as 1000 PFU of poliovirus. Therefore, column purification improved detection sensitivities at least ten-fold in all inhibited samples except the Ala Wai canal.

TABLE 3: Evaluation of Inhibitory Compounds on RT-PCR Sensitivity in Marine Water Concentrates

		Sensitivity Of Detection (PFU/PCR Reaction):	
Sample	**Sample Code**	**Untreated Sample**	**Treated Sample[1]**
Poliovirus	control	0.1	0.1
Diamond Head	E4	0.1	0.1
Waikiki Beach	W1	1.0	0.1
Sewage Outfall	D2B	100	1.0
Ala Wai Canal	AW1	>1000	>1000

[1] E4 and W1 reconcentrates were passed through Sephadex G-25/Chelex-100 columns prior to PCR analysis. Sephadex G-200/Chelex-100 were used with S1, D2B, and AW1.

A comparison of RT-PCR versus cell culture was performed with natural environmental samples from Mamala Bay. Samples which were cell culture positive were column purified, as previously described, and directly analyzed for enteroviruses using RT-PCR (Table 4). Sample E4 was again used as a negative control for RT-PCR. E4 samples were previously found to have a direct RT-PCR detection sensitivity of 0.1 PFU, and thus were assumed to be free of compounds inhibitory to the RT-PCR. Sewage samples were determined to contain PCR inhibitory compounds even after column purification, however, virus levels were high enough to compensate for the reduction in sensitivity from inhibitory substances and the loss of virus to the column.

Therefore, neither the lower RT-PCR sensitivity nor the column recovery rates affected the overall PCR result.

Finally, two quarterly outfall samples were positive by cell culture and RT-PCR. Interestingly, 1 out of 4 outfall samples was not inhibitory to RT-PCR. Although this event rarely occurred, these results suggest that the water quality at a given site can vary over PCR. Each of these samples did, however, contain relatively low levels of enteroviruses per RT-PCR and showed evidence of inhibition.

TABLE 4: Detection of Enteroviruses in Mamala Bay Using RT-PCR.

Sample (collection date)	Sample Code	Equivalent Volume Examined (mL)[1]	Estimated MPN/PCR [2]	PCR Results
Offshore control	E4	82	0	0
Sewage	S1			
4/21/94		4.8	24	3+
5/02/94		2.6	2.9	3+
Ala Wai Canal	AW1			
10/25/93		88	0.002	0
02/14/94		34	0.002	0
Ala Moana Beach	AM1			
07/05/94		105	0.004	0
Hanauma Bay	HB1			
08/15/94		116	0.001	0
Waikiki Beach	W1			
08/15/94		120	0.025	0
Sewage Outfall	D2B			
10/28/93		104	0.005	3+
02/16/94		107	0.004	3+

[1] Equivalent volume of original seawater examined per 100 mL RT-PCR.

[2] Based on cell culture data.

DISCUSSION

Evaluation of samples from the areas surrounding Mamala Bay indicate that human enteric viruses were present in all primary sewage effluent and detectable 50% of the time at the discharge outfall. Previous studies suggested that the viability of viruses was not a limiting factor in their dispersion in marine water, since they are known to survive up to three months (Loh et al., 1979). Therefore, the presence and survival of enteroviruses in outfall samples suggests the possibility of a potential public health hazard.

The offshore marine water sample, E4, thought to be unimpacted by the sewage outfall and canal discharges, large numbers of bathers, or nonpoint source runoff, was repeatedly found to be negative by cell culture. This study also considered the impact of surface runoff entering into Mamala Bay as a source of virus. The streams of Oahu are small and clear, but many do not meet federal standards for fecal coliforms and enterococci (Fujioka, 1994). In contrast, no viruses were isolated from any of the quarterly stream samples, suggesting that this nonpoint source was not a source of enterovirus contamination in Mamala Bay. Although only four stream samples were collected, three were taken during large storm events, which is the worst case scenario where runoff and bacterial indicator levels are highest. Enteroviruses were found in 50% and 17% of all outfall and canal samples, respectively; however, samples were positive at the beaches potentially impacted by these sources only 8% of the time. Although Waikiki Beach is a greater distance from the outfall and the canal compared to Ala Moana Beach, it had the highest level of contamination of any beach tested (21 PFU/100 L seawater). The level of contamination at Waikiki Beach could be due to the fact that it is more heavily frequented by bathers than any other beach in Hawaii (Hawaii Department of Business, Economic Development and Tourism, 1995).

Furthermore, Hanauma Bay, which is assumed to be unimpacted by any point sources, was found to have the highest frequency of virus isolations of any of the beaches. This may also be due to the heavy bather load, being the second most frequented beach in Hawaii, or other nonpoint sources (Cabelli, 1989; Suk et al., 1987). In fact, previous studies of Hanauma Bay water quality, pointed out the potential for microbial contamination from runoff of nearby public showers, cesspools, restrooms and rain in addition to high bather density (Fujioka, et al., 1990).

Evidence of virus transmission via swimmers and recreational waters continues to cause concern (Turner et al., 1987; Cubill, 1991; Grabow, 1991). Epidemiological data has determined that a statistically significant correlation exists between exposure to recreational waters and increased risk of enteroviral disease, with greater risks associated with beaches than swimming pools. (D'Allesio, 1980). Keswick et al., 1981 found enteroviruses in 10/14 swimming pool samples. Three of the samples were positive for virus in the presence of residual free chlorine. From these results, and supporting epidemiological data (D'Angelo, 1979), swimming pools were established as a transmission route and swimmers were indicated as the source of the viruses. Early studies suggested that viruses entered the pool via fecal material and nasal and pharyngeal secretions, and subsequent transmission occurred between swimmers in pools and other recreational swimming areas (Hawley, 1973; Bryan, 1974).

Cabelli, 1989 suggested that high density of bathers coupled with areas of poor water exchange may intensify bather effects. Other studies implied that bathers may only appear to contribute to fecal pollution in recreational waters by stirring up previously introduced pathogens and indicators in the sediments (Winslow, 1976). Therefore the pollution sources responsible for the presence of enteroviruses in multi-impacted environments is difficult to determine.

Double semi-nested PCR, enables us to detect as few as 0.1 plaque forming units (PFU) of poliovirus in concentrates of marine waters, a ten fold improvement in detection sensitivity compared to cell culture. PCR detection sensitivity in Sand Island outfall samples was estimated to be $\leq$0.004 PFU. Such sensitivity is possible with PCR since as many as 10^6-10^9 viral particles may be present for every infectious PFU in environmental isolates (Sharp, 1965; Schwab et al., 1991; Abbaszadegan et al., 1993; Straub et al., 1994). Although PCR sensitivity is much greater than cell culture, it is limited by small equivalent reaction volumes (Ma et al., 1995). In addition, the concentration of inhibitory compounds may further limit the equivalent volume that is able to be examined using RT-PCR.

Tsai et al., 1993 and 1994 utilized vortex flow filtration followed by centrifugal microconcentration (Centriprep-100 and Centricon-100) to concentrate enteroviruses from 15 L of marine water for RT-PCR analysis. Although they reported elimination of inhibitory compounds and positive PCR amplification, no information was available relating positive PCR results to infectious viral units. Cell culture information was not obtainable due to the small volume filtered.

Therefore, development of methods for filtration and concentration of large volumes of marine water is necessary to evaluate molecular methods relative to conventional cell culture methodology. Sephadex/Chelex resin columns proved useful for improving the PCR detection sensitivities of marine water samples which were slightly inhibitory to RT-PCR. However, those which had high levels of inhibition and low levels of viruses were not successfully amplified. Previous studies in our laboratory determined that smaller sizes of Sephadex resin beads (G-200) were more efficient at removing inhibition, however virus recovery efficiency from the column decreases with smaller bead sizes. Therefore, the current methodology requires that samples with high concentrations of inhibitory compounds must also have a high concentration of viruses to be detected by RT-PCR.

In conclusion, PCR may be useful for routine monitoring of sites with low levels of inhibition, such as offshore marine waters and sites with high level of viruses, such as sewage, where the inhibitory compounds may be diluted to reduce their effects, while detectable levels of viruses remain. Therefore, detection is dependent on the specific samples collected and their inherent levels of inhibition to RT-PCR. Detecting a low concentration of virus in large equivalent volumes of marine water, using RT-PCR, is still only a random occurrence.

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E. DETECTION OF INFECTIOUS ENTEROVIRUSES USING AN INTEGRATED CELL CULTURE/PCR PROCEDURE

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ABSTRACT

Reverse transcriptase-polymerase chain reaction (RT-PCR) is a rapid, sensitive detection technique that has been used effectively for direct detection of enteroviruses in a variety of environmental samples. The implication of positive results, however, has been difficult to evaluate due to amplification of nonviable particles. Traditional cell culture detects viable virions but, may take 14-21 days before cytopathic effects (CPE) are confirmed. In this study we have combined the advantages of both methodologies for more rapid detection of cell culture infective enteroviruses using RT-PCR. Buffalo green monkey kidney cells were inoculated with poliovirus type 1 (strain LSc-2ab) or primary sewage, and observed up to 28 days for cytopathic effect. Replicate flasks were frozen and thawed 3 times, freon extracted and saved for RT-PCR analysis. Poliovirus inoculums of <1 PFU/flask demonstrated CPE after 3 days, however RT-PCR was positive after only one day of incubation on BGM cells. The integrated cell culture/PCR methodology therefore allowed for more rapid detection of enteroviruses than cell culture alone. Furthermore, in a comparison of primary cell culture assay, RT-PCR and the integrated cell culture/RT-PCR, the combined methodology was at least ten fold more sensitive for detecting infectious poliovirus type 1 (strain LSc-2ab) than either method alone. Similarly, enteroviruses were detected by primary cell culture in dilutions of primary sewage samples after 10-14 days of incubation. Using the combined method of cell culture inoculation followed by RT-PCR, enteroviruses were detected after only 1-5 days. Therefore, the integrated cell culture/PCR method was nearly 5 times more rapid for the detection of cell culture infectious enteroviruses than currently used methodologies.

INTRODUCTION

In 1974, concern over the transmission of enteric viruses in recreational and drinking waters prompted an international conference to evaluate recent advances for routine monitoring techniques and propose standards for acceptable levels of virus contamination (Berg et al., 1976). Since then, contamination of recreational marine waters and shellfish harvesting areas has continued to create public health concerns (Richards, 1985; Gerba, 1988; Gerba et al., 1988). Conventional methods of infectious enterovirus analysis have prevented routine monitoring due to

the fact that they are laborious, costly and time consuming. Bacterial indicators have proven ineffective for predicting the risk of viral infections (Berg et al., 1978; Cabelli et al., 1982; Goyal et al., 1979; Deetz et al., 1984; National Research Council, 1991), thus attention has currently focused on alternative molecular-based methods, such as the polymerase chain reaction (PCR), for direct and rapid detection of viruses in the environment (DeLeon et al., 1990; Bej et al., 1991; Kopecka et al., 1993; Tsai et al., 1993). Although molecular methods are less expensive and more rapid than conventional virus detection methods, they are complicated due to inhibitory substances in environmental samples, large volumes that are often assayed, and the inability to differentiate between infective and non-cell culture infective particles (Abbaszadegan et al., 1993; Graft et al., 1993; Kopecka et al., 1993; Schwab et al., 1993; 1995; Tsai et al., 1993; Straub et al., 1994; Ma et al., 1995; Reynolds et al., 1995).

This study focuses on the use of an integrated cell culture/PCR methodology, utilizing the advantages of both conventional and molecular techniques for the detection of infectious enteroviruses in distilled water inoculated with Poliovirus type 1 (LSc-2ab strain) and primary sewage concentrates. This combined methodology offers an analysis for direct monitoring of environmental samples for pathogens that pose a significant threat to public health. Furthermore, this method allows for definitive detection of infectious enteroviruses in days compared to the weeks necessary with cell culture alone. In addition, the integrated approach eliminates the traditional flaws of direct PCR analysis, which is limited by small reaction volumes, inhibitory factors, and the inability to distinguish between infectious versus noninfectious virus. The objective of this study, therefore, was to investigate a more efficient, specific, and rapid technique to evaluate large equivalent volumes of seawater for infectious enteroviruses using an integrated cell culture/PCR approach.

MATERIALS AND METHODS

Poliovirus type 1 (strain LSc-2ab, supplied by Dr. Charles P. Gerba, University of Arizona) was initially used to evaluate detection limits and sensitivity of RT-PCR on infected cell culture lysates. Primary sewage effluent from the Sand Island treatment plant serving Honolulu, HI, was also evaluated for viable enteroviruses using RT-PCR on cell culture lysates before and after visible lysis of the cellular monolayer.

Filtration and Reconcentration

Twenty liters of primary sewage samples was adjusted to pH 3.5 with 1 M HCl and $AlCl_3$ was added to a final concentration of 0.0015 M (Farrah et al., 1978). The sample was then filtered under positive pressure using a 10 inch, 0.45 um porosity Filterite electronegative pleated cartridge filter (Gerba et al., 1978), with a total surface area of 2800 cm^2 (Filterite Corp., Timonium, MD). One L of 0.1 M NaCl (pH 3.5) was passed through the filter to enhance virus elution. Subsequently, viruses adsorbed to the filters were recovered by passage of two 1 L volumes of 1.5% beef extract V-0.05 glycine, pH 9.5 (BEV). Eluents were stored at -20°C and reconcentrated within 48 h.

In order to reduce the volume of beef extract from the primary eluates from 1 L to approximately 20-30 mL, samples were reconcentrated by organic flocculation (Katzenelson, 1976; Wallis et al., 1979; Goyal et al., 1982). Eluate pH was lowered to 3.5 by dropwise addition of 1 M HCl and the mixture was stirred for 15 min. The flocculant and adsorbed virus was centrifuged for 30 min. at 3000 x g. The pellet was resuspended in 20-30 mL of Na_2HPO_4 and the pH adjusted to 7.2 followed by stirring for 5 min. Reconcentrated samples were vigorously mixed for 15 min. with equal volumes of freon (1,1, 2-2-trichlorotrifluorethane: Aldrich, Milwaukee, WI) to reduce toxicity and other microbial contaminants to cell culture, followed by 15 min. of centrifugation at 3000 x g. The aqueous phase was collected and treated with antibiotics (penicillin/ streptomycin, mycostatin, kanamycin, and gentimycin: 100 ug/mL) for 30 minutes at 37°C prior to storage at -80°C until further processing.

Cell Culture

Poliovirus type 1 (strain LSc-2ab) stocks were diluted and a 1.0 mL inoculum from each dilution was added to 5 replicate, 25 cm^2 flasks of 4 day old buffalo green monkey kidney continuous cell line cultures (BGM). Four dilutions were assayed with estimated virus concentrations of 28, 2.8, 0.28, and 0.028 PFU. Tris buffered saline (Sigma Chemicals, St. Louis, MO) was used as a negative control inoculum. After allowing adsorption for 60 min., 1X Eagles minimal essential maintenance media was added to the flasks (Irvine Scientific Co., Santa Ana, CA: 2% fetal bovine serum; 200 mM glutamine; penicillin/streptomycin, 100 U; mycostatin, 10^4 IU; kanamycin, 100 ug/mL; sodium bicarbonate; and 1 M Hepes, (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid). Flasks were incubated at 37°C and

observed for up to 10 days for cytopathic effect (CPE). Replicate flasks for each dilution were collected for early growth detection using RT-PCR on days 1, 2, 3, 5 and 10. Flasks to be used for RT-PCR amplification were frozen and thawed at -80°C and 37°C respectively. Poliovirus was purified from lysed cells using either Sephadex 200/Chelex 100 columns as previously described (Straub et al., 1994) or equal volume freon extraction followed by low speed centrifugation (3000 x g for 15 min.).

Primary sewage concentrates were diluted to 10^{-3} and assayed on BGM monolayers as previously described. Flasks were incubated for 1, 2, 3, 5, 10, and 14 days for each dilution prior for RT-PCR analysis. On the appropriate day, incubating flasks were frozen and thawed three times, to lyse the potentially infected cells, followed by equal volume freon extraction and centrifugation (3000 x g for 15 min.). Samples were then ready for RT-PCR or secondary cell culture analysis. Lysates which were negative by primary cell culture and positive by RT-PCR were assayed a second time on BGM cells. One mL of primary lysate was added to fresh 25 cm^2 flasks of BGM monolayers and observed for another 14 days for CPE.

The most probable number technique was used to determine the concentration of virus in cell culture samples (APHA, 1992).

RT-PCR

Specific enterovirus sequences present in the cell culture lysates were amplified by RT-PCR. Fifty uL of total PCR reaction volume was used to accommodate a lysate sample volume of 5 uL per reaction (5 uL=0.0071 % of the total lysate volume). For the RT reaction, a master mix of 1.5 uL of 10X buffer, 3.5 uL of 25 mM $MgCl_2$, and 4 uL of 10 mM dNTP was made for each 50 uL reaction. Potential target viral RNA was extracted from PCR samples by heating at 99°C for 5 min. In order to transcribe the released RNA to cDNA, 0.5 uL each of reverse transcriptase, random hexamers and RNase inhibitor were added to each reaction mix (Perkin Elmer, Norwalk, CT). Each sample was submitted to 1 cycle of: 24°C for 10 min.; 44°C for 50 min.; 99°C for 5 min.; and 5°C for 5 min. For cDNA amplification, a master mix of 3.5 uL of 10X buffer, 1.5 uL of 25 mM $MgCl_2$, 28.75 uL double distilled water, 0.25 uL of AmpliTaq DNA polymerase (5 U/uL; Perkin Elmer, Roche Molecular Systems, Branchburg, NJ), and 0.25 uL (0.5 ug/uL) of the downstream (base pairs 577-594: 5'> TGT CAC CAT AAG CAG CC <3') and upstream (base pairs 445-465: 5'> TCC GGC CCC TGA ATG CGG CT <3') primers. Primer sequences were

synthesized from the vpg region of poliovirus positive sense RNA and are highly conserved among enteroviruses; polio, coxsackie, and echo (Abbaszadegan et al., 1993). Samples were subjected to 30 cycles of denaturation at 94°C for 1 min., primer annealing at 55°C for 45 sec, and primer extension at 72°C for 45 sec. After 30 cycles were completed, a final extension cycle was performed at 72°C for 7 min. Samples which were negative by single PCR were analyzed using double, seminested PCR by adding 5 uL of the single PCR volume to 50 uL reactions of fresh reagents with the upstream external primer, previously described, and a downstream primer internal to the sequence amplified in single PCR (base pairs 531-550: 5'> CCC AAA GTA GTC GGT TCC GC <3') (Straub et al., 1994). The amplification conditions for second cycle PCR were identical to those for single PCR.

Amplified PCR products were visualized by 1.6% low EEO agarose gel electrophoresis (Fisher Scientific, Fair Lawn, NJ). Twenty uL of the PCR product was combined with 2 uL of ficol loading buffer (20% ficol, 1% SDS, 0.25% bromphenol blue, and 0.1 M EDTA pH 8.0) and electrophoresed at 100 V for 1.5 h. The gel was stained with ethidium bromide solution for 15 min. and destained in distilled water for 45 min. Presumptive and confirmed product bands were visible at the 149 and 105 standard base pair marker (123 bp ladder; Gibco BRL, Gaithersburg, MD) when exposed to a 302-nm UV-transilluminator. Amplifications were rated on a scale of 0-3 according to band intensity, with three being the greatest intensity and zero implying no amplification.

RESULTS

Table 1 compares the ability to detect poliovirus type 1 (strain LSc-2ab) by CPE, PCR and the integrated cell culture/PCR procedure. RT-PCR on day zero of cell culture assay was negative for all inoculums. Although no CPE was observed for low virus concentrations (0.28 and 0.028 PFU) in primary cell culture assay, the virus could be detected by RT-PCR. Cell culture supernatants which were positive by PCR but negative after 14 days in primary cell culture, were reinoculated onto fresh BGM cells for another 14 days. Representatives of each dilution series which did not correlate to PCR results after primary cell culture were eventually positive by CPE after secondary passages. Therefore, RT-PCR analysis of cell culture monolayers consistently produced more rapid detection of enteroviruses. Furthermore, results were confirmed positive in several hours, using seminested PCR, compared to days with cell culture alone. Poliovirus type 1

(strain LSc-2ab) inoculums greater than 2.8 PFU were detected by primary cell culture, however, CPE was not observed until day 3 of the assay compared to only 1 day for positive detection using the integrated approach. Therefore, using primary cell culture alone, no poliovirus could be detected in ten-fold poliovirus (strain LSc-2ab) dilutions below 2.8 PFU/flask, however, RT-PCR analysis on cell culture flasks inoculated with 0.28 PFU were randomly positive beginning on day 2 of the cell culture assay. These low level samples were only positive by CPE after >2 weeks of incubation, involving two cell culture passages.

The integrated cell culture technique produced similar results with primary sewage effluent inoculated with poliovirus type 1 (strain LSc-2ab) (Table 2). Primary sewage effluent samples were PCR negative for all dilutions (10^{-3}, 10^{-2}, 10^{-1}, 10^{-0}) prior to cell culture inoculation and on day zero of incubation, presumably due to the presence of PCR inhibitory factors. After primary assay on BGM cells, the samples were confirmed positive by CPE at 10^{-0}, 10^{-1}, and 10^{-2} dilutions after 10, 14, and >14 days respectively. Using the integrated technique, presence of enterovirus was confirmed after only 1 day for the 10^{-0} dilution, compared to a maximum of 10 days for a presumptive positive with cell culture alone. Furthermore cell culture analysis detected no enteroviruses in the 10^{-2} dilution after 14 days of primary assay but were positive after secondary passage. This same sample was confirmed positive for enteroviruses using the integrated technique, after 5 days of assay. Therefore indirect PCR was able to detect enteroviruses in samples which appeared negative by primary cell culture or direct PCR alone. Although all dilutions which were positive by RT-PCR were eventually detected during the secondary assay, the integrated approach could detect infectious enteroviruses up to 9 days faster than cell culture alone.

The integrated cell culture/PCR approach allows for more rapid and sensitive detection of low levels of enteric viruses in large volumes of water concentrates, compared to primary cell culture alone. Furthermore, the reduced time that samples are contacted with cells may help to minimize toxicity effects, commonly observed in cell culture, thereby reducing costs of further purification and repeat analysis. Incubation of the sample on cells allows for multiplication of infectious viruses only and may overcome the initial presence of noninfectious particles and inhibitory compounds which decrease the effectiveness of PCR. In addition, PCR analysis on cell culture harvests allows us to indirectly evaluate a larger sample volume than using PCR alone. Therefore, the integrated technique has the potential to greatly reduce the time involved for routine assay of water samples for infectious viruses.

TABLE 1: Integrated Cell Culture/RT-PCR Analysis of Distilled Water Inoculated With Poliovirus[1]

Estimated PFU/Flask[2]	Days Of Incubation	Cell Culture	PCR[3]
tris buffered saline	1, 2, 3, 5, 10, >14[4]	-	0
0.028	0	-	0
"	1	-	0
"	2	-	0
"	3	-	0
"	5	-	0
"	10	-	3+
"	>14	+	3+
0.28	0	-	0
"	1	-	2+
"	2	-	0
"	3	-	3+
"	5	-	0
"	10	-	3+
"	>14	+	3+
2.8	0	-	0
"	1	-	2+
"	2	-	3+
"	3	+	2+
"	5	+	2+
"	10	+	3+
28.0	0	-	0
"	1	-	3+
"	2	-	3+
"	3	+	1+
"	5	+	1+
"	10	+	1+

[1]Poliovirus type 1 strain LSc-2ab. [2]Summary of two replicate trials.

[3]Integrated cell culture/PCR. [4]Secondary cell culture passage.

TABLE 2:Integrated Cell Culture/PCR Analysis of Primary Sewage Concentrates

Primary Sewage Dilution	Days of Incubation	Cell Culture	PCR[3]
tris buffered saline[1]	1, 2, 3, 5, 10, 14, >14[2]	-	-
10-3	0	-	0
"	1	-	0
"	2	-	0
"	3	-	0
"	5	-	0
"	10	-	0
"	14	-	0
10^{-2}	0	-	0
"	1	-	0
"	2	-	0
"	3	-	0
"	5	-	1+
"	10	-	1+
"	14	-	1+
"	>14	+	2+
10^{-1}	0	-	0
"	1	-	0
"	2	-	0
"	3	-	0
"	5	-	1+
"	10	-	1+
"	14	+	2+
10^{-0}	0	-	0
"	1	-	2+
"	2	-	1+
"	3	-	1+
"	5	-	0
"	10	+	3+
"	14	+	2+

[1]Negative control. [2]Secondary cell culture passage. [3] Integrated cell culture/PCR.

DISCUSSION

Increased sensitivity and more rapid results for enterovirus detection, are two main advantages of the integrated cell culture/RT-PCR analysis. Other advantages are also inherent. Previous studies in our laboratory have revealed that inhibition can be overcome with higher target concentrations (Straub et al., 1994). However an integrated cell culture/PCR technique may be useful in samples with low levels of enteroviruses and high levels of RT-PCR inhibitory factors. Initial sample dilution by the cell culture media, coupled with an increase in virus concentration, reduces the effects of toxic substances. Commonly used column purification methodologies are not always 100% efficient at recovering enteroviruses and purifying samples from PCR inhibitory compounds (Ma et al., 1995). In this experiment, combined procedures for RT-PCR analysis on cell culture lysates did eliminate problems of PCR inhibition in primary sewage samples.

The issue of whether or not PCR detected viruses are viable is important. The public health significance of direct RT-PCR positive results are difficult to evaluate since PCR has the ability to detect noninfectious as well as infectious target sequences. For every enterovirus PFU, there may be 10 to 1000 particles in propagated laboratory stocks (Lonberg-Holm et al., 1974) and more than 10^6 in environmental samples (Sharp, 1969). Furthermore, the ratio of particles to PFU is variable amongst each virus type and even for those of the same family (Heinz, et al., 1986). It is therefore essential for a routine virus analysis technique to target only those viruses which are infectious and potentially capable of causing deleterious public health effects.

With the integrated cell culture/RT-PCR analysis technique no RT-PCR amplification was detectable at time zero, indicating that the level of enteroviral sequences, from either particles or PFU, was beyond the limit of detection. This may be due either to dilution of the sample by cell culture media, since only 0.125 uL equivalent volume of the original sample is being examined per PCR reaction, or to inhibitory factors reducing RT-PCR sensitivity. Samples containing enterovirus sequences were positive by RT-PCR only after >24 hours of incubation in cell culture, indicating that RT-PCR detection was due to infectious virus replication and subsequent increase in the target levels.

BGM cells are one of the most efficient cell lines for isolating enteroviruses in waters and wastewaters (Morris, 1985; Guttman-Bass, 1987). However, the efficiency of infection varies with virus source, level of concentration, and methods of culture and enumeration (Bachrach et al.,

1958; Dahling et al., 1984; Benton et al., 1990; Payment et al., 1985; Richards et al., 1985). Since the integrated cell culture/PCR technique is ultimately dependent on the efficiency of the cell culture system, it is important to note some of the major problems inherent to cell culture. The combined PCR based-technology may circumvent some of the traditional problems of cell culture analysis. For example, sample toxicity will be less detrimental since cultural assay times are greatly reduced. Furthermore, samples may be analyzed and confirmed in up to two weeks faster than cell culture alone. Samples with very low theoretical levels of viruses (<1 PFU) were positive by cell culture only after a secondary passage onto fresh BGM cell monolayers. The integrated cell culture/PCR approach, however, was capable of detecting enteroviruses in primary sewage after a minimum of 5 days of incubation in primary cell assays. Another advantage of the integrated system is the increased chance that an infectious unit is being detected. Prior to incubation in cell culture, both poliovirus type 1 (strain LSc-2ab) and primary sewage samples were determined negative by RT-PCR but were positive after >24 hours of incubation. In addition, more sample volume is ultimately represented in the PCR since a larger sample volume (up to 3 mL) may be placed on cells which allows any infectious viruses present to replicate and be detected by PCR. Given these advantages, this rapid method of integrated cell culture/RT-PCR may eventually allow for routine monitoring for infectious enteroviruses in a variety of environmental samples.

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F. SURVIVAL OF ENTERIC PATHOGENS IN THE MARINE WATERS OF MAMALA BAY

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INTRODUCTION

To be a potential waterborne threat enteric pathogenic microorganism must survival long enough in the environment to reach the host. The rate of pathogen death or inactivation is useful to assess the likely concentrations to reach areas of exposure. Survival of a pathogen is dependent on many environmental factors such as sunlight, temperature, chemical composition of the water, and the native microflora. This study was designed to assess the survival of Salmonella enteric viruses, and Cryptosporidium in the marine waters of Mamala Bay.

MATERIAL AND METHODS

Test Organisms.

Cultures of *Salmonella typhimurium* (ATTCC 14028) grown overnight in Trypticase soy broth (Difco, Detroit, MI) at 37^{0}C were removed from suspension by centrifugation and resuspended in phosphate buffered saline. This procedure was repeated one additional time. this suspension was added to the test waters to achieve a final concentration of 10^6 to 10^7 colony forming units (CFU) per ml. Samples were assayed by spread plating onto Hectoen Enteric Agar (Difco) at 35^{0}C for 24-48 hrs.

The enteric viruses poliovirus type 1 (stain LSc2ab) was propagated and assayed in the BGM cell line. Cell debris from the virus suspension were removed by low-speed centrifugation, followed by extraction twice with Freon (Trichlorotrifluoroethane). These preparations were frozen and stored at -20^{0}C until needed. Assays were by the plaque forming unit method (Ma et al, 1994).

Giardia muris (Shiwagi Ramalingan, Beverton, OR)and *Giardia lamblia* cysts were obtained from Waterborne, Inc. (New Orleans, LA). *Giardia lamblia* was obtained from the feces of infected gerbils and *G. muris* from the feces of infected white mice. They were purified by a sheather's density gradient. Samples (300 ml) of marine water were placed in plastic beakers. At the

time intervals indicated 40 ml samples were removed for assay. These were centrifuged to at 2500 x g to pellet the cysts and the supernatant aspirated to a volume of 0.2 ml. Next excystation media was added to each sample and the samples added incubated at 37^{0}C for 30 minutes. The cysts were then pelted again and washed once in trypsin-tyrode's solution and then incubated at 37^{0}C for 45 minutes. Ten µL aliquot were removed and placed onto a hemocytometer (Baxter Health Care Corp., McGraw Park, IL). Samples were then examined under 400X magnification with a phase-contrast microscope (BH-2, Olympus, Japan). Empty, partially excysted tropozoites, intact cysts and tropozoites were enumerated. Additional details of the methods for *G. muris* and *G. lamblia* are found in the Guidance Manual (EPA,1990) and Schaefer, 1988.

Survival of the *Salmonella* and poliovirus type 1 is reported as the $\log_{10} N_t/N_0$ which expresses the reduction in microbial numbers at each time interval, where N_0 and N_t are the initial titer and final viral or bacterial titers, respectively. *Giardia* inactivation was assessed as the percent viable at any time interval and is expressed as a percent reduction in viability.

Survival experiments

One experiment was conducted on the survival of *G. muris*, poliovirus, and *S. typhimurium* were conducted at the University of Hawaii on the island of Oahu. The two marine waters used in this experiment were obtained from Black Point and the Ala Wai Canal. The salinity of both waters was 35 parts per thousand. The control water used was phosphate buffered saline with a salinity of zero parts per thousand. There one liter polypropylene beakers were filled with 300 ml of the test waters and placed in the laboratory in the dark at room temperature (23^{0}C). A duplicate of each type of water in another three beakers was set up in a similar fashion and was placed on the roof of a building at the University of Hawaii in a water bath with constant stirring. The test organisms were then inoculated into each of the 500 ml of water in all six beakers. For those beakers stored in the sunlight, ice was added to the water bath to keep the water temperature in the beakers between 23-27^{0}C. Samples were collected at regular interval for assay. *G. muris* and *S. typhimurium* were assayed at the University of Hawaii.

A similar experiment was collected with both *G. muris* and *G. lamblia* with water collected at Black point. The water was returned to the University of Arizona and stored at 4^{0}C until need. This experiment was conducted to compare the inactivation of *G. muris* to *G. lamblia*. The identical survival experiments performed in the beakers was repeated at the University of Arizona.

RESULTS AND DISCUSSION

All of the pathogens were inactivated at a more rapid rate in the presence of sunlight than in the dark. This effect was most dramatic with the bacterial pathogen were seven logs were loss within four hours in the presence of sunlight (Figure 1 and 2). No viable *Giardia* could be detected (a greater than 99% decrease) after three hours in the presence of sunlight although the same degree of inactivation required 77 hours in the dark (Figure 3-5) . Both *S. typhimurium* and *G. muris* survived longer in PBS in the presence of sunlight than when the organisms were placed in seawater suggesting that substances present in the seawater in the presence of sunlight are antagonistic to the inactivation of these organisms.The presence of ultraviolet light in sunlight is probably the cause of the more rapid inactivation of these organisms in the presence of daylight (Fujioka et al, 1981).

Poliovirus was inactivated at the slowest rate of the pathogens studied in either the dark or in the presence of sunlight (Figure 6 and 7). Less than a 99% loss of poliovirus occurred between 6 and 8 hours in the two marine waters studied.

The longer survival of enteric viruses in marine waters than enteric bacteria has been previously observed (Goyal, 1984) and is probably due to their lack of metabolic enzymes and greater resistance to inactivation by the ultraviolet light found in sunlight.

No significant difference in the rate of inactivation between Black Point and the Ala Wai canal for all of the organisms tested. For example, poliovirus declined in titer at a rate of 0.097 $\log_{10}$/hr in water from Black Point in sunlight and 0.092 $\log_{10}$/hr in water collected from the Ala Wai canal (Table 1).

Table 1. Log_{10} decrease in titer of *S. typhimurium* and Poliovirus type 1 in water

Microorganism	Black Point		Ala Wai Canal	
Salmonella typhimurium	1.96	0.101	1.87	0.030
Poliovirus type 1	0.097	0.038	0.092	0.043

Giardia muris was used for most of the survival experiments because it exhibits a greater degree of excystation and results in more reproducible excystation than *G. lamblia* (Schaefer, 1988).

However, comparative survival experiments were conducted at the University of Arizona using water from Black Point. The results of these experiments. However, no greater than 30% of the fresh cysts excysted under the experimental conditions of the study (data not shown). When added to marine water from Black Point not excystation occur after 90 minutes in the presence of sunlight and none after 90 minutes in the dark (Figure 5). Although, excystation of *G. lamblia* was low the limited data suggests that they did not survive longer than the *G. muris*.

CONCLUSIONS

1. All groups of pathogens were more rapidity inactivated in the presence of sunlight.

2. Poliovirus had the slowest rate of inactivation of all the pathogens studied.

3. *Giardia muris* and *G. lamblia* appear to be rapidity inactivated in marine waters with greater than 99.9.% reduction in viability in 3 hours in the presence of sunlight.

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Figure 1

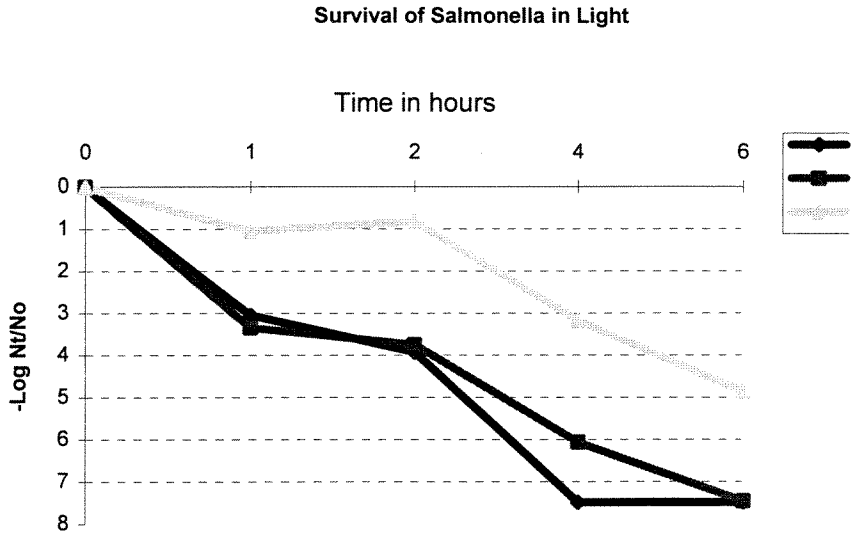

Survival of Salmonella in Light
Time in hours
0
1
2
4
6
-Log Nt/No
0
1
2
3
4
5
6
7
8

Figure 2

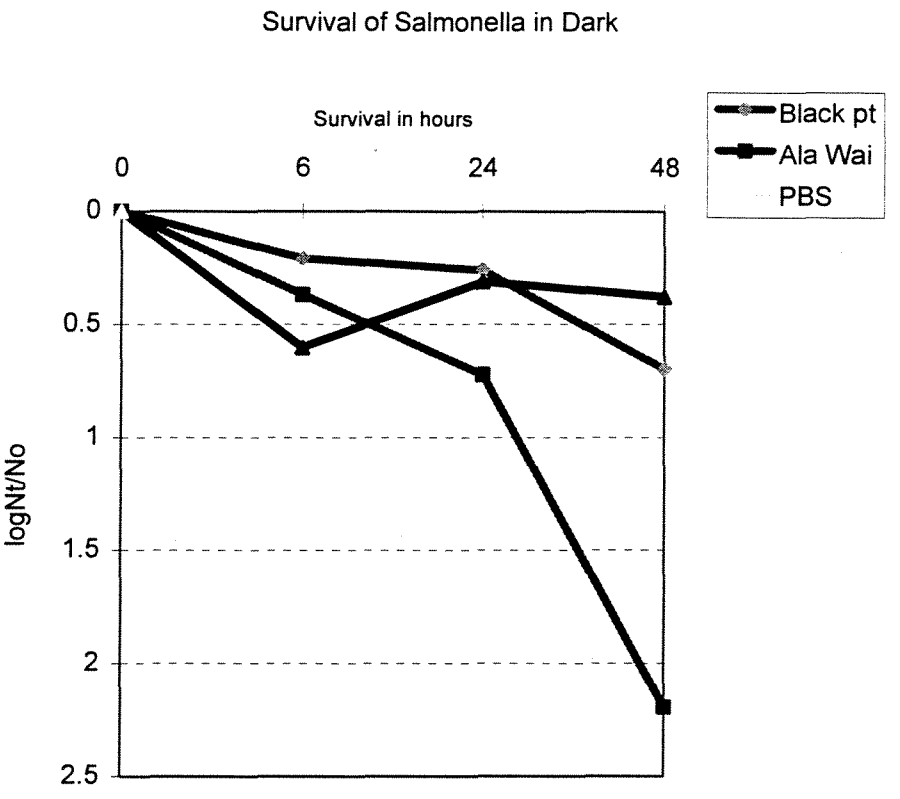
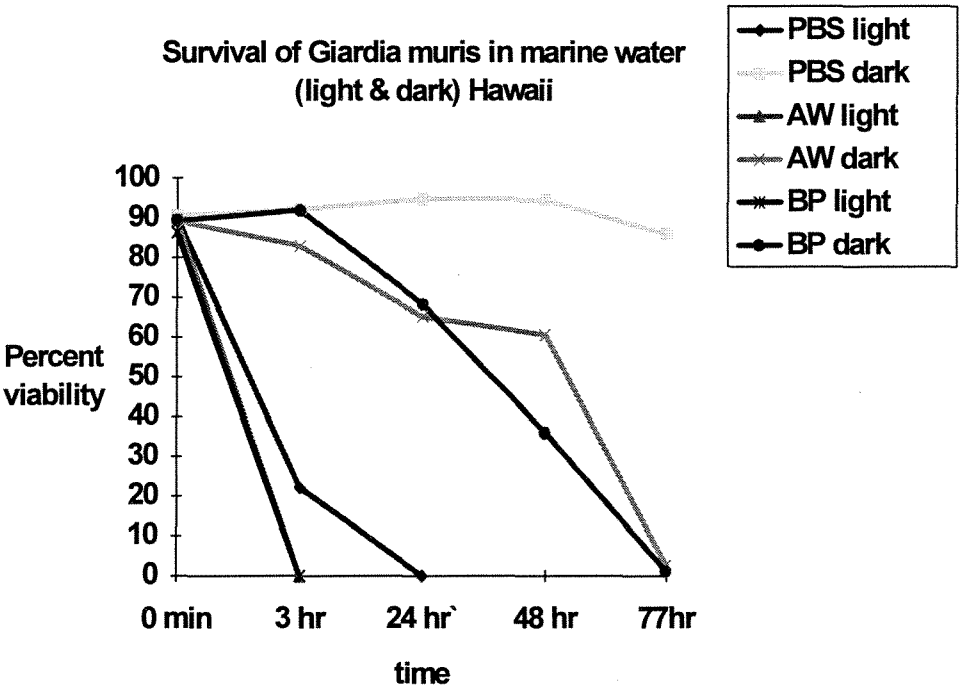

Survival of Salmonella in Dark
Survival in hours
0
6
24
48
0
0.5
1
1.5
2
2.5
logNt/No
Black pt
Ala Wai
PBS

Figure 3



PBS = phosphate buffered saline, pH 7.2
AW = Ala Wai canal
BP = Black point beach

Figure 4

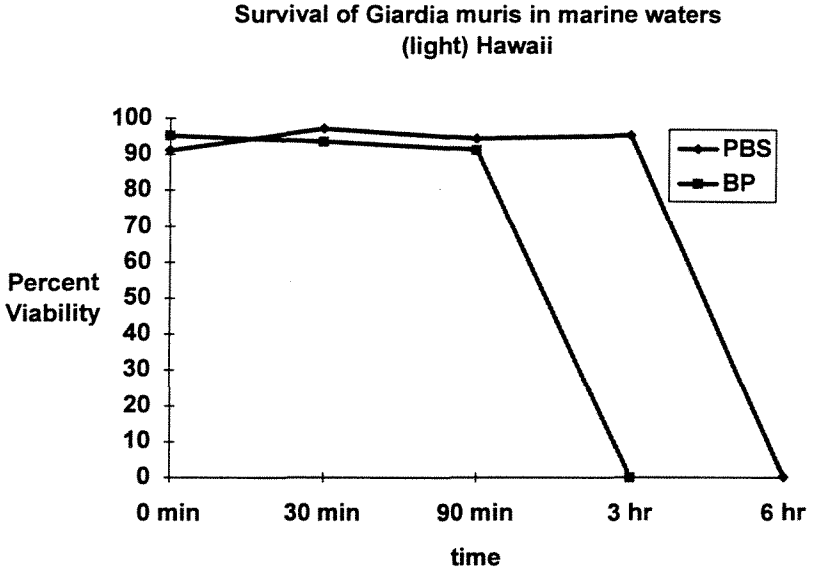
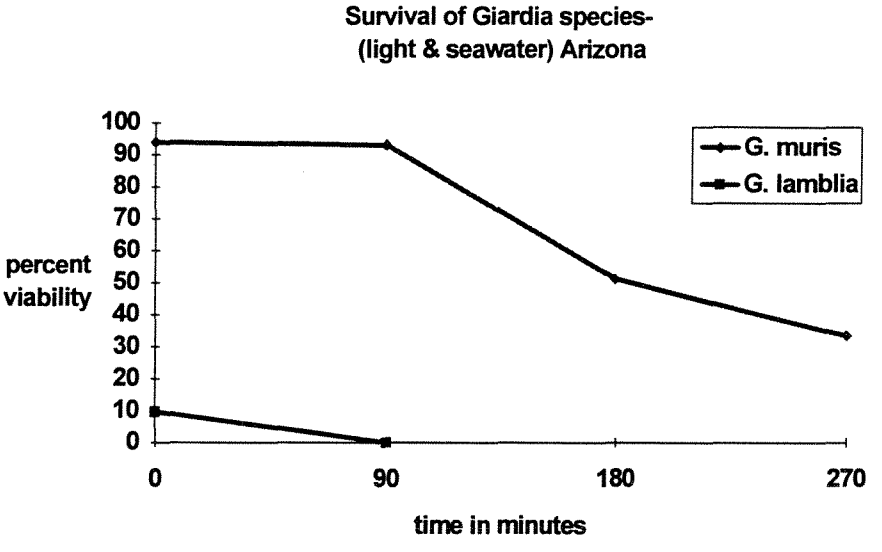

Survival of Giardia muris in marine waters
(light) Hawaii
Percent Viability
100
90
80
70
60
50
40
30
20
10
0
0 min
30 min
90 min
3 hr
6 hr
time
PBS
BP

Figure 5



Temperature range 17-20 C, pH Blackpoint seawater 8.1, PBS 7.4

Figure 6

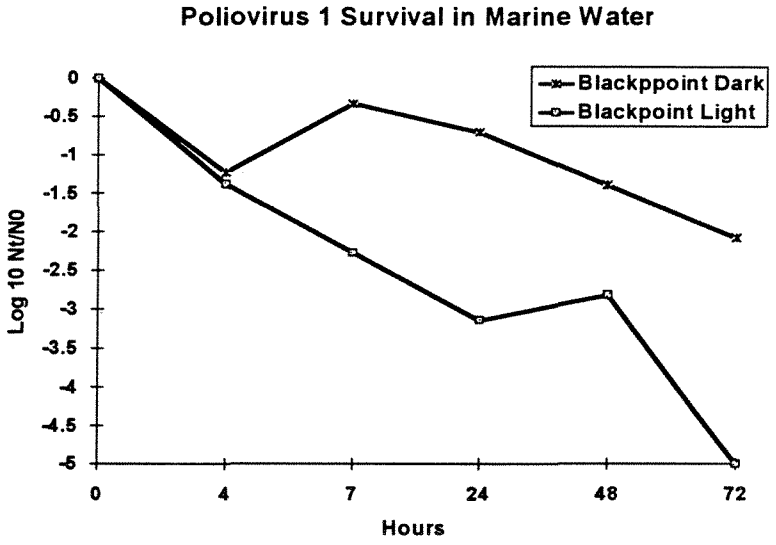
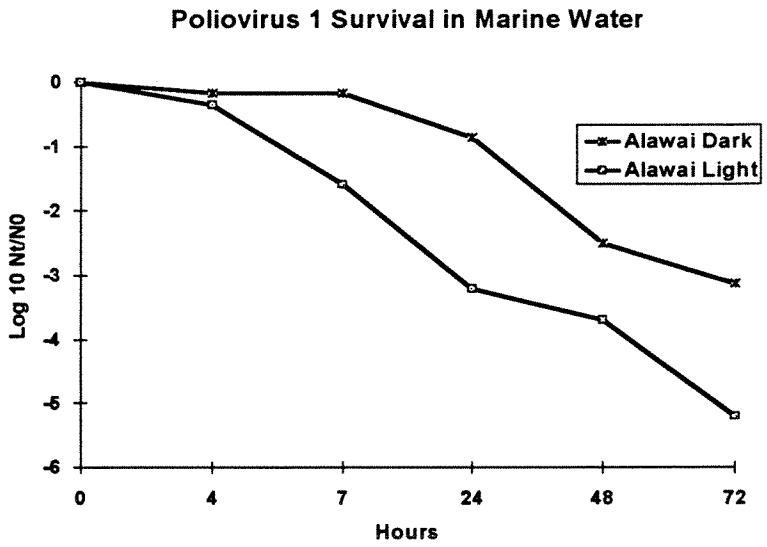

Poliovirus 1 Survival in Marine Water
Blackppoint Dark
Blackpoint Light
Log 10 Nt/N0
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
-4.5
-5
0
4
7
24
48
72
Hours

Figure 7


Poliovirus 1 Survival in Marine Water
Log 10 Nt/N0
0
-1
-2
-3
-4
-5
-6
0
4
7
24
48
72
Hours
Alawai Dark
Alawai Light