FINAL REPORT
NWRI GRANT WQ-91-01

Development and Application of Molecular Techniques to Detect Indicator and Pathogenic Microorganisms in Treated Wastewater

Dr. Carol J. Palmer, Principal Investigator
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ENVIRONMENTAL SCIENCES LABORATORY
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INTRODUCTION

Wastewater effluent from municipal plants is commonly discharged into local marine or freshwater environments. Most wastewater treatment facilities in the U.S. monitor their effluent for biochemical oxygen demand (BOD), suspended solids, and hydrogen-ion concentration. Microbial analysis of effluent is usually limited to coliforms. Coliforms are used as indicator or sentinel organisms for fecal contamination and the potential presence of other pathogenic microorganisms. Specific pathogen monitoring is not performed due to the lack of rapid and specific methodologies for their detection in water.

Due to an increase in concern over the public health safety of ocean water and/or surface waters receiving treated effluent, it is important that methods are developed to allow rapid and specific identification of pathogenic microorganisms in water. The goals of this two year study were to develop and evaluate better detection methodologies for coliforms and to develop genetic detection methods for specific pathogenic microorganisms.

COLIFORMS

Since the turn of the century, coliforms have been used as indicator organisms for water quality. Although, it has been shown that they do not always correlate with the presence or absence of pathogens, they remain the indicator organism of choice for all Federal and local regulations pertaining to microbiological analysis of water. This is because a better indicator organism has not yet been found and pathogen specific testing is not economically feasible at this time.

Most water utilities detect coliforms using either membrane filtration or Most Probable Number (MPN) methodologies. The MPN method is more frequently used since it is does

not take a high level of expertise to perform or interpret the test. The drawback of this method is that it takes from 48 to 72 hours to obtain results. It is difficult to alert the public to potential contamination problems when you find a problem in water that they were exposed to (swimming or drinking)four days ago.

As part of our study we evaluated a new product, Colilert Marine Water (Colilert-MW), developed to test ocean water for coliforms. This product uses an MPN format to detect both total coliforms and, more specifically, *E. coli* in under 24 hours. In our study, we paralleled the standard MPN with the Colilert-MW product on tests of coastal ocean water. Results showed a strong correlation between the traditional MPN method and the Colilert-MW product for recovery of both total coliforms ($r = 0.95$) and *E. coli* ($r = 0.89$) from ocean water samples. We concluded that since the Colilert-MW product is easy to use and interpret and takes only 24 hours to complete, it would be a useful tool with which to monitor coastal beach waters and provide important public health information in a real time fashion. A manuscript detailing this study was published in Applied and Environmental Microbiology in March, 1993(59:786-790)and is included in the appendix of this report.

A more technically advanced methodology was also developed during our study to detect *E. coli* in sewage and sludge. Using the polymerase chain reaction (PCR) we developed a rapid and specific genetic detection method for quick testing or screening of this type of environmental sample. In the past, it was impossible to perform genetic techniques such as PCR on sewage and sludge due to the high humic and fulvic acid content of these samples. The humic material interfered with the PCR enzymes and made genetic amplification difficult. We developed a DNA extraction and subsequent clean-up procedure

that allowed us to successfully perform PCR technology on these worst case scenario samples. A manuscript detailing this study was published in Applied and Environmental Microbiology in February, 1993 (59:353-357) and is included in the appendix of this report.

TOXIGENIC *E. COLI*

Although most *E. coli* are harmless, some strains are able to cause infection in humans. One of the more notable disease causing strains are the enterohemorrhagic *E. coli* (EHEC) which cause hemorrhagic colitis and hemolytic uremic syndrome. The EHEC organisms produce potent cytotoxins similar to Shiga toxin. Clinical symptoms include bloody and copious diarrhea along with abdominal pain and cramping. These organisms were the cause of the recent Jack-in the Box outbreaks in which several children became ill after eating hamburgers containing this strain of *E. coli*. A Shiga like toxin 1 and 2 (SLT1 & SLT11) have been identified in EHEC organisms.

We were interested in determining the proportion of enterohemorrhagic *E. coli* entering and surviving the different phases of sewage treatment. Since an important part of this grant was to encourage and provide training for others interested in water quality, we supported a graduate student in civil engineering, Chris Pendroy, at the University of California, Irvine. Mr. Pendroy, working under the direction of Dr. Stanley Grant, provided an in-depth study of the percentage of EHEC organisms in the CSDOC treatment plant plus studied sewage retention times. Dr. Grant and Mr. Pendroy integrating a civil engineering perspective to the sampling methodology in order to optimize sampling regimes.

In their study, fecal coliforms and heterotrophic bacteria were isolated from the treatment plant using membrane filtration with a set of selective and nonselective agars.

Isolates were screened for EHEC strains by colony hybridization and polymerase chain reaction. For every sampling run, the detention times of the primary and secondary treatment processes were considered so that samples were taken from the same element of fluid as it moved through the treatment plant. A total of 4,396 colonies were isolated from samples of raw (1385 isolates), primary (1675 isolates), and secondary (1,336 isolates) treated sewage. They found that in each stage of the treatment process, fecal coliforms represented about 10% of the total heterotrophic counts. Total heterotrophic bacterial concentrations in primary and secondary treated samples were 50% and 99%, respectively. below those concentrations found in the raw sewage. Out of 4,396 isolates tested, only a single isolate hybridized to a probe specific for DNA encoding the A subunit of the verotoxin protein. When this single isolate was subjected to PCR analysis using two primers flanking a 346bp fragment within the gene for the A subunit, no amplicon was found, suggesting that this isolate was not producing shiga-like toxin. Overall, results indicate an extremely low incidence of shiga-like toxin producing bacteria in the CSDOC treatment plant during this year and a half study. A manuscript detailing this study is under preparation and will be submitted for publication consideration to Applied and Environmental Microbiology.

LEGIONELLA

In the summer of 1976 an outbreak of an acute febrile respiratory illness struck 2,500 participants of an American Legion convention in Philadelphia, infecting 221 and killing 34 individuals. The new infectious agent was identified as a bacterium and given the name *Legionella.* These organisms are now known to be transmitted through aerosolization of water droplets and outbreaks have been traced to a number of potable water sources including

showerheads, swimming pools, jacuzzis, public fountains, and a supermarket vegetable misting machine.

It is thought that these organisms are able to survive water treatment barriers and enter distribution systems. The extent of the occurrence of *Legionella* in raw surface waters and in treated water distribution systems has not been fully investigated. This research has been hampered by the lack of a simple technique for detecting and quantifying *Legionella* in environmental waters.

The Perkin-Elmer Cetus corporation has recently developed a methodology to detect these pathogens in water. The test relies on the use of PCR to amplify a portion of DNA found only in *L. pneumophila*, the macrophage infectivity potentiator or MIP gene. The test also contains primers that amplify a conserved region in the 5S ribosomal RNA gene to detect 25 other species of *Legionella* but it is not able to speciate them. We tested this method on sewage influent, primary and secondary sewage effluent, and both coastal and outfall ocean water. Our results showed that *Legionella* survive through all stages of the sewage treatment process and that their numbers do not decline in any stage of the process. This could be attributed to the ability of these organisms to "hide" inside protozoans or algae or to become part of the biofilm in sewage pipes. Further experiments were completed to determine how long *Legionella pneumophila* whole cells and extracted DNA would survive in ocean water. It was shown that *L. pneumophila* whole cells survived six weeks in ocean water but that extracted DNA was quickly degraded. A manuscript detailing this study was published in Applied and Environmental Microbiology in November, 1993 (59:3618-3624) and is included in the appendix of this report.

ENTEROVIRUS

The enteroviruses (EVs) are the most common and one of the most important viral pathogens of humans. Despite the name, enteric disease is not a prominent manifestation of EV infection, although diarrhea and vomiting may be significant manifestations of certain outbreaks of "summer flu" due to the EVs. The EV's are responsible for a wide array of clinical diseases affecting all major body systems. Acute clinical EV syndromes of significance include encephalitis, poliomyelitis (polioviruses), myocarditis (coxsackievirus), and hepatitis.

There are compelling reasons for seeking a rapid method for detection of EVs. Since its inception 40 years ago, tissue culture continues to be the only method available to detect EVs. There are well-recognized limitations with this technique. Tissue culture is labor intensive and as many as 25 to 35% of EV serotypes, particularly coxsackievirus A and Norwalk virus, do not grow at all in cell culture. Also, mean isolation times for EVs using tissue culture are seven or more days.

One of the major problems associated with recovery of virus from water samples is that, unlike clinical samples, viruses typically occur in low numbers in the environment. The lack of an easy methodology with which to concentrate the large volumes of water necessary to locate viruses in the environment has been a hindrance to environmental virus recovery. In our study we developed a methodology which quickly concentrates large volumes of water and allows for sample processing using genetic technology. This technique, coupled with our environmental sample clean-up method, allowed us to examine water samples for the presence of enteric viruses. Using a published pan-specific primer that detects 17 different

enteroviruses and a primer set to detect hepatitis A virus (HAV), we examined sewage and coastal water for the presence of these organisms. We also performed standard tissue culture methods in parallel with the genetic recovery.

Results from genetic testing indicated that 80% (4/5) of sewage influent, 60% (3/5) of primary effluent and 40% (2/5) of secondary effluent contained enterovirus. These results suggest that viruses are reduced during the treatment process. However, HAV was present in 25% (1/4) of all sewage samples with no reduction observed. Coastal water was positive for enterovirus in 17% (1/6) of the samples and positive for HAV in 25% (1/4) of the samples. Positive samples were also found from water from the Santa Ana River (SAR) and a nearby flood control channel. Since both of these waters emptied into our coastal study area, it may be theorized that coastal water was adversely impacted by urban run-off. Traditional tissue culture did not always correlate with genetic detection. This is most likely due to the fact that the genetic amplification method detects more types of enterovirus than does tissue culture. It could also mean that the virus detected by PCR were not infective virus. More research is needed in this area to better answer these questions. A manuscript on this study was published in the proceedings of the Water Environmental Federation National Conference and is included in the appendix of this report. Also, another manuscript comparing PCR and tissue culture methodologies is in preparation at this time. In addition, we have recently developed a triplex PCR technique that allows for simultaneous genetic detection of three specific viruses (polio, HAV, and rotavirus) from one amplification and detection procedure. A manuscript detailing this research has been submitted to Applied and Environmental Microbiology for publication consideration.

SUMMARY

This study has met all of the goals set forth in the original grant proposal. We have: **1)** evaluated and developed new technologies for the detection of indicator coliform bacteria; **2)** determined the prevalence of pathogenic *E. coli*, *Legionella* species, and enteroviruses present in different stages of the CSDOC sewage treatment plant using genetic technology; **3)** developed a database for use in evaluating the safety of treated wastewater for land reuse using non-radioactive gene probe technology; **4)** established a state-of-the-art molecular water quality laboratory at CSDOC; and **5)** provided the results of our work to the scientific community through publication of our studies in peer-reviewed journals.

In addition, we presented our results at the American Society for Microbiology National Conference both last year and have several accepted abstracts for presentation this year. Accepted abstracts (which are published in the conference proceedings) are in the appendix of this report. We also gave talks at the Water Environment Federation National Conference and participated in a pre-conference workshop on ocean water quality. Outreach activities also include work with UCLA on detecting viruses in water from Los Virgenes treatment plant in Malibu, and collaborative work with Dr. Christine Paszko Kolva of Advanced Technologies Laboratories on *Legionella* detection in dental water and in laboratory eyewash stations and showers.

ACKNOWLEDGEMENTS

The principal investigators would like to thank the National Water Research Institute (NWRI) for supporting this research. In addition, we wish to thank Ronald Linsky and Kris Lindstrom for their insightful comments and stimulating suggestions which helped us focus these studies on the water quality issues relevant to regulators and public health officials.

We also would like to thank and acknowledge the invaluable help received from members of the CSDOC Molecular Microbiology team and the CSDOC bacteriology monitoring group.

Molecular Microbiology
Cynthia Mayer
A. Lee Lang
Moyasar Yahya
Marilyn Koval
Steven Parker
G. Fred Bonilla
Bich Tran
Charles Reed
Ken Sakamoto

Bacteriology
Charles McGee
Kim Patton
Mike von Winckelmann
Linda Kirchner

APPENDIX I

PUBLISHED ABSTRACTS AND PRESENTATIONS: 1993

№ 176058

93rd General Meeting, Atlanta, Georgia

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3. Mail abstract to:
General Meeting Abstracts
American Society for Microbiology
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Detection of Pathogenic Escherichia coli Isolated from Ocean Water using Non-Radioactive Oligonucleotide Probes. CAROL J. PALMER, YU-LI TSAI, A. LEE LANG*, KIM C. PATTON, & LOUIS R. SANGERMANO. County Sanitation Districts of Orange County, Fountain Valley, CA 92728,

The presence of pathogenic microorganisms in nearshore coastal water is an important public health concern. Some strains of E. coli are known to cause diarrheal episodes of varying severity and can enter coastal water through urban and stormdrain runoff. Enterohemorrhagic E. coli (EHEC) produce potent cytotoxins called shiga-like toxins (SLT) that are closely related to the toxin produced by Shigella dysenteriae. Enterotoxigenic E. coli (ETEC) can produce a labile toxin (LT) and/or a stable toxin (ST). We examined 171 E. coli isolated from coastal water to determine the incidence of potentially pathogenic strains. DNA was extracted using the G-NOME kit (Bio 101) with modifications on the manufacturers instructions. DNA extracts were blotted onto nylon membranes and hybridized with digoxigenin-11-ddUTP labeled oligonucleotide probes which hybridized to the internal sequences of the LT, ST, SLTI, and SLTII genes. Pathogenicity of positive isolates was tested by bioassay.

4. Full name and professional mailing address of the author who will present the paper (must be typed).

Lee Lang

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Fountain Valley CA 92708 USA

ABSTRACT AUTHORS (Type EXACTLY as they appear on original abstract) List complete names (first, middle initial, last).

Signing Author: Kim C Patton

Presenting Author: Lee Lang

To aid in the appropriate scheduling of abstracts into sessions, please provide the following information: List 3 key words or suggested session titles for this abstract (highest priority first). Try to select words in common usage such as those used in Medline and Index Medicus.

Escherichia coli pathogenic ocean water

Abstract Title: Detection of Pathogenic Escherichia coli Isolated from Ocean Water using Non-Radioactive Oligonucleotide Probes

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SIGNED Kim C. Patton (You may sign only one abstract) TYPED NAME Kim C. Patton MEMBER # 5052378

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Rapid Method using Microconcentrators and Polymerase Chain Reaction to Detect Enteroviruses in Sewage and Ocean Water.

YU-LI TSAI*, CAROL J. PALMER, MOYASAR T. YAHYA, CHARLES D. McGEE, and LOUIS R. SANGERMANO. County Sanitation Districts of Orange County, Fountain Valley, CA 92728.

A rapid method was developed to detect the presence of enteroviruses in sewage and ocean water. Sewage samples were filtered through 0.45 μm filters and the filtrates were concentrated by Centriprep-100 and Centricon-100 at 800 x g. Ocean samples collected from an offshore sewage outfall area and nearshore coastal area were concentrated by vortex flow filtration and Centricon-100. Reverse transcriptase-polymerase chain reaction (RT-PCR) using pan-enterovirus primers was used to detect enteroviruses in all concentrated samples. A nonradioactive internal probe was used to confirm the amplified products. In addition, the sensitivity of RT-PCR and tissue culture was compared using concentrates from vortex flow filtration and absorption/elution. RT-PCR was found to be at least 100 fold more sensitive than tissue culture. Results were obtained within 8 hours as compared to the two to six weeks required using tissue culture.

4. Full name and professional mailing address of the author who will present the paper (must be typed).

Yu-Li Tsai 4182069
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Fountain Valley CA 92728-8127 U.S.A.

ABSTRACT AUTHORS (**Type** EXACTLY as they appear on original abstract) List complete names (first, middle initial, last).

Signing Author: Yu-Li Tsai

Presenting Author: Yu-Li Tsai

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Polymerase chain reaction Microconcentrators Enteroviruses

Abstract Title Rapid method using microconcentrators and polymerase chain reaction to detect enteroviruses in sewage and in ocean water

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SIGNED Yu-Li Tsai (You may sign only one abstract) TYPED NAME Yu-LI Tsai MEMBER # 4182069

№ 209364

93rd General Meeting, Atlanta, Georgia

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Detection of Legionella in Sewage and Ocean Water using Polymerase Chain Reaction (PCR), Direct Fluorescent Antibody (DFA) Staining, and Culture Techniques.

CAROL J. PALMER*, YU-LI TSAI, CHRISTINE PASZKO-KOLVA, CYNTHIA L.MAYER, AND LOUIS R. SANGERMANO.
County Sanitation Districts of Orange County, Fountain Valley, CA 92728 and Advanced Technology Lab., Alta Loma, CA. 91701

Legionella are opportunistic pathogens responsible for increasing morbidity and mortality. The major route of infection is through inhalation of contaminated aerosols. Little is known of their survival through the sewage treatment process or in ocean waters receiving treated sewage. This study used three techniques to determine both survival and viability of Legionella. Results from the Perkin Elmer Roche EnviroAmp PCR kit indicated that Legionella were present at greater than 10^3/ml in primary influent, primary effluent, and secondary effluent, suggesting that Legionella may survive these steps during the sewage treatment process. Speciation (for non-pneumophila) was completed using DFA and viability determined using BCYE culture media. Ocean water, at both the offshore sewage outfall area and nearshore coastal areas, was negative for Legionella. Ocean water seeded with sewage containing Legionella showed that Legionella could be recovered for several weeks suggesting that they can survive the higher salinity of ocean water.

4. Full name and professional mailing address of the author who will present the paper (must be typed).

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ABSTRACT AUTHORS (**Type** EXACTLY as they appear on original abstract) List complete names (first, middle initial, last).

Signing Author: Carol J. Palmer

Presenting Author: Carol J. Palmer

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Legionella Sewage Ocean

Abstract Title: Detection of Legionella in Sewage and Ocean Water using Polymerase Chain Reaction (PCR), Direct Fluorescent Antibody (DFA) Staining, and Culture Techniques

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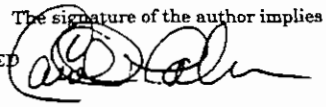
SIGNED  (You may sign only one abstract) TYPED NAME Carol J. Palmer MEMBER # 5131685

APPENDIX II

PUBLISHED ABSTRACTS AND PRESENTATIONS: 1994

93rd General Meeting, Las Vegas, Nevada

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Analysis of Efficiency of Colilert-Marine Water and Ammonia Concentrations in Tracking Ocean Outfall Plume Movement

CAROL J. PALMER*, FRED J. O'BRIEN, RICHARD V. SANTANGELO, YU-LI TSAI, MICHAEL ROSENGURT, IRWIN HAYDOCK, and LOUIS R. SANGERMANO. Orange County Sanitation Districts, Fountain Valley, CA.

Tracking treated sewage effluent discharged from ocean outfalls is a primary public health issue for wastewater agencies. Previous studies have attempted to track effluent with rhodamine dye, chlorophyll fluorescence, salinity, and/or ammonia concentrations. Fecal coliforms (specifically *E. coli*) would be useful indicators in plume tracking, however, the time and labor required to perform traditional recovery methods have made this technique inadequate for use in large scale plume tracking studies. This study tested a newly developed product, Colilert-Marine Water (MW) coupled with ammonia measurements to trace the path of effluent discharged at an ocean outfall located five miles offshore in 200 ft of water. Colilert-MW is a medium utilizing a chromogenic substrate detection format to identify both total coliforms and *E. coli* in a single tube within 24 hours. In this study, the Colilert-MW medium was used in a 5 tube MPN format. Ammonia levels were measured using Standard Method 4500-NH_3- H. Samples were collected daily for five days over both flood and ebb tides. Fourteen sampling stations surrounding the outfall were included and all sites were tested at 3 to 5 different depths twice daily. Results showed that NH_3 levels quickly dropped to non-detectable levels a short distance from the outfall site. Coliform numbers, however, gave a clear picture of plume movement away from the outfall. These data suggest that coliform levels are more efficient than ammonia concentration for tracking effluent in ocean water. The Colilert-MW formulation yielded fast and simple determination of coliforms numbers within 24 h and allowed for effective plume tracking.

4.Full name and professional mailing address of the author who will present the paper (must be typed).

First Name: Carol | MI: J. | Last Name: Palmer | Member Number: 5131685
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Is the presenting author a student? ☐ yes ☒ no

If yes, please complete the following: Type of student ☐ Undergraduate ☐ Graduate ☐ Postdoctoral

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ocean water quality | sewage effluent | coliform detection methods

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93rd General Meeting, Las Vegas, Nevada

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Detection of Poliovirus, Hepatitis A Virus, and Rotavirus from Sewage and Ocean Water by Using Triplex Reverse Transcriptase-Polymerase Chain Reaction. YU-LI TSAI*, BICH TRAN, and CAROL J. PALMER. County Sanitation Districts of Orange County, Fountain Valley, CA 92728.

A triplex reverse transcriptase-polymerase chain reaction (RT-PCR) was developed to detect poliovirus, hepatitis A virus (HAV) and rotavirus simultaneously in sewage and ocean water. Seeded or unseeded sewage and ocean water was concentrated by vortex flow filtration and/or ultrafiltration. A random hexamer and a rotavirus oligonucleotide primer were used to initiate the reverse transcription on the concentrated samples. Three different sets of primers specific for poliovirus, HAV and rotavirus cDNA were mixed in the PCR reaction mixture to amplify each target virus. Three distinct amplified DNA products representing poliovirus, HAV and rotavirus were identified on an agarose gel as a 394 bp, a 192 bp, and a 278 bp fragment, respectively. Dot blot and Southern analysis with a nonradioactive internal probe were used to confirm the PCR products for each virus. The sensitivity of triplex RT-PCR was found to be similar to that of monoplex RT-PCR for single target virus. Because of sensitivity, timesaving and cost effectiveness, the triplex RT-PCR has greater advantages over the monoplex for multiple virus detection. In this study, the triplex RT-PCR technique successfully and specifically detected three different viruses from environmental water samples within two days.

4.Full name and professional mailing address of the author who will present the paper (must be typed).

First Name: Yu-Li | MI: | Last Name: Tsai | Member Number: 4182069
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Address: 10844 Ellis Avenue
City: Fountain Valley | State: CA | Zip Code: 92728 | Country: U.S.A.
Telephone: (714)962-2411
Fax: (714)962-2591

Is the presenting author a student? ☐ yes ☒ no

If yes, please complete the following: Type of student ☐ Undergraduate ☐ Graduate ☐ Postdoctoral

To aid in the appropriate scheduling of abstracts into sessions, please provide the following information: List 3 key words or suggested session titles for this abstract (highest priority first). Try to select words in common usage such as those used in Medline and Index.

Multiplex RT-PCR | Environmental Virology | Enteric Virus Detection

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Comparison of RT-PCR and Tissue Culture Techniques for the Detection of Enteroviruses and Hepatitis A Virus in Seawater. M.T. YAHYA, Y.L. TSAI, M.E. KOVAL, B. TRAN, and L.R. SANGERMANO, and C.J. PALMER. County Sanitation Districts of Orange County, Fountain Valley, CA 92728.

Seawater samples were collected monthly from four locations surrounding an ocean outfall receiving treated sewage effluent. Enteroviruses (EV) and hepatitis A virus (HAV) were concentrated from 15 L seawater by vortex flow filtration and microconcentrated to 5-10 mL for tissue culture assays. Samples were further concentrated to 100 μL for reverse transcriptase-polymerase chain reaction (RT-PCR). Nonradioactive probes were used to confirm the amplified products. Samples were also tested for total coliform and Escherichia coli using Colilert Marine Water medium. Less than 30% of samples collected from areas close to the sewage outfall pipe tested positive for EV (30-50 PFU/15 L) and HAV with both RT-PCR and tissue culture. Most surface water samples tested negative by both RT-PCR and tissue culture. However, some were positive with RT-PCR. Higher sensitivity for detection of EV and HAV was observed with RT-PCR in seawater samples. Neither total coliform nor E. coli numbers were found to correlate with the presence of EV and HAV.

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Start ▸ Isolation and Identification of Protozoa and Other Microbial Contaminants of Emergency Shower and Eyewash Stations.

C. PASZKO-KOLVA*[1], T. K. SAWYER[2], C. J. PALMER[3], K. A. SAKAMOTO[3], T. A. NERAD[4], AND R. FAYRE[5]. [1]Advanced Technology Laboratories, Inc. Alta Loma, CA., [2]Rescon Assoc. Royal Oak, MD., [3]Orange County Sanitation District, Fountain Valley, CA., [4]American Type Culture Collection, Rockville, MD., and [5]U. S. Department of Agriculture, Beltsville, MD.

Emergency eyewash and shower stations are relied upon to provide a cleansing stream of water in personal injuries resulting from chemical spills, burns or other accidents. Water from fourty stations was cultured for the presence of protozoa, heterotrophic bacteria, and *Legionella* species. Total heterotrophic bacteria were enumerated by membrane filtration followed by plating on R2A and acridine orange direct counts (AODC). The Perkin Elmer EnviroAmp polymerase chain reaction (PCR) kit was used to detect *Legionella* species in water samples together with culture on buffered charcoal yeast extract agar. Chemical parameters measured included pH, turbidity, specific conductivity, hardness, and total alkalinity. Protozoal isolates identified from the stations included *Hartmannella sp., Vexillifera sp., Vahlkampfia sp., and Vanella sp.* Heterotrophic plate counts ranged from 10^3 to 10^6 CFU/ml and AODC counts from 10^5 to 10^6 cells/ml. PCR and gene probe analysis showed that 88% of the stations were positive for *Legionella* species by although culture results were negative. These results highlight the need for regular maintenance and monitoring of emergency eyewash and shower stations.

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Legionella PCR Eye wash stations

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Examination of Dental Units for the presence of *Legionella* species by the EnviroAmp Polymerase Chain Reaction (PCR)

C. PASZKO-KOLVA*[1], M. SHAHAMAT[2], H. WILLIAMS[3], C. PALMER[4], AND C. PETTIS[2]. [1]Advanced Technology Laboratories, Alta Loma, CA., [2]University of Maryland at College Park, MD., [3]University of Maryland Dental School, Baltimore, MD., and [4]Orange County Sanitation District, Fountain Valley, CA.

Private dental offices, university, and hospital dental clinics were included in a study which examined the water delivered by the dental unit air-water syringe [specifically, dental turbines] for the presence of *Legionella* species. *Legionella* bacteria are ubiquitous in freshwater aquatic systems and the primary route of infection is through inhalation of contaminated aerosols. The typical temperature of dental waterlines (23°C) combined with the ability of *Legionella* to form biofilms, the potential for stagnation of the water in the lines and a low chlorine residual, all provide a unique niche for this microorganism. Water samples from dental units [turbines] were analyzed for *Legionella* by PCR, culture and Direct Fluorescent Antibody (DFA) staining. Sixty-eight percent of the samples examined by PCR were positive for *Legionella* species. Of these 52% percent were positive at concentrations less than 10^3 cells/ml by PCR and 16% were positive at concentrations greater than 10^3 cells/ml. Direct fluorescent antibody staining revealed that 52% of the samples tested were positive. Despite repeated isolation attempts culturable *Legionella* were not recovered, this may have been due to inhibition by heterotrophic bacteria.

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PUBLICATIONS: Applied and Environmental Microbiology

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Evaluation of Colilert-Marine Water for Detection of Total Coliforms and *Escherichia coli* in the Marine Environment

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A test that allows for early detection of fecally contaminated coastal water would enhance public health protection. Colilert-Marine Water (Colilert-MW; Environetics, Branford, Conn.) is a rapid 24-h test that has recently been developed to detect total coliforms and *Escherichia coli* in coastal water. We performed a premarketing evaluation of the Colilert-MW product, testing it in parallel with the multiple tube fermentation (MTF) method for 86 coastal water samples in southern California. Statistical analysis was performed by using paired *t* tests and linear regression. Bacterial isolates were evaluated by biochemical and genetic analysis. The results of this study showed a strong correlation between the traditional MTF and the Colilert-MW method for detection of total coliforms ($r = 0.95$) and *E. coli* ($r = 0.89$) in ocean water samples. Paired *t*-test results indicated that the Colilert-MW and MTF were equivalent in detecting *E. coli* and that the Colilert-MW may be more sensitive in the detection of total coliforms. We conclude that Colilert-MW would be a useful tool with which to monitor coastal beach water.

A new product, Colilert-Marine Water (Colilert-MW), has been developed by Environetics (Branford, Conn.) to simultaneously detect total coliforms and *Escherichia coli* in marine water within 24 h. Similar to the Colilert product developed for fresh water, the new marine water test relies on the substrates *O*-nitrophenyl-β-D-galactopyranoside (ONPG) and 4-methylumbelliferyl-β-D-glucuronide (MUG) to detect total coliforms and *E. coli*, respectively. The presence of coliforms is indicated by a change in the medium from clear to yellow, while the presence of *E. coli* is determined by fluorescence under long-wave (366-nm) UV light. The new formulation has a reduced amount of sodium chloride in the medium in order to accommodate marine water samples.

The freshwater Colilert has been extensively tested. National studies conducted by Edberg et al. (5, 6) tested the Colilert at water utilities in different geographical areas and found that the product accurately detected total coliforms and *E. coli* in drinking water. Rice and coworkers determined that 95.5% of *E. coli* strains from humans, cows, and horses fluoresced in the medium and that the test distinguished *E. coli* from other *Escherichia* species (14, 15). Clark et al. (3) found that the Colilert was equivalent to membrane filtration in the detection of *E. coli* from untreated surface water but that it did not effectively recover *E. coli* from chlorine-treated water samples. More recently, the Colilert method and *E. coli* medium with MUG (ECMUG) were both found to be useful for the detection of chlorine-exposed *E. coli* (4, 12).

The ability of the Colilert to detect total coliforms and *E. coli* in fresh water has been established. The Colilert for fresh water was approved by the U.S. Environmental Protection Agency for total coliforms in 1989 (8) and for detection of *E. coli* in June 1992 (9). These approvals have provided water utilities and public health agencies with the opportunity to quickly and efficiently determine the quality of fresh water. The development of a rapid method for detecting coliforms in ocean water would provide similar benefits to agencies that monitor the marine environment and provide quick results that could aid in protecting public health. We evaluated the new Colilert-MW product in parallel with standard multiple tube fermentation (MTF) testing to determine the level of agreement and its potential usefulness for ocean coliform monitoring. The presence of total coliforms and *E. coli* was confirmed by using biochemical identification and genetic analysis.

MATERIALS AND METHODS

Sampling. Over a period of several weeks, 86 water samples were collected in sterile bottles from 17 nearshore beach surf zone stations in southern California. All samples were kept on ice and processed within 4 h after collection. Initially, 26 ocean samples were tested in parallel with the MTF monitoring. An additional 60 samples were tested in February 1992, when southern California received heavy rains. These rains contributed to minor sewage overflows and caused storm drain overloads to enter coastal waters, resulting in a 1-mile beach closure in the monitored area. This presented a unique opportunity to test the Colilert-MW under harsh environmental conditions.

MTF. Fifteen-tube MTF tests were performed as described in *Standard Methods* prepared (1). Briefly, 1:10, 1:100, and 1:1,000 dilutions were prepared, inoculated into lauryl tryptose broth (LTB), and incubated at 35°C for 24 to 48 h. During the rainy period, 1:100, 1:1,000, and 1:10,000 dilutions were used for sites that had high coliform counts. A loopful of broth from tubes containing LTB that showed positive results (positive LTB tubes) was transferred to brilliant green bile broth (BGB) and ECMUG. BGB tubes were incubated at 35°C for 24 to 48 h. ECMUG tubes were incubated at 44.5°C for 24 h.

Colilert-MW. Concurrent studies comparing the Colilert-MW with the traditional MTF were completed by rehydrating Colilert-MW tubes with 9 ml of sterile distilled water and 1 ml of coastal water sample. The other Colilert-MW tubes were inoculated from the same dilution tubes that were

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used for the 1:100, 1:1,000, and 1:10,000 dilutions in the MTF procedure. Tubes were incubated at 35°C for 24 h. An additional test was completed for 20 separate samples by using 10 ml of undiluted ocean water to rehydrate the Colilert-MW tubes.

Bacterial analysis. The first 26 samples were subjected to intensive examination. Samples from all 1:10 dilution tubes (plus positive tubes from other dilutions) were streaked onto MacConkey agar plates. In addition, 11 negative and 3 positive ECMUG tubes were streaked onto MacConkey agar plates. Samples showing representative morphologies were streaked for isolation on Trypicase soy agar (TSA). Bacterial isolates were identified biochemically with the API20E (Analytab Products, Plainview, N.Y.).

Since two isolates of *Vibrio cholerae* non-O1 were recovered from one of the first 17 sites tested, thiosulfate-citrate-bile salts-sucrose (TCBS) medium was included during testing of the last 9 sites. Samples from the Colilert-MW tubes that tested positive were streaked onto TCBS (in addition to MacConkey agar) and incubated at 35°C for 24 h. Representative morphologies were identified as described above.

Genetic analysis. The UAR-900 oligonucleotide (5′-ACGCGTGGTTACAGTCTTGCG-3′), described by Bej et al. (2), detects a portion of the *uidA* gene. The *uidA* gene codes for β-glucuronidase, a 1.8-kb single-copy gene responsible for fluorescence in *E. coli* when MUG is metabolized.

Genomic DNA was extracted from bacterial isolates by using the G-NOME extraction kit (Bio 101, Inc., La Jolla, Calif.) and resuspended in TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) buffer. One microgram of extracted DNA was diluted in 100 μl of TE, heat denatured at 100°C for 10 min, and placed on ice for 5 min. One hundred microliters of 20× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate [pH 7.0]) was added to each sample. The samples were immediately slot blotted onto Hybond-N^+ membranes (Amersham, Arlington Heights, Ill.) with a Minifold II filtration apparatus (Schleicher & Schuell, Keene, N.H.). The DNA was cross-linked for 2 min with a Stratalinker 2400 (Stratagene, La Jolla, Calif.).

The oligonucleotide probe was synthesized (Genosys Biotechnologies, Woodlands, Tex.) and labeled by using the Genius 5 kit (Boehringer Mannheim, Indianapolis, Ind.). The membrane was prehybridized for 2 h at 58°C in prehybridization solution (5× SSC, 0.1% *N*-lauroylsarcosine, 0.02% sodium dodecyl sulfate [SDS], 2% Boehringer Mannheim blocking reagent) and hybridized with the UAR-900 probe (2 pmol/ml) for 12 h at 58°C. The membranes were washed twice in 2× SSC–0.1% SDS at 58°C for 15 min and twice in 0.5× SSC–0.1% SDS at 51°C for 15 min. Probe hybridization was detected by using the Genius 3 (Boehringer Mannheim) chemiluminescence kit per the manufacturer's instructions.

E. coli isolates were also screened to determine the presence of any enterohemorrhagic strains. *E. coli* cells that did not fluoresce in Colilert-MW or ECMUG medium were tested for the somatic O157 antigen by using the *E. coli* O157:H7 latex test reagent kit (Pro-Lab Diagnostics, Round Rock, Tex.).

Statistics. Statistical analysis was completed by an independent company, EcoAnalysis (Ojai, Calif.). The results from the Colilert-MW and MTF methods were compared by paired *t* tests. In addition, Pearson correlation coefficients and principal-axis regression slopes were computed to quantify the relationships between the two methods. Principal-axis regression analysis was used instead of the standard least-squares regression tests to compensate for the sampling error associated with both methods. The standard regression method assumes that there is no error in one of the variables. Since analyses with raw data values were dominated and distorted by a small number of relatively large values, all analyses were performed with values transformed by a natural logarithm.

RESULTS

Comparison of standard MTF and Colilert-MW results for recovery of total coliforms and *E. coli*. Overall results for the total coliform test showed that 605 tubes with BGB were positive and 622 tubes were positive in the Colilert-MW. With ECMUG, which identifies *E. coli*, 249 tubes fluoresced and produced gas while 247 Colilert-MW tubes fluoresced, indicating the presence of *E. coli*.

We compared the most-probable-number values obtained with the Colilert-MW with the most-probable-number values obtained by the traditional MTF test. For purposes of analysis, the data were compared in three different groups. Group 1 included all 86 samples, group 2 included the 66 samples in which the standard dilutions (1:10, 1:100, and 1:1,000) were used, and group 3 included only the high-dilution (1:100, 1:1,000, and 1:10,000) data.

Paired *t*-test analysis of *E. coli* recovery (Table 1) strongly indicated that there was no difference between the two tests for the three groups since none of the *P* values were significant. Results from paired *t* tests for total coliform recovery showed that the Colilert-MW method gave significantly higher most-probable-number values for groups 1 ($P = 0.02$) and 2 ($P = 0.01$). The mean ln differences for groups 1 and 2 indicated that the results from the Colilert-MW were 22 and 27% higher, respectively ($e^{0.20} = 1.22$ and $e^{0.24} = 1.27$). However, the results of the total coliform sample analyses for group 3 were not significant ($P = 0.75$), indicating there was no difference between the Colilert-MW and MTF tests during storm conditions.

The data were also analyzed by standard linear regression analysis. The principal-axis slopes were close to 1.0 for all *E. coli* and total coliform groups. The principal-axis slope confidence limits for all analyses, except for *E. coli* standard dilutions, covered the value of 1.0. Strong overall *r* values for comparison of recovery of total coliforms ($r = 0.95$) and *E. coli* ($r = 0.89$) were obtained.

The facts that groups 1 and 2 of total coliforms had significant *t*-test values and slopes of 1.0 are not a contradiction. These results may be due to the fact that the *y* intercept of the regression equation is less than zero (the *y* intercepts for the two analyses are −0.69 and −0.72, respectively).

Bacterial analysis. Twenty-six (30%) of the 86 water samples were subjected to intensive testing to determine the rate of false-positive and false-negarive results with Colilert-MW medium. For the 26 water samples, 390 tubes (15 tube dilutions × 26 samples) were tested; 83 turned yellow and 41 also fluoresced. In the total coliform portion of the test, a false-positive result was defined as a Colilert-MW tube that turned yellow but contained no coliforms, and a false-negative result was a tube that remained clear but contained coliforms. For the *E. coli* portion of the test, a false-positive result was a Colilert-MW tube that turned yellow and fluoresced, but from which no *E. coli* organisms were recovered. A false-negative result was defined as a tube that turned yellow, contained *E. coli*, but did not fluoresce.

Total coliforms. Coliforms were not recovered from 16 (19%) of the 83 yellow Colilert-MW tubes (Table 2). *Aeromonas hydrophila*, *V. cholerae* non-O1, *Kluyvera* species,

TABLE 1. Comparison of Colilert-MW and MTF results

Organism and group	No. of samples	Mean ln difference[a]	t[b]	P[c]	r[d]	PA slope[e]	95% confidence limits[f]
Total coliform							
1	86	−0.20 (22%)	−2.32	0.02	0.95	1.071	0.998–1.150
2	66	−0.24 (27%)	−2.62	0.01	0.95	1.079	0.995–1.170
3	20	−0.07	−0.32	0.75	0.73	1.048	0.675–1.640
E. coli							
1	86	−0.04	−0.43	0.67	0.89	1.079	0.966–1.199
2	66	0.03	0.28	0.78	0.90	1.192	1.059–1.344
3	20	−0.25	−1.06	0.30	0.63	1.060	0.602–1.903

[a] Mean difference between the paired ln-transformed data values (MTF and Colilert). For tests with a significant t value, the percent difference corresponding to the mean ln difference is given in parentheses.
[b] t value from the paired t test.
[c] Type-1 probability associated with the t test.
[d] Pearson correlation coefficient.
[e] Slope of the principal-axis regression equation.
[f] 95% confidence limits of the principal-axis slope.

and 10 organisms that were not identified in the API20E data base were recovered from these tubes. No false-negative results were found. Tubes that remained colorless and did not fluoresce contained *Providencia rettgeri*, *Vibrio alginolyticus*, *Acinetobactor calcoaceticus*, and several organisms that were not identified in the API20E data base.

E. coli. *E. coli* was not recovered from 6 (15%) of the 41 Colilert-MW tubes that turned yellow and fluoresced (Table 2). *Serratia liquefaciens*, *Klebsiella pneumoniae*, and four isolates that were not identified in the API20E data base were recovered from these tubes. The above six isolates were all uegative for fluorescence upon reinoculation, suggesting that they were not the cause of the fluorescence during the original test. Five (12%) of 42 yellow Colilert-MW tubes contained *E. coli* but did not fluoresce. *E. coli* isolates from four of these five tubes fluoresced upon reiuoculation into Colilert-MW medium.

Eleven negative ECMUG tubes that were tested for accuracy included five that produced gas but did not fluoresce and six that fluoresced but did not produce gas. Organisms recovered from tubes that only produced gas included *Serratia odericii*, two *E. coli* isolates, *Serratia* spp., and one organism that was not identified by the API20E. Organisms recovered from ECMUG tubes that fluoresced but did not produce gas included two organisms that were not in the API data base, *Serratia* spp., two *E. coli* isolates, and *Escherichia fergusonii*. In summary, 4 (36%) of the 11 tubes contained *E. coli* isolates but would not have been considered positive. The *E. coli* isolates did not fluoresce or produce gas when reinoculated into ECMUG.

***Vibrio* spp.** Two tests were performed to determine whether *Vibrio* spp., a predominant group in ocean waters, caused interference. In one test, nine Colilert-MW tubes, rehydrated with 9 ml of sterile distilled water and inoculated with 1 ml of undiluted seawater, were plated on both MacConkey agar and the TCBS medium. Vibrio growth on TCBS medium was observed and verified by the API20E. No vibrios were recovered from MacConkey agar plates. Other coliforms, including *E. coli*, were recovered from MacConkey agar plates.

In a separate experiment, 20 Colilert-MW tubes were inoculated with 10 ml of undiluted ocean water. Fourteen (70%) of 20 were yellow and fluoresced. Samples from the positive tubes were streaked in duplicate onto MacConkey plates. Where possible, five isolates per plate were streaked for isolation on TSA and identified by the API20E. Of 91 isolates identified by the API20E, 54 were *Vibrio* spp. (*V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus*, and *V. fluvialis*), 36 isolates were oxidase positive but their profiles were not in the API20E data base, and one isolate was identified as *E. coli*.

Molecular analysis. DNA was extracted from isolates recovered from the first 26 water samples and hybridized with the UAR-900 probe. *E. coli* ATCC 43886 was used as a positive control, and *E. fergusonii* and *Escherichia hermannii*, recovered from other ocean water samples, were used as negative controls. The presence of DNA that hybridized to the UAR-900 probe was detected in 17 of 17 *E. coli* isolates from yellow, fluorescent Colilert-MW tnbes and from 4 of 5 *E. coli* isolates (Fig. 1, lane A, 4, 7, and 10, and lane B, 3 and 4) recovered from yellow, nonfluorescent Colilert-MW tubes. One of two *E. coli* isolates from BGB tubes hybridized with the probe (lane B, 9 and 10). No hybridization was detected for *V. fluvialis*, *V. cholerae*, *K. pneumoniae*, *Citrobacter freundii*, or five isolates that were not identified by the API20E.

Four of four *E. coli* isolates recovered from negative ECMUG tubes hybridized with the UAR-900 probe (data not shown). None of the *E. coli* isolates from Colilert-MW and ECMUG tubes agglutinated when tested with the latex O157 antigen detection kit.

TABLE 2. False-positive and false-negative test results

Organism	No. (%) of results:			
	Positive		Negative	
	True	False	True	False
Total coliforms	67 (81)	16 (19)	20 (100)	0 (0)
E. coli	35 (85)	6 (15)	37 (88)	5 (12)

DISCUSSION

Pollution of nearshore coastal environments is a growing concern for public health agencies, regulators, and the general public. Coastal areas can be adversely impacted by a variety of events, including (i) heavy rains that cause storm drain overflow, (ii) agricnltural and nrban runoff, (iii) rup-

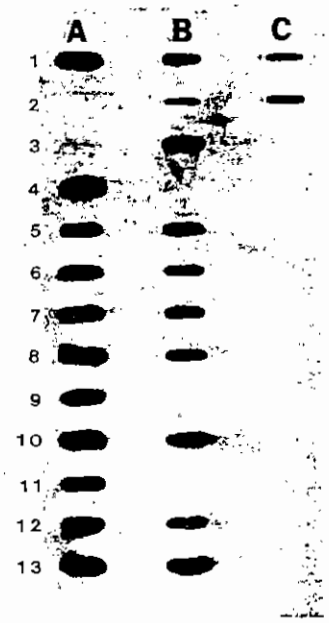

FIG. 1. Hybridization signals of bacterial DNA hybridized with the UAR-900 oligonucleotide probe. A1, *E. coli* ATCC 43886; A2, *E. fergusonii*; A3, *E. hermannii*; A4 to B10, *E. coli*; B11, isolate not identified by API20E; B12 to C2, *E. coli*; C3, *Klebsiella oxytoca*; C4 and C5, *V. cholerae*; C6, *K. pneumoniae*; C6 and C7, *C. freundii*; C8 to C10, isolates not identified by API20E.

tures in sewage discharge pipes, and (iv) boats illegally discharging human waste. Many coastal states monitor ocean water by using the standard MTF to detect total or fecal coliforms. The MTF with LTB and BGB media takes up to 4 days to obtain results, is labor intensive, and unless ECMUG is used, does not identify *E. coli*. A rapid and efficient method to test marine water and the development of specific federal regulations requiring all coastal states to monitor beach waters on a regular basis are needed to ensure that beaches are safe for human contact.

This study evaluated a new, rapid, 24-h product proposed for testing marine waters, the Colilert-MW, and compared the results with those of the traditional MTF method. Statistical analysis using paired *t* tests indicated that there was no difference between the two tests for the detection of *E. coli*. For the detection of total coliforms, however, the Colilert-MW gave higher numbers than did the MTF method. Bacterial analysis showed recovery of three species that are known to be ONPG positive, *A. hydrophila*, *V. cholerae*, and *Kluyvera* species, from the Colilert-MW tubes. Thus, depending on the relative abundance of these organisms in a given coastal area, the Colilert-MW test could yield falsely elevated numbers of total coliforms. In an earlier study, Ellgas et al. (7) also reported higher total coliform recovery in the marine environment when the original Colilert was used. They hypothesized that the Colilert medinm did a better job of resnscitating coliforms stressed by exposure to highly saline bay water than does conventional recovery medium. This possibility cannot be overlooked.

Bacterial analysis of the first 26 water samples revealed false-positive rates of 19 and 15% for total coliform and *E. coli* detection, respectively. False-positive reactions obtained by the traditional MTF method applied to marine water have been previously documented (11). Non-*E. coli* isolates recovered from yellow, fluorescent tubes did not fluoresce upon reinoculation, suggesting that they were not the original cause of fluorescence. Additionally, only one loopful was streaked, and it is highly possible that the target coliforms were present in the positive Colilert-MW tubes but were not recovered. Thus, the false-positive rates are not alarming.

Importantly, coliforms were not recovered from Colilert-MW tubes that remained clear. Previous work has shown that the traditional MTF method may have a 50% false-negative rate for ocean water (13). Therefore, the lack of false-negative results with the Colilert-MW product is encouraging.

Biochemical identification studies have shown that 10 to 20% of environmental *E. coli* strains are negative in MUG medium (3, 4, 16). In our study, *E. coli* isolates were identified in 5 of 41 yellow, nonfluorescent Colilert-MW tubes, indicating a MUG-negative rate of 12%. Genetic analysis indicated that four of these *E. coli* isolates contained sequences that hybridized with the UAR-900 probe. This suggests that the *uidA* gene, although present, was not expressed. This phenomenon has also been observed by Feng et al. (10) and Bej et al. (2). Interestingly, false-negative results were found in 4 of 11 ECMUG tubes that showed atypical results, suggesting that the standard recovery medium for *E. coli* may also be inadequate for ocean monitoring.

The number of *Vibrio* isolates is normally higher than the number of coliforms in coastal water. Typically, we recovered 80 to 100 presumptive *Vibrio* organisms per 10 ml of southern California coastal water while the number of coliforms is less than seven per 10 ml. For this reason, we needed to determine how *Vibrio* spp. would react in the Colilert-MW medium. The fact that *Vibrio* species inhibited coliforms in the Colilert-MW medium was apparent when over 50% of the organisms recovered from 90 Colilert-MW tubes that were rehydrated with undiluted seawater were *Vibrio* spp. This was not the case for tubes rehydrated with 9 ml of sterile distilled water and 1 ml of undiluted marine water since coliforms were easily recovered.

Since marine water is the natural habitat for *Vibrio* bacteria, undiluted ocean water provides vibrios with a competitive advantage over the coliform group. Conversely, rehydrating Colilert-MW tubes with sterile fresh water and seawater dilutions may provide coliforms with an advantage in that marine vibrios do not survive in fresh water and may lyse upon entering these tubes. Therefore, it is recommended that the Colilert-MW not be rehydrated with undiluted seawater but that dilutions, starting with 1:10, are made to perform the test.

In summary, the new Colilert-MW prodnct is eqnivalent to the traditional MTF method for the detection of *E. coli* but not for the detection of total coliforms. The higher recovery of total coliforms may be attributed to increased sensitivity or interference from other ONPG-positive organisms in coastal water. However, testing of coastal water for the presence of total coliforms may not be appropriate. Many members of the total coliform group live in the environment and wash into coastal waters on a daily basis. Therefore, the total coliform group does not accurately indicate sewage pollution. The presence of *E. coli*, although not a perfect indicator organism, is a better indicator of possible sewage contamination since the number of *E. coli* organisms in the mammalian gut is higher than in the environment in the continental United States. Since the Colilert-MW method

accurately determined the levels of *E. coli* within 24 h, we feel that it a powerful tool with which to rapidly assess possible fecal contamination in marine waters. This product could provide agencies that monitor coastal waters with a simple and rapid method for assessing the microbial conditions in nearshore beach and bathing waters.

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REFERENCES

1. **American Public Health Association.** 1989. Standard methods for the examination of water and wastewater, 17th ed. American Public Health Association, Washington, D.C.
2. **Bej, A. K., S. C. McCarty, and R. M. Atlas.** 1991. Detection of coliform bacteria and *Escherichia coli* by multiplex polymerase chain reaction: comparison with defined substrate and plating methods for water quality monitoring. Appl. Environ. Microbiol. **57**:2429–2432.
3. **Clark, D. L., B. B. Milner, M. H. Stewart, R. L. Wolfe, and B. H. Olson.** 1991. Comparative study of commercial 4-methylumbelliferyl-β-D-glucuronide preparations with the *Standard Methods* membrane filtration fecal coliform test for the detection of *Escherichia coli* in water samples. Appl. Environ. Microbiol. **57**:1528–1534.
4. **Covert, T. C., E. W. Rice, S. A. Johnson, D. Berman, C. H. Johnson, and P. J. Mason.** 1992. Comparing defined substrate coliform tests for the detection of *Escherichia coli* in water. J. Am. Water Works Assoc. **84**:98–104.
5. **Edberg, S. C., M. J. Allen, D. B. Smith, and The National Collaborative Study.** 1988. National field evaluation of a defined substrate method for the simultaneous enumeration of total coliforms and *Escherichia coli* from drinking water: comparison with the standard multiple tube fermentation method. Appl. Environ. Microbiol. **54**:1595–1601.
6. **Edberg, S. C., M. J. Allen, D. B. Smith, and The National Collaborative Study.** 1989. National field evaluation of a defined substrate method for the simultaneous detection of total coliforms and *Escherichia coli* from drinking water: comparison with presence-absence techniques. Appl. Environ. Microbiol. **55**:1003–1008.
7. **Ellgas, W. M., M. J. Kenney, K. E. Osborn, and A. E. Greenberg.** 1989. Evaluation of autoanalysis Colilert in wastewater. *In* Proceedings of the Water Pollution Control Federation Speciality Conference on Microbial Aspects of Surface Water Quality, 30 May 1989, Chicago, Ill.
8. **Federal Register.** 1989. National primary drinking water regulations: total coliforms (including fecal coliforms and *E. coli*). Fed. Regist. **54**:27544–27567.
9. **Federal Register.** 1992. National primary drinking water regulations: analytical techniques, coliform bacteria, final rule. Fed. Regist. **57**:24744.
10. **Feng, P., R. Lum, and G. W. Chang.** 1991. Identification of *uidA* gene sequences in β-D-glucuronidase-negative *Escherichia coli*. Appl. Environ. Microbiol. **57**:320–323.
11. **Hussong, D., J. M. Damaré, R. M. Weiner, and R. R. Colwell.** 1981. Bacteria associated with false-positive most-probable-number coliform test results for shellfish and estuaries. Appl. Environ. Microbiol. **41**:35–45.
12. **McCarty, S. C., J. H. Standridge, and M. C. Stasiak.** 1992. Evaluating a commercially available defined-substrate test for recovery of *E. coli*. J. Am. Water Works Assoc. **84**:91–97.
13. **Olson, B. H.** 1978. Enhanced accuracy of coliform testing in seawater by a modification of the most-probable-number method. Appl. Environ. Microbiol. **36**:438–444.
14. **Rice, E. W., M. J. Allen, D. J. Brenner, and S. C. Edberg.** 1991. Assay for β-glucuronidase in species of the genus *Escherichia* and its applications for drinking water analysis. Appl. Environ. Microbiol. **57**:592–593.
15. **Rice, E. W., M. J. Allen, and S. C. Edberg.** 1990. Efficacy of β-glucuronidase assay for identification of *Escherichia coli* by the defined-substrate technology. Appl. Environ. Microbiol. **56**:1203–1205.
16. **Shadix, L. C., and E. W. Rice.** 1991. Evaluation of β-glucuronidase assay for the detection of *Escherichia coli* from environmental waters. Can. J. Microbiol. **37**:908–911.

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Detection of *Escherichia coli* in Sewage and Sludge by Polymerase Chain Reaction

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A method in which the polymerase chain reaction (PCR) was used was developed to amplify either a *uidA* gene fragment or a 16S rRNA gene fragment from *Escherichia coli* in sewage and sludge. Because of interference caused by humic acidlike substances, crude DNA extracts were purified with a Sephadex G-200 spun column before the PCR was begun. A Southern analysis in which a nonradioactive chemiluminescent method was used was performed to confirm the presence of PCR products. The sensitivity of detection for PCR products when the chemiluminescent method was used was determined to be 30 ag of *E. coli* genomic DNA template. In seeded sludge, the PCR amplified the target DNA from 80 *E. coli* cells per g of sludge and 50 *Shigella dysenteriae* cells per g of sludge. Because only 0.05 aliquot of a sludge extract was used for the PCR, we deduced that the PCR detected target DNA equivalent to the DNA of 2.5 to 4 cells in the extract. The PCR amplified the *uidA* fragment from diluted sewage influents and effluents containing *E. coli* cells. Therefore, the PCR performed with a chemiluminescent gene probe can be used to detect the presence of potentially pathogenic microorganisms in sewage and sludge. This technique can be expanded to permit direct detection of pathogenic microorganisms in water samples, thus leading to enhanced public health protection.

The polymerase chain reaction (PCR) has recently become a powerful tool with which to explore microbial activities and identities in environmental microbiology. Because of its superior sensitivity and specificity, the PCR has been used to (i) detect specific microorganisms in water, soils, and sediments without the need for cell cultivation (2, 3, 6, 9, 11, 15, 16, 18), (ii) detect viable but nonculturable organisms, such as *Vibrio vulnificus*, from a microcosm (4), and (iii) amplify mRNA (by an RNA PCR) to distinguish live *Giardia* cysts from dead cysts (10) in water samples.

The use of the PCR for identifying specific organisms obtained from environmental samples has been problematical because of the presence of various interfering substances, such as humic acids, metal ions, and other substances. Methods such as diluting crude extract templates (18), performing the PCR with preamplified products (11), and removing interfering substances (19) have been developed to overcome these problems when soil or sediment samples are used for the PCR.

Raw sewage and raw sludge contain relatively high concentrations of humic acidlike substances compared with soils. This has prevented the use of the PCR with sewage and sludge samples. If the interfering substances could be removed from these samples, the PCR could be used to detect low numbers of pathogenic microorganisms, such as *Legionella* spp., *Mycobacterium* spp., and enteric viruses, including hepatitis A virus, that are of public health concern.

The ability to detect low numbers of microorganisms would be valuable for wastewater agencies that reuse treated wastewater for irrigation of parks, golf courses, and other public greenways. In the future, if sludge is proven to be microbiologically safe, it may be used as a fertilizer for important food crops. Monitoring treated wastewater and sludge by the PCR would provide increased public health protection since the PCR is much more sensitive for detecting specific pathogenic microorganisms than traditional culture techniques are.

In this study, we adapted a rapid DNA extraction method (17) and a rapid purification procedure (19) and combined them with the PCR to detect *Escherichia coli* in sewage and sludge. The PCR was successfully used to amplify from both sewage and sludge a fragment of the *E. coli uidA* gene that codes for β-D-glucuronidase.

MATERIALS AND METHODS

Sampling and DNA extraction. Raw sludge, digested sludge, primary influent, and effluent sewage samples were collected from the treatment facilities of the County Sanitation Districts of Orange County, Fountain Valley, Calif., and were processed within 4 h after sampling. The raw sludge contained 95% water, 2.7% total solids, and 1.6% volatile solids. The digested sludge consisted of 90% water, 6.5% total solids, and 4.6% volatile solids. The digested sludge was obtained from a primary digester that was fed with raw sludge.

Total DNA was extracted from 1 g (wet weight) of sludge by a rapid freeze-thaw, phenol-chloroform extraction method (17) and was resuspended in 200 μl of sterile TE buffer (10 mM Tris-HCl, 1.0 mM EDTA; pH 8.0). Portions (10 ml) of primary influent, primary effluent, and dilutions (10^{-3} to 10^{-6}) were filtered through 0.5-μm-pore-size FHLP Teflon filters (diameter, 13 mm) by using a Swinnex filter holder (Millipore, Bedford, Mass.). Phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) was used as the diluent. Crude DNA extracts from bacterial cells were obtained as described by Bej et al. (3).

Organisms and culture media. *E. coli* ATCC 35346 and *Shigella dysenteriae* ATCC 49552 were obtained from the American Type Culture Collection, Rockville, Md., and were maintained on nutrient agar (Difco, Detroit, Mich.). Mid-log-phase *E. coli* and *S. dysenteriae* cells were collected, washed twice with phosphate-buffered saline, and

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serially diluted (10^{-2} to 10^{-9}); the serial dilutions of each strain were seeded into sterile 1-g sludge samples and enumerated by using heterotrophic plate count techniques (1). Xylose lysine desoxycholate agar (Becton Dickinson Microbiology Systems, Cockeysville, Md.) and mFC agar (Difco) were used as selective media for *Shigella* spp. and *E. coli*, respectively. Environmental isolates were identified by using the API 20E system (Analytab Products, Plainview, N.Y.). Genomic DNA was extracted from *E. coli* cultures by using a G NOME kit (Bio 101, La Jolla, Calif.) according to the manufacturer's instructions.

DNA purification by gel filtration chromatography. Crude DNA extracts obtained from sludge and sewage were purified with Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N.J.) spun columns by using a previously described method (19). Briefly, 100 μl of a crude DNA extract was loaded onto a Sephadex G-200 column, which was then centrifuged twice for 10 min at 1,100 × *g* to obtain 100 μl of eluent.

PCR. Primers UAL-754 (5'-AAAACGGCAAGAAAAAG CAG-3') and UAR-900 (5'-ACGCGTGGTTACAGTCTT GCG-3'), which have been described previously by Bej et al. (2), were used to amplify a 147-bp coding region of the *uidA* gene. All of the oligonucleotides were synthesized commercially (Genosys Biotechnologies, The Woodlands, Tex.). Eluents (10-μl aliquots) from Sephadex G-200-purified sewage and sludge samples were used as templates for the PCR. DNA amplification was completed by using native *Taq* polymerase (Perkin-Elmer, Norwalk, Conn.) and the protocol described by Bej et al. (2). The PCR products were analyzed by electrophoresis on a 2% SeaKem GTG agarose (FMC BioProducts, Rockland, Maine) gel stained with ethidium bromide (0.5 μg/ml) and were identified by using a gel image analysis system (Ultra-Violet Products, San Gabriel, Calif.).

DNA hybridization. Internal oligonucleotide probe UA-IN (5'-TGCCGGGATCCATCGCAGCGTAATG-3') was used to verify the identities of the PCR products. The oligonucleotide was 3' end labeled with digoxigenin-11-ddUTP by using a Genius 5 nonradioactive DNA labeling kit (Boehringer Mannheim, Indianapolis, Ind.). The amplified DNA was transferred onto Hybond-N^+ positively charged nylon membranes (Amersham, Arlington Heights, Ill.) by using a Minifold II slot blotter (Schleicher & Schuell, Keene, N.H.) (5) or a PosiBlot pressure blotter (Stratagene, La Jolla, Calif.) for the Southern analysis (14). DNA was fixed to the nylon membranes by UV irradiation at 254 nm for 2 min by using a UV Stratalinker 2400 apparatus (Stratagene). Hybridization was performed at 60°C in the presence of a labeled oligonucleotide internal probe (2 pmol/ml). The hybridized filters were washed twice with a high-salt solution (2× SSC in 0.1% sodium dodecyl sulfate [1× SSC is 0.15 M NaCl plus 0.015 trisodium citrate, pH 7.0]) at room temperature and then twice with a low-salt solution (0.1× SSC in 0.1% sodium dodecyl sulfate) at 50°C. A Genius 3 nucleic acid detection kit (Boehringer Mannheim) was used to prepare the hybridized filters for chemiluminescent detection. The hybridization signals were visualized on X-OMAT film (Eastman Kodak, Rochester, N.Y.) by using autoradiography (12).

RESULTS

The results obtained with the total DNAs extracted from primary sewage influent and raw sludge are shown in Fig. 1A. Less fragmentation of DNA was found in the sewage extracts (Fig. 1A, lanes 2 and 3) than in the sludge extracts (lanes 4 and 5). DNA extracts from the samples were either pale yellow or brownish, indicating the presence of impurities, mainly humic acidlike substances. The concentration of the impurities in the sludge extracts was approximately 5 μg (dry weight) per μl of extract. A 1-μl portion of sludge extract was enough to block the PCR, as reported previously (19). After Sephadex G-200 column purification, the purified extracts (eluents) were colorless and suitable for the PCR. Figure 1B shows the inhibitory effects on PCR amplification observed with unpurified crude DNA extracts containing 5 μg of humic acidlike substances (Fig. 1B, lanes 2 through 5). A distinct PCR product (371 bp) from sewage and sludge (Fig. 1B, lanes 6 through 9) was observed when the purified extracts were used as templates. The amplified target fragment (positions 1169 through 1539 on the *E. coli* 16S rRNA gene) indicated that *E. coli* was present in the sewage and sludge samples.

The heterotrophic plate counts were $2.6 \times 10^8 \pm 0.2 \times 10^8$ CFU/ml for raw sewage and $1.6 \times 10^{10} \pm 0.8 \times 10^{10}$ CFU/g for raw sludge. The *E. coli* counts on mFC agar were $8.6 \times 10^3 \pm 6.0 \times 10^3$ CFU/ml and $1.2 \times 10^6 \pm 0.4 \times 10^6$ CFU/g for raw sewage and raw slndge, respectively. *Shigella* spp. were not recovered from either sample.

The PCR detection limit for the *uidA* gene was determined by using *E. coli* genomic DNA as the template. Figure 2A shows 147-bp fragments amplified from the *uidA*-coding region in *E. coli* genomic DNA when amounts of DNA ranging from 3 ng to 3.ag were used. The amplified products were visualized on an ethidium bromide-stained agarose gel under UV light when the imitial concentration of the DNA template was as low as 30 fg per reaction. A Southern blot analysis showed that the PCR products were detected when 30 ag of genomic DNA was used as the template (Fig. 2B). Long PCR fragments (147 to 400 bp) were found at template concentrations greater than 3 fg per reaction.

Figure 3A shows the hybridization signals of amplified 147-bp *uidA* fragments from raw sludge and digested sludge when the internal probe was used. Since *E. coli* was abundant in both samples (data not shown), positive PCR results were expected. However, amplified products were observed only when purified extracts were used as templates (Fig. 3A, slots 2, 4, 6, 8, 10, and 12). The heterotrophic plate counts for raw sludge and digested sludge in this experiment were $4.0 \times 10^8 \pm 2.0 \times 10^8$ and $4.1 \times 10^5 \pm 2.3 \times 10^5$ CFU/g, respectively.

The results of an experiment to determine the sensitivity of detection of *E. coli* in seeded sterile sludge when the PCR was used are shown in Fig. 3B. *E. coli* genomic DNA was used to amplify the 147-bp *uidA* fragment. The amplified product was detected in sludge seeded with as few as 80 cells per g. No amplified products were detected in sterile unseeded sludge (Fig. 3B, slot 1). Similarly, the amplified *uidA* fragment was detected in sterile sludge seeded with a minimal density of 50 *S. dysenteriae* cells per g (data not shown). Because only 5% (10 of 200 μl) of each purified extract was used for the PCR, the sensitivities of detection were assumed to be 4 and 2.5 cells for *E. coli* and *S. dysenteriae*, respectively.

Membrane filtration followed by freeze-thaw cycles (3) was used to extract total DNAs from influent and effluent sewage samples. Prior to the PCR, the crude DNA extracts were pnrified with a Sephadex G-200 spun column. No PCR products were detected when unpurified extracts were nsed for amplification. The hybridization signals revealed the presence of an amplified *uidA* fragment in diluted sewage

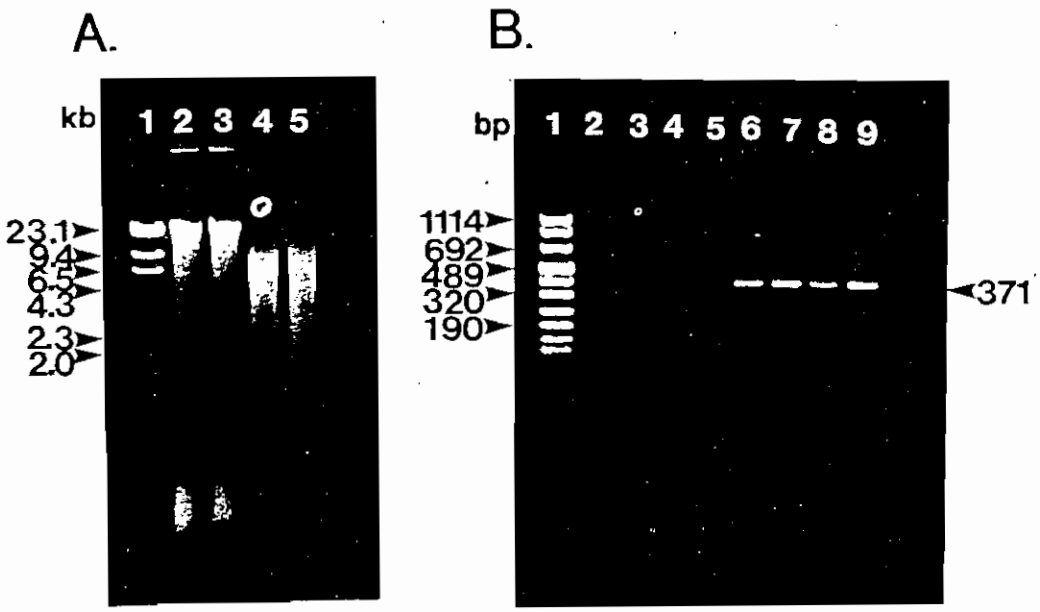

FIG. 1. Gel electrophoresis analysis of DNA extracts and PCR products from raw sewage and sludge. (A) The gel was a 0.7% SeaKem agarose gel. Lane 1, lambda *Hin*dIII-digested molecular weight marker (0.5 μg); lanes 2 and 3, 0.1 aliquots of crude DNA extracts obtained from 30 ml of primary sewage influent; lanes 4 and 5, 0.025 aliquots of crude DNA extracts obtained from 1 g of raw sludge. (B) *E. coli* 16S rRNA gene fragments (371 bp) amplified from unpurified and purified DNA extracts. The volumes indicated in parentheses below were the volumes used as templates in the reactions; the gel was a 2% SeaKem agarose gel. Lane 1, low-molecular-weight DNA marker (0.5 μg); lanes 2 and 3, unpurified sewage extracts (1 μl); lanes 4 and 5, unpurified sludge extracts (1 μl); lanes 6 and 7, Sephadex G-200-purified sewage extracts (10 μl); lanes 8 and 9, Sephadex G-200-purified sludge extracts (10 μl). The samples loaded into lanes 2 through 9 were 0.2 aliquots of amplified reaction mixtures.

samples at dilutions ranging from 10^{-3} to 10^{-6}. No amplification was detected when we examined the Sephadex G-200 column eluents that did not contain DNA templates (Fig. 3C, slots 1 and 6). The heterotrophic plate counts for the influent and effluent samples were $7.1 \times 10^6 \pm 2.1 \times 10^6$ and $5.1 \times 10^6 \pm 1.9 \times 10^6$ CFU/ml, respectively. No *Shigella* spp. were recovered from influent and effluent sewage samples. The *E. coli* counts were not determined.

DISCUSSION

The initial problem associated with using the PCR with sewage and sludge samples (i.e., interfering substances) was eliminated. A Sephadex G-200 spun column was used to purify all crude extracts before amplification. The removal of interfering substances allowed the use of larger volumes (10 to 50 μl) of extracts as templates during the PCR (total volume, 100 μl), resulting in increased sensitivity. Spun columns have been used successfully by other researchers to remove interfering components from beef extract-glycine-ferric chloride virus concentrates before the PCR was performed (6, 13). This illustrates the efficacy of using gel filtration chromatography to purify environmental samples used for PCR amplification. Because *E. coli* is commonly found in sewage and sludge samples, detection of *E. coli* 16S rRNA genes or the *uidA* gene by the PCR was expected. Although the *uidA* gene may be found in some *Shigella* spp. (2), it is most commonly present in *E. coli* (8).

An ethidium bromide-stained gel could detect the PCR products amplified from 30 fg of *E. coli* genomic DNA (seven copies of the *uidA* gene). The sensitivity of detection was increased to 30 ag (less than one copy) when the chemiluminescent probe was used. The chemiluminescent detection limit is at the same order of magnitude as the detection limit reported by Bej et al. (3), who used radioactive ^{32}P-labeled oligonucleotide probes with *Legionella pneumophila* genomic DNA. Because of its safety, low cost, and sensitivity equivalent to the sensitivity of the ^{32}P method, the chemiluminescent method has distinct advantages over radioisotopic detection. Long fragments with defined primer ends were produced during the PCR (7). In the sensitivity test, the long fragments were found only when higher template concentrations were used for amplification. This suggests that optimization of template concentration is necessary to reduce the production of long fragments and to increase the amplification efficiency.

The hybridization results showed that the presence of humic acidlike substances completely inhibited the PCR. Amplified DNA was detected only after sample extracts were purified with Sephadex G-200 spun columns. The results of the sensitivity tests performed with seeded sterile sludge samples suggest that 80 *E. coli* cells per g and 50 *S. dysenteriae* cells per g can be detected by using the PCR and gene probe methods. Because mid-log-phase cells were used for seeding, amplified DNA from nonviable cells was minimized. However, the PCR products of unseeded native samples could be amplified from free DNA or from DNA from dead cells. The detection limit for unseeded sludge samples needs further investigation. Only the DNA direct extraction method (17) was used for obtaining the PCR DNA templates from sludge samples. The filtration method (3) was not suitable for determining PCR sensitivity for sludge samples because dilution procedures, which reduced the detection sensitivity, were necessary to prevent the filters from clogging.

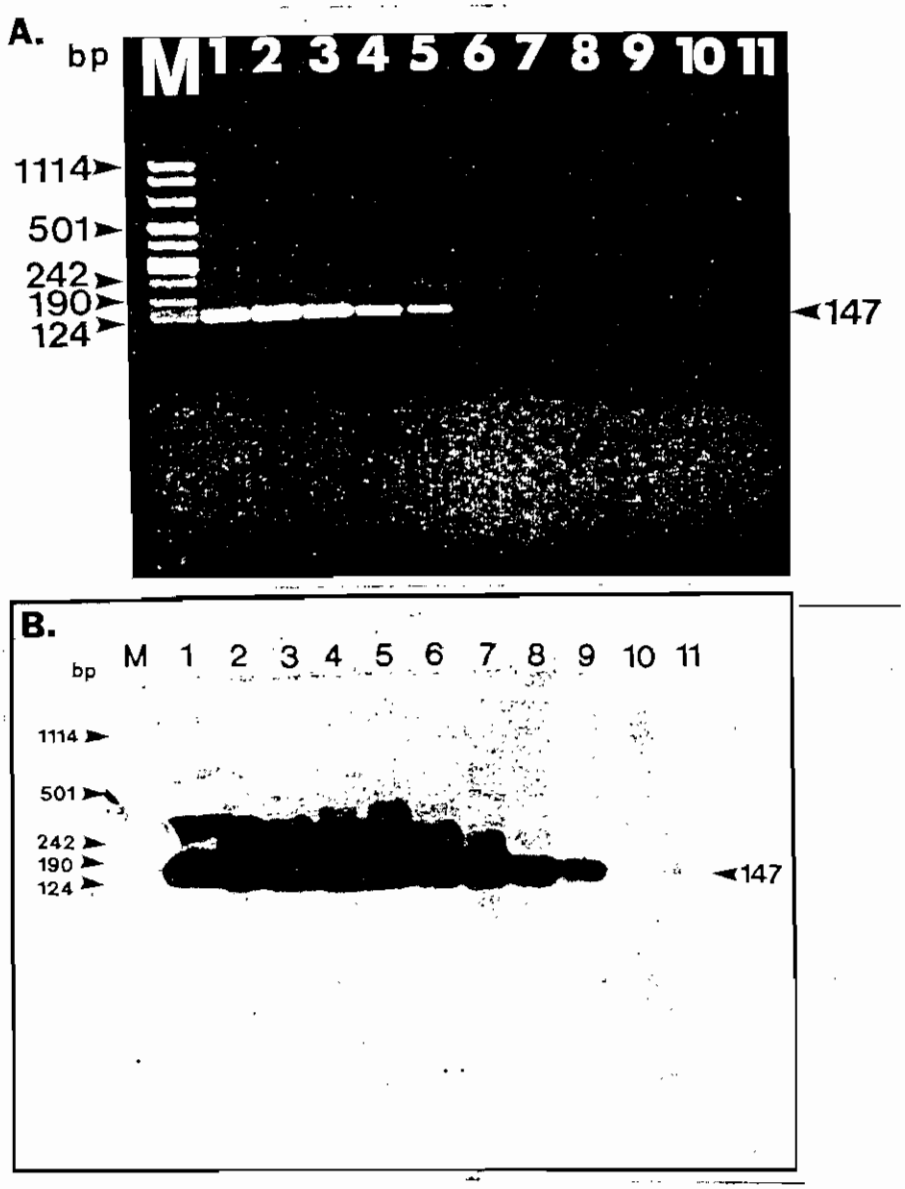

FIG. 2. Sensitivity of detection of the *E. coli uidA* gene fragment when the PCR was used. Lanes M, low-molecular-weight DNA marker; lanes 1, 3 ng of *E. coli* genomic DNA; lanes 2, 300 pg; lanes 3, 30 pg; lanes 4, 3 pg; lanes 5, 300 fg; lanes 6, 30 fg; lanes 7, 3 fg; lanes 8, 300 ag; lanes 9, 30 ag; lanes 10, 3 ag; lanes 11, reaction mixture (no DNA template). (A) PCR products on an ethidium bromide-stained 2% SeaKem agarose gel. (B) Autoradiogram of panel A produced by using a chemiluminescent internal probe.

Because raw sewage also contains other particulate materials, diluting the samples is necessary before filtration. Alternatively, centrifugation at 6,000 × *g* for 10 min was used to obtain cell pellets from large volumes (50 to 100 ml) of undiluted sewage samples. Crude DNA extracts obtained by a freeze-thaw, phenol-chloroform extraction method (17) must be purified prior to the PCR.

Our results showed that DNA shearing was more prominent in sludge than in sewage after extraction. This could be caused by different components, such as more particulate matter present in sludge than in sewage, or by the action of contaminated DNase during the extraction. The filtration methods require an additional Sephadex G-200 purification step in order to obtain positive PCR results. *Shigella* spp. were not recovered from influent and effluent samples, suggesting that the amplified *uidA* gene fragments originated from *E. coli*.

In conclusion, the PCR and nonradioactive gene probe methods were successfully used to detect target microorganisms present in sewage and sludge samples. The PCR interfering substances were removed by using a Sephadex G-200 spun column. This purification procedure is crucial for environmental DNA extracts before PCR amplification. Because of their sensitivity and specificity, the PCR and gene probe techniques can be used to detect potentially pathogenic microorganisms in raw sewage and sludge. This should allow wastewater industries to evaluate the efficiency of treatments for removing pathogens.

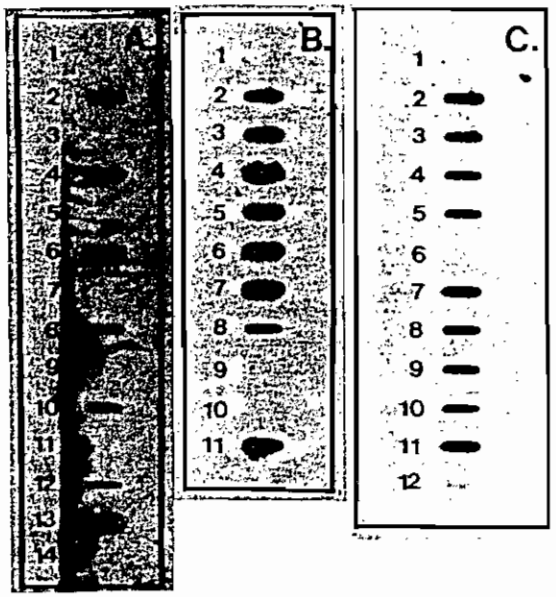

FIG. 3. Slot blot hybridization signals for amplified *uidA* DNA obtained from sludge and sewage samples. (A) Raw sludge and digested sludge. Slots 1, 3, and 5, unpurified raw sludge extracts; slots 2, 4, and 6, purified raw sludge extracts; slots 7, 9, and 11, unpurified digested sludge extracts; slots 8, 10, and 12, purified digested sludge extracts; slot 13, *E. coli* genomic DNA control; slot 14, reaction mixture (no template). (B) Seeded sterile sludge. Slot 1, unseeded extracts; slot 2, preparation seeded with 8.0×10^7 cells per g; slot 3, preparation seeded with 8.0×10^6 cells per g; slot 4, preparation seeded with 8.0×10^5 cells per g; slot 5, preparation seeded with 8.0×10^4 cells per g; slot 6, preparation seeded with 8.0×10^3 cells per g; slot 7, preparation seeded with 8.0×10^2 cells per g; slot 8, preparation seeded with 8.0×10^1 cells per g; slot 9, preparation seeded with 8.0 cells per g; slot 10, reaction mixture (no template); slot 11, *E. coli* genomic DNA. (C) Primary influent (slots 2 through 5) and effluent (slots 7 through 10). Slots 1 and 6, unseeded control (sterile diluent); slots 2 and 7, 10^{-3} dilution; slots 3 and 8, 10^{-4} dilution; slots 4 and 9, 10^{-5} dilution; slots 5 and 10, 10^{-6} dilution; slot 11, *E. coli* genomic DNA; slot 12, reaction mixture (no remplate).

ACKNOWLEDGMENTS

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REFERENCES

1. **American Public Health Association.** 1989. Standard methods for the examination of water and wastewater, 17th ed. American Public Health Association, Washington, D.C.
2. **Bej, A. K., J. L. DiCesare, L. Haff, and R. M. Atlas.** 1991. Detection of *Escherichia coli* and *Shigella* spp. in water by using the polymerase chain reaction and gene probes for *uid*. Appl. Environ. Microbiol. 57:1013–1017.
3. **Bej, A. K., M. H. Mabbubani, J. L. DiCesare, and R. M. Atlas.** 1991. Polymerase chain reaction-gene probe detection of microorganisms by using filter-concentrated samples. Appl. Environ. Microbiol. 57:3529–3534.
4. **Brauns, L. A., M. C. Hudson, and J. D. Oliver.** 1991. Use of the polymerase chain reaction in detection of culturable and nonculturable *Vibrio vulnificus* cells. Appl. Environ. Microbiol. 57:2651–2655.
5. **Davis, L. G., M. D. Dibner, and J. F. Battey.** 1986. Basic methods in molecular biology, p. 147–149. Elsevier Science Publishing Co., New York.
6. **De Leon, R., C. Shieh, R. Baric, and M. Sobsey.** 1990. Detection of enteroviruses and hepatitis A virus in environmental samples by gene probes and polymerase chain reaction, p. 833–853. *In* Advances in water analysis and treatment. Proceedings of the Water Quality Technology Conference, San Diego, Calif. American Water Works Association, Denver.
7. **Gibbs, R. A.** 1990. DNA amplification by the polymerase chain reaction. Anal. Chem. 62:1202–1214.
8. **Jochimsen, B., P. Nygaard, and T. Vestergaard.** 1975. Location on the chromosome of *Escherichia coli* of genes governing purine metabolism. Mol. Gen. Genet. 143:85–91.
9. **Mahbubani, M. H., A. K. Bej, R. Miller, L. Haff, J. DiCesare, and R. M. Atlas.** 1990. Detection of *Legionella* with polymerase chain reaction and gene probe methods. Mol. Cell. Probes 4:175–187.
10. **Mahbubani, M. H., A. K. Bej, M. Perlin, F. W. Schaefer III, W. Jakubowski, and R. M. Atlas.** 1991. Detection of *Giardia* cysts by using the polymerase chain reaction and distinguishing live and dead cysts. Appl. Environ. Microbiol. 57:3456–3461.
11. **Pillai, S. D., K. L. Josephson, R. L. Bailey, C. P. Gerba, and I. L. Pepper.** 1991. Rapid method for processing soil samples for polymerase chain reaction amplification of specific gene sequences. Appl. Environ. Microbiol. 57:2283–2286.
12. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Schwab, K. J., R. De Leon, R. S. Baric, and M. D. Sobsey.** 1991. Detection of rotaviruses, enteroviruses and hepatitis A virus by reverse transcriptase-polymerase chain reaction, p. 475–491. *In* Advances in water analysis and treatment. Proceedings of the Water Quality Technology Conference, Orlando, Fla. American Water Works Association, Denver.
14. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503–517.
15. **Starnbach, M. N., S. Falkow, and L. S. Tompkins.** 1990. Species-specific detection of *Legionella pneumophila* in water by DNA amplification and hybridization. J. Clin. Microbiol. 27:1257–1261.
16. **Steffan, R. J., and R. M. Atlas.** 1988. DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. Appl. Environ. Microbiol. 54:2185–2191.
17. **Tsai, Y.-L., and B. H. Olson.** 1991. Rapid method for direct extraction of DNA from soil and sediments. Appl. Environ. Microbiol. 57:1070–1074.
18. **Tsai, Y.-L., and B. H. Olson.** 1992. Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. Appl. Environ. Microbiol. 58:754–757.
19. **Tsai, Y.-L., and B. H. Olson.** 1992. Rapid method for separation of bacterial DNA from humic substances for polymerase chain reaction. Appl. Environ. Microbiol. 58:2292–2295.

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Detection of *Legionella* Species in Sewage and Ocean Water by Polymerase Chain Reaction, Direct Fluorescent-Antibody, and Plate Culture Methods

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***Legionella* spp. are ubiquitous in most environmental water sources; however, sewage treatment plants have not been examined as potential environmental reservoirs for these bacteria. This study used polymerase chain reaction, direct fluorescent-antibody staining, and culture methods to examine raw and treated sewage, ocean-receiving waters, and nearshore coastal environments for the presence of *Legionella pneumophila* and other *Legionella* spp. The study concluded that *Legionella* spp. are present in all phases of sewage treatment and that population numbers do not significantly decline through the treatment process. Ocean-receiving waters located 5 miles offshore, where the treated sewage is discharged, were found to contain *Legionella* spp., but ocean water between the discharge site and coastal bathing beaches was negative. This suggests that the *Legionella* spp. from the ocean discharge site were not reaching the nearshore beach waters. A flood control channel and river that entered the ocean were found to contain *Legionella* spp., and a nearby beach swimming area was also found to be positive, suggesting that land runoff from the flood control channel and river were the source of the *Legionella* spp. in the beach water samples that tested positive.**

Legionellae are well known as the etiological agent of both Legionnaires' disease and Pontiac fever. Forty-eight species and more than 51 serogroups of *Legionella* have been identified. Eighteen species have been associated with either fatal pneumonia, i.e., Legionnaires' disease, or a noupneumonic self-limiting flu-like illness, Pontiac fever (6). *Legionella* species are ubiquitous in environmental waters and capable of surviving extreme ranges of environmental conditions (8, 14–16). Human infection occurs through the inhalation of aerosols contaminated with *Legionella* spp. Potential sources of legionellosis include *Legionella*-contaminated water in cooling towers and air conditioners (4, 19), hot tubs (10), showerhead water (18), and public fountains (7).

Sewage treatment plants represent an environment where *Legionella* spp. possibly may exist. This potential environmental reservoir of *Legionella* spp. has not been studied or characterized. The objective of this 1-year study was to determine whether *Legionella* spp. were present in the influent of a major metropolitan sewage treatment plant, and if present, to determine how well these organisms survived the different stages of sewage treatment. Since the treatment facility discharges treated sewage to a location 5 miles offshore, it was also important to determine whether *Legionella* spp. were present in the ocean outfall area and whether they reached beach swimming areas. In addition, a storm water drainage channel and river water that entered a coastal beach area were tested, as was water from a beach adjacent to the river mouth, to determine whether *Legionella* spp. were entering coastal water from land runoff. This study used three methods: polymerase chain reaction (PCR), direct fluorescent-antibody (DFA) staining, and plate culture. These methods were used to determine the occurrence of *Legionella* spp. in these diverse water bodies.

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MATERIALS AND METHODS

Sample collection. Primary influent, primary effluent, and secondary effluent samples were collected twice monthly for 1 year in 250-ml bottles and processed immediately. Primary effluent treatment samples were collected after sewage went through settling basins, while secondary treatment samples were collected after trickling filtration and/or activated sludge treatment.

Ocean surfzone samples were collected in sterile 500-ml bottles. Ocean outfall samples were collected at the surface and 30 m below the surface. Surface samples were collected by submersing a hand-held sterile bottle. Subsurface samples were collected with a rosette sampler (General Oceanics, Miami, Fla.). Ocean samples were placed on ice and processed within 6 h of collection. Confirmation of wastewater plume sampling was completed by using the Colilert-Marine Water product as described previously (12). Salinity was determined with a refractometer.

PCR. For genetic detection of *Legionella* spp., the EnviroAmp test kit, recently developed by Perkin-Elmer Roche (Alameda, Calif.), was used as described in the manufacturer's instructions with some exceptions. While the manufacturer recommends filtering unmodified samples, many of our water samples required dilutions to eliminate interference caused by undefined substances in both sewage and ocean water. Environmental genetic detection of *Legionella* species and *Legionella pneumophila* was accomplished by filtering 100 ml of diluted ocean water (1:10 or 1:100) or 10 ml of diluted sewage (1:10 or 1:100) and using PCR to amplify DNA sequences in the conserved regions of the 5S rRNA gene (2, 9, 20) and sequences from the *mip* (macrophage

infectivity potentiator) gene, which is unique to *L. pneumophila* (3, 5). The kit included these specific probes immobilized on nylon membranes (a reverse dot blot technique). PCR, with biotinylated primers, allowed for detection with streptavidin-horseradish peroxidase conjugate. A blue "L" dot indicated the presence of *Legionella* species and a blue "P" dot indicated the presence of *L. pneumophila*. Twenty *Legionella* species can be identified by the species dot. Positive and negative controls are also built into the strip. The test is semiquantitative since the intensity of the blue positive control dot is equivalent to 10^3 cells per ml. In this study, when the sample L or P dot was darker than the control dot, the sample was considered to contain $>10^3$ cells per ml in the original sample. When the sample L or P dot was lighter than the positive control dot, the sample was considered to contain $<10^3$ cells per ml in the original sample. Importantly, the filtration system in this kit is designed to allow free DNA to pass through the filter while still capturing intact *Legionella* cells.

DFA. One hundred milliliters of ocean water or 10 ml of sewage was filtered through a 0.45-μm-pore-size HVLP Millipore (Bedford, Mass.) filter. The filter was placed into 2 ml of deionized water and vortexed for 30 s to dislodge cells from the filter. The filter was removed from the vial, and formalin was added to a final concentration of 2%. Twenty microliters of sample was added to an eight-well toxoplasmosis slide (Bellco Biotechnology, Vineland, N.J.). The slide was air dried and heat fixed. Twenty microliters of one of two fluorescein isothiocyanate-labeled *Legionella* polyclonal antibodies (SciMedX, Denville, N.J.) was added per well. The species-specific polyclonal antibody identifies *Legionella micdadei*, *Legionella bozemanii*, *Legionella longbeachae* serogroups 1 and 2, *Legionella dumoffii*, *Legionella gormanii*, and *Legionella jordanis*. Positive samples were further tested with 20 μl of polyclonal antibody specific to *L. pneumophila* serogroups 1 to 6 (Genetic Systems, Seattle, Wash.). Slides were incubated for 30 min at 37°C in a humidity chamber. Slides were rinsed with phosphate-buffered saline (pH 7.6) with a final deionized water rinse and then dried. Cells were counted with an epifluorescent microscope (Olympus, Lake Success, N.Y.).

Plate culture. After concentration (as described for DFA), samples were pretreated with acid (1) to eliminate most nontarget organisms. After acid treatment, 0.1 ml was spread plated onto buffered charcoal-yeast extract agar containing α-ketoglutarate and glycine (3 g/liter) and supplemented with cycloheximide (80 mg/liter), vancomycin (5 mg/liter), and polymyxin B (100 IU/ml). The plates were incubated at 37°C in a humid 4% CO_2 environment for 5 to 7 days. *Legionella* colonies were identified by the colony blot protocol described by Steinmetz et al. (17). This method identifies the organisms by using a monoclonal antibody that recognizes a 60-kDa heat shock protein present in all *Legionella* species.

Seeding experiments: whole cell. A suspension of 10^3 cells of *L. pneumophila* (ATCC 33155) per ml was added to one flask each of filter-sterilized and nonsterile ocean water that tested negative for the presence of *Legionella* spp. and then incubated at 16°C with stirring. This temperature approximates the normal ocean temperature of water from the sampling area. Water was pretested for the presence of *Legionella* spp. by PCR as described above. After seeding, 100 ml was removed at time zero, 24 h, 2.5 days, and weeks 1, 2, 3, 4, 5, and 6 and tested by PCR.

A second seeding experiment in which 3×10^3 *L. pneumophila* cells per ml was added to unfiltered and filter-sterilized secondary sewage effluent and to filter-sterilized ocean water was performed. These waters were tested by both PCR and colouy culture at time zero and days 1, 4, 5, 6, and 7. Plant retention time of sewage is less than 24 h; therefore, the 7-day experiment adequately covered this time frame. PCR was performed as described above. For colony culture, 10 ml of sample was filtered and acid treated. At time zero and day 1, 0.1 ml of the samples was plated at a 1:10 dilution. All other samples were plated with 0.1 ml of undiluted sample.

Seeding experiments: DNA. *Legionella* DNA was extracted by the method of Woo et al. (21). One hundred eighty nanograms of *Legionella* DNA was added to a 2-liter plastic carboy containing sterile ocean water, a carboy containing 2 liters of nonsterile ocean water that tested negative for *Legionella* spp., and to 2 liters of filter-sterilized secondary sewage effluent. The DNA-seeded water was incubated at 16°C. After seeding with the extracted DNA, 100 ml was removed from the container at time zero and at 24, 48, 72, and 96 h postseeding. The water was processed by centrifugation for 15 min at $1,000 \times g$ in a Centriprep 100 concentrator (Amicon, Beverly, Mass.). The remaining water was further concentrated in a Centricon 100 (Amicon). Approximately 100 μl was recovered from the Centricon 100 and placed into 2 ml of Chelex provided in the *Legionella* detection kit. A 20-μl aliquot was removed, and PCR and detection were performed as described in the instructions in the *Legionella* kit.

Recovery efficiency. Ocean water was seeded with 10^2, 10^3, and 10^4 *L. pneumophila* (ATCC 33155) cells per ml. The water was tested by PCR, DFA, and culture methods (as described above) 1 h after seeding to determine the recovery efficiency of these three *Legionella* detection methods.

RESULTS

Recovery of *Legionella* spp. from sewage by PCR, DFA, and plate culture methods. Sewage samples, tested regularly for nearly 1 year by PCR, indicated that *Legionella* species were always present in all phases of the sewage treatment process, including the secondary effluent that was discharged through an ocean outfall (Table 1). The *Legionella* spp. in sewage samples were predominantly non-*L. pneumophila* species with two exceptions. PCR detection indicated that *Legionella* species were present at concentrations of $>10^3$ cells per ml throughout the treatment process with no noticeable reduction of population numbers.

Approximately 35% of the samples underwent additional testing by the DFA method. Analysis showed that the DFA method detected *L. pneumophila* with much greater frequency than the PCR method did. Similarly, *Legionella* species were shown to be present in greater numbers by the DFA method than by PCR. The DFA method, however, showed a slight reduction in the count of *Legionella* spp. after primary treatment and a 10-fold reduction after secondary treatment but agreed with the PCR method in that $>10^3$ cells per ml was observed after secondary treatment.

Twenty-five percent of the sewage samples underwent additional analysis by colony culture and monoclonal identification. This method did not distinguish *L. pneumophila* from other *Legionella* species. Only one of the sewage samples yielded *Legionella* colonies. Viable counts of *Legionella* spp. were well below counts obtained with the DFA and PCR methods but indicated that the number of viable *Legionella* spp. increased during the treatment process.

Recovery of *Legionella* spp. from ocean water by PCR,

TABLE 1. Recovery of *Legionella* spp. from sewage and ocean water

Method	Sample source	*L. pneumophila*		*Legionella* species	
		%	Avg. no. of cells/ml	%	Avg. no. of cells/ml
PCR	Sewage				
	Primary influent	0 (0/19)[a]	0	100 (19/19)[a]	$>10^3$
	Primary effluent	10 (2/21)	$<10^3$	100 (21/21)	$>10^3$
	Secondary effluent	5 (1/20)	$<10^3$	100 (20/20)	$>10^3$
	Ocean outfall				
	Outfall surface	20 (1/5)	$<10^3$	40 (2/5)	$<10^3$
	Outfall deep	50 (2/4)	$<10^3$	75 (3/4)	10^3
	Perimeter	0 (0/11)	0	0 (0/11)	0
	Surfzone				
	River mouth	0 (0/11)	0	45 (5/11)	$<10^3$
	Channel	0 (0/13)	0	46 (6/13)	$<10^3$
	Beach	7 (1/14)	$>10^3$	14 (2/14)	10^3
DFA	Sewage				
	Primary influent	100 (7/7)	5.1×10^2	100 (7/7)	3.5×10^4
	Primary effluent	100 (7/7)	7.1×10^2	100 (7/7)	1.7×10^4
	Secondary effluent	86 (6/7)	6.3×10^1	86 (6/7)	5.9×10^3
	Ocean outfall				
	Outfall surface	40 (2/5)	28	40 (2/5)	6
	Outfall deep	33 (1/3)	17	33 (1/4)	14
	Perimeter	Not tested			
	Surfzone				
	River mouth	66 (4/6)	8	66 (4/6)	4
	Channel	50 (3/6)	4	50 (3/6)	27
	Beach	50 (3/6)	2	16 (1/6)	18
Colony culture	Sewage				
	Primary influent			25 (1/4)	10
	Primary effluent			25 (1/4)	33
	Secondary effluent			25 (1/4)	500
	Ocean outfall				
	Outfall surface			0 (0/4)	0
	Outfall deep			0 (0/4)	0
	Perimeter			Not tested	
	Surfzone				
	River mouth			0 (0/4)	0
	Channel			0 (0/4)	0
	Beach			0 (0/4)	0

[a] Values in parentheses indicate number of positive samples/total number of samples.

DFA, and plate culture methods. Although 100% of treated sewage samples entering the ocean outfall contained *Legionella* spp. in concentrations of $>10^3$ cells per ml, only 75% of the outfall deep-water samples contained *Legionella* spp., and concentrations determined with the PCR dropped to 10^3 cells per ml (Table 1). Only 40% of outfall surface water samples were positive, and the count of *Legionella* spp. dropped to $<10^3$ cells per ml in outfall surface water. In two samples, *L. pneumophila* was detected in the deep ocean outfall water and once in the outfall surface water. These correlated with the two times that sewage samples were culture positive for *L. pneumophila*. Ocean water from the area between the outfall and the surfzone (labeled outfall perimeter) was always negative for *Legionella* spp. Open-ocean salinity was 33 to 35‰.

PCR results indicated that surfzone beach water (salinity 33‰) was positive for *Legionella* spp. in 2 of 14 (14%) samples and contained *L. pneumophila* in only one sample (7%). The beach areas that were positive were close to a river and flood control channel that drained into the ocean. The river mouth and flood control channel were positive for *Legionella* spp. in nearly 50% of the samples tested. The salinities of the river mouth and flood control channel were 2 and 18‰, respectively.

DFA results indicated that the ocean outfall water was positive for *Legionella* spp., but lower overall numbers were detected by DFA than by PCR. Although the *Legionella* count was low (less than 30 cells per ml), surfzone, river mouth, and flood control channel waters were positive in over 50% of the samples. Both *L. pneumophila* and *Legionella* spp. were detected. Colony culture methods did not detect any *Legionella* spp. in any ocean water samples.

Recovery efficiency. The recovery efficiencies of PCR, DFA, and culture methods are shown in Table 2. All three methods recovered the seeded *Legionella* spp. from the sample with different degrees of success. Although semi-

TABLE 2. Efficiency of recovery of *Legionella* spp. by PCR, DFA, and plate culture methods

Method	Recovery from seeded amt of:		
	1.5×10^2 cells/ml	1.7×10^3 cells/ml	3×10^4 cells/ml
PCR	$<10^3$ cells/ml	10^3 cells/ml	$>10^3$ cells/ml
DFA	3.5×10^1 cells/ml	1.1×10^2 cells/ml	1.1×10^3 cells/ml
Plate culture	90 CFU	870 CFU	ND[a]

[a] ND, not done.

quantitative, the PCR method yielded the best recovery and was able to detect *Legionella* spp. in the approximate seeded log concentrations. The DFA method detected approximately 10-fold fewer cells than the amount seeded in all three dilutions. The plate culture method detected approximately half of the seeded concentrations of 10^2 and 10^3 cells per ml.

Seed experiments: whole-cell survival. The PCR method detected whole *Legionella* cells inoculated into both sterile and nonsterile ocean water at 10^3 cells per ml for 6 weeks postseeding. The original seeding population numbers appeared to be maintained and did not decline after 6 weeks in the ocean water (Fig. 1). At the end of the experiment, DFA counts were 99 cells per ml, a result consistent with the recovery efficiency experiment.

In a second experiment, it was shown that seeded *L. pneumophila* (3×10^3 cells per ml) was easily recovered by PCR 7 days after seeding into unfiltered secondary sewage effluent and filter-sterilized secondary effluent and ocean water. Detection by PCR showed that all samples contained $>10^3$ cells per ml (Table 3).

Since sewage was always positive for *Legionella* spp., it was not surprising that the secondary unfiltered effluent was shown, by PCR, to contain $>10^3$ *L. pneumophila* cells per ml prior to seeding. Colony culture, however, did not show growth from the preseeded sample. Additionally, only 100 colonies were recovered on medium at time zero, and no colony growth was observed from day 1. The day-1 sample, however, was plated at a 1:10 dilution in anticipation of high colony numbers, and thus *Legionella* spp. could have been missed. The day-4 sample, plated without dilution, showed 30 colonies, and samples on days 5, 6, and 7 showed no growth.

In filtered secondary effluent, no growth was observed from the 1:10 diluted time zero sample. Samples from day 1 showed 340 colonies, the day-4 sample contained 70 colonies, and samples on days 5, 6, and 7 had no growth. Three hundred colonies were detected from filtered ocean water at time zero, 90 colonies were counted on day 1, and then no growth was observed on days 4, 5, 6, and 7.

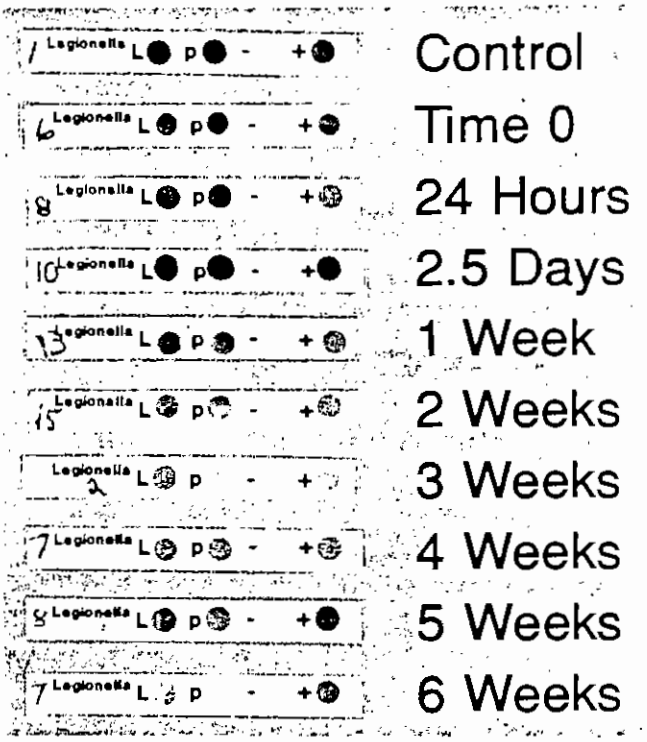

FIG. 1. Detection of whole-cell *L. pneumophila* (ATCC 33155) seeded at 10^3 cells per ml into 16°C ocean water. Water was sampled at time zero and at 24 h, 2.5 days, and 1, 2, 3, 4, 5, and 6 weeks postseeding. *L. pneumophila* was detected by PCR with the EnviroAmp test kit.

Seed experiments: *Legionella* DNA. *Legionella* DNA, as detected by PCR, degraded after only 4 days in both sterile and nonsterile ocean water but was still recovered from sewage (Fig. 2). A 2% agarose gel was run to confirm that the DNA was disintegrating. The agarose gel clearly confirmed the PCR results since it is evident that the bands amplified from the samples rapidly lose intensity after *L. pneumophila* is seeded into ocean water (Fig. 3). The 135-bp band indicating amplification of the internal positive control is seen in

TABLE 3. Recovery of seeded *L. pneumophila* from unfiltered and filter-sterilized secondary effluent and filter-sterilized ocean water

Seeding time	Unfiltered secondary effluent			Filtered secondary effluent			Filtered ocean water		
	PCR (cells/ml)		Culture (CFU/ml)	PCR (cells/ml)		Culture (CFU/ml)	PCR (cells/ml)		Culture (CFU/ml)
	L[a]	P[b]		L	P		L	P	
Preseeding	>+[c]	>+	0						
Time zero	>+	>+	100	>+	>+	0	>+	>+	300
Day 1	>+	>+	0	>+	>+	340	>+	>+	90
Day 4	>+	>+	30	>+	>+	70	>+	>+	0
Day 5	>+	>+	0	>+	>+	0	>+	>+	0
Day 6	>+	>+	0	>+	>+	0	>+	>+	0
Day 7	>+	>+	0	>+	>+	0	>+	>+	0

[a] L, *Legionella* species.
[b] P, *L. pneumophila*.
[c] >+, $>10^3$.

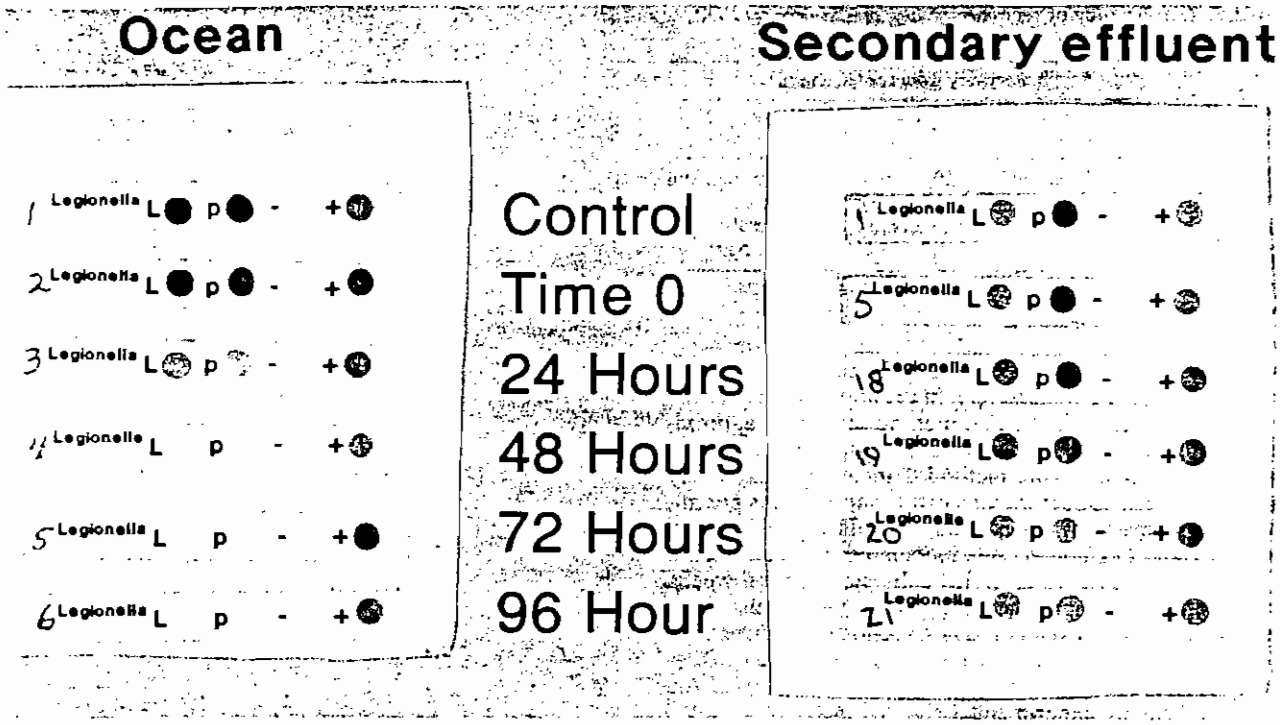

FIG. 2. Detection of extracted *L. pneumophila* DNA (180 ng/ml) seeded into 16°C ocean water and secondary effluent. Water was sampled at time zero and days 1, 2, 3, and 4. *L. pneumophila* DNA was detected with the EnviroAmp test kit.

all samples, except the negative control, indicating that the PCR performed as expected. The *mip* gene (168 bp), unique to *L. pneumophila*, is clearly seen in lane 1 (positive control) and at time zero and at 24 h. The 5S rRNA product (108 bp) which identifies *Legionella* species, can be seen in the positive control and at time zero and at 24 and 48 h.

DISCUSSION

Legionella spp. are ubiquitous in freshwater aquatic environments and have been detected in most water sources tested, including lakes, streams, and ocean waters receiving freshwater runoff (8, 11). This study determined that *Legionella* species were present throughout the treatment train of a sewage treatment plant and that concentrations were not appreciably reduced by either primary or secondary treatment processes. This finding could be related to the fact that *Legionella* species are known to reside inside protozoans (7a). This affords protection for the organism and may explain why the numbers are still high at the end of the treatment process. Additionally, they could be part of the biofilm in the pipes, either living within protozoans and/or algae or free living.

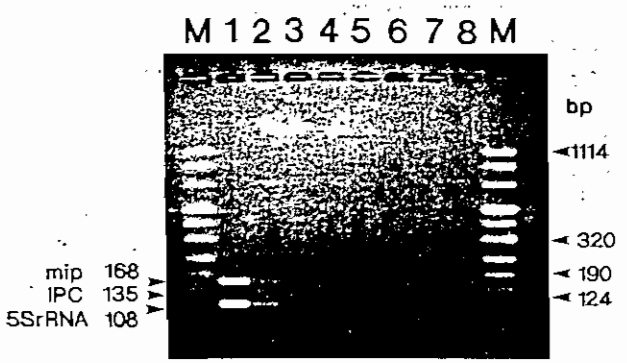

FIG. 3. Agarose gel showing samples from extracted *L. pneumophila* DNA seeded in 16°C ocean water. *L. pneumophila* DNA was detected with the EnviroAmp test kit. Lanes: 1, control *L. pneumophila* DNA; 2 to 7, samples from time zero and from 24, 48, 72, 96, and 120 h, respectively; 8, a negative control (electrophoresis running buffer). Molecular size markers (M) are shown on both the far left and right wells.

The PCR method recovered *Legionella* spp. in 100% of the sewage samples tested. However, *L. pneumophila*, the organism responsible for the majority (80%) of legionellosis outbreaks, was recovered in only 10% of primary effluent samples and 5% of secondary effluent samples. The DFA method recovered both *Legionella* spp. and *L. pneumophila* in almost 100% of the sewage samples tested; however, it is unclear whether the DFA method actually detected *L. pneumophila* since the PCR and plate culture methods did not support the DFA method findings. The DFA polyclonal antibodies could have cross-reacted with other organisms in the sewage. These antibodies were developed for clinical samples and have not been widely tested for cross-reactivity with other environmental microorganisms. Additionally, damaged *Legionella* cells, devoid of nucleic acid and thus nonvirulent, would be detected by the DFA method but not by PCR.

Colony culture detected *Legionella* spp. in only 25% of the sewage samples, and counts were at least 20-fold lower than that detected by the PCR or DFA method. This may be attributed to competition from faster-growing organisms that were not eliminated by the acid pretreatment. In addition, Paszko-Kolva et al. (13) reported the inhibitory effect of

heterotrophic bacteria on the cultivation of *Legionella* spp. They showed that many organisms, including *Pseudomonas* spp., secrete bactericidal products into surrounding media which dramatically inhibit *Legionella* growth. Exposure to these bactericidal products produced by similar organisms found in sewage or ocean water could explain why it was so difficult to culture *Legionella* spp. during this study. The bactericidal products could also have remained after filter sterilization and thus continued to inhibit colony formation. Further confirmation of the inability of the plate culture method to recover *Legionella* spp. was observed during seeding experiments of secondary effluent with whole-cell *L. pneumophila*. The PCR easily detected the seeded *L. pneumophila* in approximately the same concentrations as that seeded throughout the 7-day study, but plate culture detected relatively few organisms and was unable to detect any *Legionella* spp. after day 4.

Ocean outfall water was found to contain *Legionella* spp. in both surface and subsurface waters by both the PCR and DFA methods. The *Legionella* spp. detected in the outfall water did not appear to impact nearshore coastal bathing waters since ocean water between the outfall and the surf-zone waters was negative for *Legionella* spp. The major impact on nearshore coastal water was found to come from a river and flood control channel that emptied into coastal water. Both an adjacent river and channel contained *Legionella* spp. in nearly 50% of the samples tested. Adjacent beach water tested positive in several samples when the river and channel were positive for *Legionella* spp.

Only one other study has detected *Legionella* spp. in saline ocean environments. Ortiz-Roque and Hazen used the DFA method to detect *Legionella* spp. in tropical ocean water (11) surrounding Puerto Rico. This present study is the first report of *Legionella* spp. detected from temperate high-salinity ocean water. Although the PCR method used in this study did not distinguish between live and dead organisms, seeding experiments showed that whole-cell *L. pneumophila* organisms were recovered by PCR 6 weeks after seeding into ocean water, while *Legionella* DNA seeding experiments showed that the *Legionella* DNA, detected by PCR, rapidly disintegrated in ocean water in just 4 days. Results of gel electrophoresis showed that the 5S rRNA band was present for a day or two longer than the *mip* gene product was. This may be because the 5S rRNA target is amplified more efficiently than the *mip* gene target since the 5S rRNA is present in multiple copies while the *mip* gene is thought to be single copy. The recovery of the whole-cell seeded *L. pneumophila*, however, suggests that *Legionella* spp. may survive for extended periods in the high-salinity water of the open ocean.

In conclusion, the three methods used to detect *Legionella* (PCR, DFA, and plate culture) gave different results. The colony culture may be the least sensitive of the methods for use when working with environmental samples. This may be due to large numbers of other organisms that can outcompete *Legionella* spp. on the media from the sewage aud ocean samples, the inability of injured or nouculturable *Legionella* spp. to form colonies on the media, or inhibition owing to bactericidal products produced by other microorganisms. Moreover, in many cases, acid treatment of the water sample combined with plating on antibiotic media did not inhibit non-*Legionella* organisms in the sample. However, acid treatment may have had detrimental effects upon *Legionella* recovery. The DFA method detected *L. pneumophila* and other *Legionella* species more frequently than the PCR method, but these results could be questioued as explained previously. The PCR method may have been the most sensitive and specific detection method since the PCR detected nucleic acid specific to these organisms. Also, from a procedural viewpoint, the DFA method is labor intensive, since reading slides under the microscope is time consuming and tedious, and the results are subjective. The PCR required more sample processing time, but the test was easy to perform, easy to interpret, and yielded an unbiased result.

Finally, this study clearly showed that sewage treatment plants may provide an environmental reservoir for *Legionella* spp. Moreover, it was shown that *Legionella* spp. can survive in ocean waters for an extended period of time but that *Legionella* DNA quickly degrades in saline water. These two environmental sources of *Legionella* spp., sewage treatment plants and ocean water, require further study to determine whether a public health risk is possible.

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REFERENCES

1. **Bopp, C. A., J. W. Sumner, G. K. Morris, and J. G. Wells.** 1981. Isolation of *Legionella* spp. from environmental water samples by low-pH treatment and use of a selective medium. J. Clin. Microbiol. **13**:714–719.
2. **Chumakov, K. M., I. S. Tartakovsky, O. A. Ogarkova, and S. V. Prozorovsky.** 1986. Use of the 5S ribosomal RNA nucleotide sequence analysis for the study of the phylogeny of the genus *Legionella*. Mol. Genet. Mikrobiol. Virusol. 7:38–40.
3. **Cianciotto, N. P., J. M. Bangsborg, B. I. Eisenstein, and N. C. Engleberg.** 1990. Identification of *mip*-like genes in the genus *Legionella*. Infect. Immun. **58**:2912–2918.
4. **Dondero, T. J., R. J. Rendtoriff, and G. F. Malluson et al.** 1980. An outbreak of Legionnaires' disease associated with contaminated air conditioning cooling tower. N. Engl. J. Med. **302**:365–370.
5. **Engleberg, N. C., C. Carter, D. R. Weber, N. P. Cianciotto, and B. I. Eisenstein.** 1989. DNA sequenced of *mip*, a *Legionella pneumophila* gene associated with macrophage infectivity. Infect. Immun. **57**:1263–1270.
6. **Fang, G. J., D. Vlyu, and R. M. Vickers.** 1989. Disease due to the legionellaceae (other than *L. pneumophila*). Medicine **68**: 116–132.
7. **Fenstersheib, M. D., M. Miller, C. Diggins, S. Liska, L. Detweiler, S. B. Werner, D. Lindquist, W. L. Thacker, and R. F. Benson.** 1990. Outbreak of Pontiac fever due to *Legionella anisa*. Lancet **ii**:35–37.

7a. **Fields, B. S.** 1993. *Legionella* and protozoa: interaction of a pathogen and its natural host, p. 129–136. *In* J. M. Barbaree, R. F. Breiman, and A. P. Dufour (ed.), *Legionella*: current status and emerging perspectives. American Society for Microbiology, Washington, D.C.

8. **Fliermans, C. B., W. B. Cherry, L. H. Orrison, S. J. Smith, D. L. Tison, and D. H. Pope.** 1981. Ecological distribution of *Legionella pneumophila*. Appl. Environ. Microbiol. **41**:9–16.
9. **MacDonell, M. T., and R. R. Colwell.** 1987. The nucleotide sequence of the 5S rRNA from *Legionella pneumophila*. Nucleic Acids Res. **15**:1335.
10. **Mangione, E. J., R. S. Remis, K. A. Tait, G. W. Gorman, B. B.**

Wentworth, P. A. Baron, A. W. Hightower, J. M. Barbaree, and C. V. Broome. 1982. An outbreak of Pontiac fever related to whirlpool use, Michigan. JAMA **253**:535–539.

11. **Ortiz-Roque, C. M., and T. C. Hazen.** Abundance and distribution of *Legionellaceae* in Puerto Rican Waters. Appl. Environ. Microbiol. **53**:2231–2236.
12. **Palmer, C. J., Y.-L. Tsai, A. L. Lang, and L. R. Sangermano.** 1993. Evaluation of Colilert-Marine Water for detection of total coliforms and *Escherichia coli* in the marine environment. Appl. Environ. Microbiol. **59**:786–790.
13. **Paszko-Kolva, C., P. A. Hacker, M. A. Stewart, and R. L. Wolfe.** 1993. Inhibitory effect of heterotrophic bacteria on the cultivation of *Legionella dumoffii*, p. 203–205. *In* J. M. Barbaree, R. F. Breiman, and A. P. Dufour (ed.), Legionella: current status and emerging perspectives. American Society for Microbiology, Washington, D.C.
14. **Paszko-Kolva, C., M. Shahamat, and R. R. Colwell.** 1992. Long-term survival of *Legionella pneumophila* serogroup 1 under low-nutrient conditions and associated morphological changes. FEMS Microbiol. Ecol. **102**:45–55.
15. **Paszko-Kolva, C., M. Shahamat, H. Yamamoto, T. Sawyer, J. Vives-Rego, and R. R. Colwell.** 1991. Survival of *Legionella pneumophila* in the aquatic environment. Microb. Ecol. **22**:75–83.
16. **Paszko-Kolva, C., H. Yamamoto, M. Shahamat, T. K. Sawyer, G. Morris, and R. R. Colwell.** 1991. Isolation of amoebae and *Pseudomonas* and *Legionella* spp. from eyewash stations. Appl. Environ. Microbiol. **57**:163–167.
17. **Steinmetz, I., C. Rheinheimer, and D. Better-Suermann.** 1992. Rapid identification of legionellae by a colony blot assay based on a genus-specific monoclonal antibody. J. Clin. Microbiol. **30**:1016–1018.
18. **Tobin, J. O., J. Beare, and M. S. Dunnill et al.** 1980. Legionnaires' disease in a transplant unit: isolation of the causative agent from shower batbs. Lancet **ii**:307–310.
19. **Winn, W. C., Jr.** 1988. Legionnaires' disease: historical perspective. Clin. Microbiol. Rev. **1**:60–81.
20. **Wolters, J., and V. A. Erdmann.** 1988. Compilation of 5S rRNA and 5S rRNA gene sequences. Nucleic Acids Res. **16**(Suppl.): r1–r70.
21. **Woo, T. H. S., A. F. Cheng, and J. M. Ling.** 1992. An application of a simple method for the preparation of bacterial DNA. Biotechniques **13**:696–698.

APPENDIX IV

PUBLICATIONS: Water Environment Federation

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66TH ANNUAL CONFERENCE & EXPOSITION
ANAHEIM, CALIFORNIA U.S.A.
OCTOBER 3-7, 1993

DETECTION OF ENTEROVIRUSES AND HEPATITIS A VIRUS IN SEWAGE AND OCEAN WATER BY REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

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AC93-020-001

DETECTION OF ENTEROVIRUSES AND HEPATITIS A VIRUS IN SEWAGE AND OCEAN WATER BY REVERSE TRANSCRIPTASE - POLYMERASE CHAIN REACTION

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ABSTRACT

Molecular detection of enteroviruses and hepatitis A virus from sewage and ocean water was successfully achieved in this study. Sewage samples were concentrated by microconcentrators at 1,000 x *g*. Samples collected from esturine and near-shore surfzone ocean water in Southern California were concentrated by vortex flow filtration (VFF) and microconcentration. Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect enteroviruses or HAV in all concentrated samples. A nonradioactive internal probe was used to confirm the amplified products. Results of seeding experiments indicated that at 4°C, HAV was more resistant than poliovirus in seawater, and both HAV and poliovirus survived longer at 4°C than at 25°C. RT-PCR was found to be at least 500-fold more sensitive than cell culture. The RT-PCR results indicated high recovery efficiency using VFF and microconcentration to recover polio viruses from seeded sterile ocean water. Results for detection of enteroviruses or HAV were obtained within 5 hours by RT-PCR as compared to 5 days to 4 weeks required using cell culture.

Key words: reverse transcriptase-polymerase chain reaction, vortex flow filtration, microconcentration, enteroviruses, HAV, sewage, ocean water.

INTRODUCTION

Water is known to be a major vehicle for transmitting diseases. Bacterial agents found in sewage-polluted recreational and drinking waters have accounted for numerous water-borne outbreaks of illness. Although under optimum conditions, drinking water and sewage treatment facilities efficiently remove most viruses, some still escape the treatment processes (Guy et al., 1977). Enteric viruses originated from point or non-point sources have been found in natural waters (Leong, 1983). Transmission of viral diseases via surface waters contaminated with sewage discharge are also well documented (Alexander et al., 1992; Balarajan et al., 1991; Gerba et al., 1978). Because the occurrence of enteric viruses is not always related to the presence of indicator organisms (Berg and Metcalf, 1978), current microbial standards using coliform bacteria as indicators of fecal pollution may not be indicative of the presence of water-borne pathogens. It is recommended that direct monitoring for the presence of pathogenic bacteria and viruses replace the antiquated coliform indicator system in order to provide better public health protection.

Viruses have been recovered from water and wastewater by adsorption/elution on microporous filters

followed by cell culture methods (American Public Health Association, 1992). Because viruses propagate slowly on specific cell lines, it can take from 5 days to 4 weeks to detect the presence of viruses by plaque assay or observation of cytopathic effects (CPE). The sensitivity of detection using cell culture is low (Kopecka et al., 1993) and the method is labor intensive and tedious. Moreover, some enteric viruses such as Norwalk virus, rotaviruses and hepatitis A virus (HAV) are difficult or impossible to cultivate by cell culture. Recently, reverse transcriptase - polymerase chain reaction (RT-PCR) has been successfully used to detect human RNA viruses such as enteroviruses, polioviruses, rotavirus, Norwalk virus and HAV from stool, oyster and environmental samples (Atmar et al., 1993; De Leon et al., 1990, 1992; Gouvea et al., 1990; Jiang et al., 1992; Kopecka et al., 1993; Rotbart, 1990; Wilde et al., 1992). The RT-PCR is a rapid and highly sensitive detection method which can circumvent the disadvantages of traditional cell culture method.

The reported virus concentration procedures use beef extract (APHA, 1992) and polyethylene glycol (PEG) (APHA,1992; Lewis and Metcalf, 1988; Zhou et al., 1991) to elute viruses from cartridge-filters or to precipitate viruses from homogenized samples. One major problem related to the previous concentration methods is that the beef extract and PEG are not eliminated from the concentrate, RT-PCR is inhibited and further clean-up steps are required to obtain positive results (De Leon et al., 1990; Schwab et al., 1991). Sewage and surface water samples concentrated by flocculation also inhibit the RT-PCR (Kopecka et al., 1993). In addition, humic substances in environmental extracts were also found to inhibit the PCR procedure (Steffan et al., 1988; Tsai and Olson, 1992a). Although methods have been developed to overcome the problem of humic interference (Tsai and Olson, 1992b; Tsai et al., 1993), the beef extract and PEG still posed problems when trying to perform RT-PCR to detect viruses.

In the present study, we successfully applied a simple concentration method and RT-PCR to detect enteroviruses and HAV from concentrated sewage and ocean samples without the need for the beef extract or PEG elution steps. Vortex flow filtration and microconcentration were used to concentrate viruses from environmental samples. The inhibitory effects caused by humic substances, beef extract and PEG on RT-PCR were successfully eliminated.

MATERIALS AND METHODS

Sampling of sewage and ocean water. Sewage samples, including primary influent, primary effluent and secondary effluent were collected from a major metropolitan sewage treatment plant. Ocean samples were collected from near-shore ocean waters of Southern California. Sample sites included surfzone (HP), the brackish zone of a river (SAR) and of a flood control channel (D2). All samples were processed within 4 h after collection.

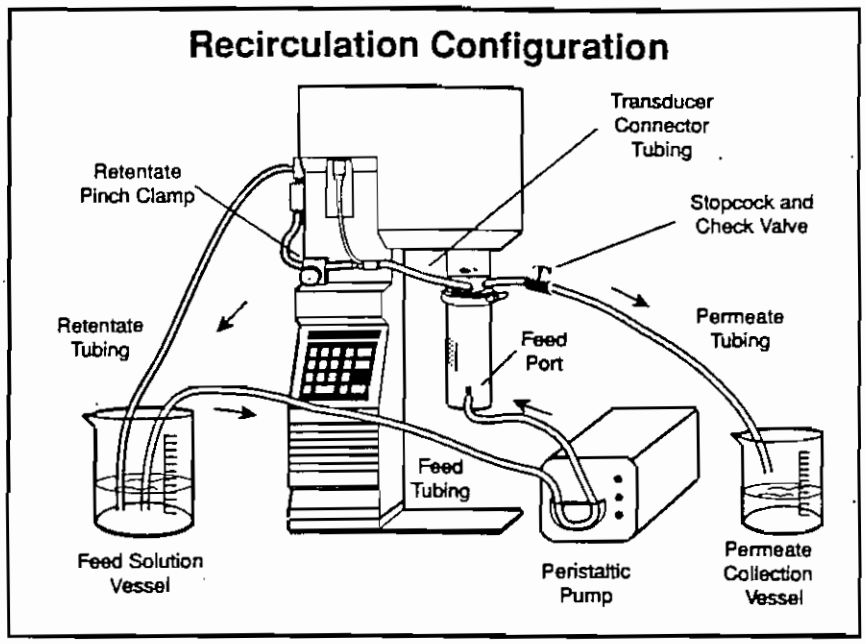
Preparation of concentrated samples. One hundred milliliters of sewage was concentrated to 4 ml using a Centriprep-100 (Amicon, Inc., Beverly, Mass.) concentrator at 1,000 x g. An equal volume (4 ml) of a chloroform-isoamyl alcohol (24:1) mixture (Amresco, Solon, OH) was added to the concentrated sample. After brief vortexing, the homogenate was centrifuged at 800 x g for 10 min to reach bi-phase. The upper aqueous phase was further concentrated to 100 μl using a Centricon-100 (Amicon) microconcentrator at 1,000 x g. All sewage samples were processed at 4°C. Concentrated samples were stored at -20°C before analysis.

Fifteen liters of each sample of ocean water were concentrated to 100 ml with the use of a vortex flow filtration (VFF) device (Figure 1) (Membrex Inc., Garfield, N.J.) at 5-6 psi with a 100 KDa filter and a rotor speed of 1,000-1,500 rpm . The preconcentrated samples were further condensed to 100 μl using Centriprep-100 and Centricon-100 as described for the sewage samples.

Plaque assays. Hepatitis A virus strain HM175 was provided by Dr. Mark Sobsey (University of North Carolina, Chapel Hill). BS-C-1 Cells (an African green monkey kidney-derived cell line) and/or FRhK-4 cells (fetal rhesus kidney derived cell line) were used for propagation HAV stocks. FRhK-4 cells were used for the enumeration of HAV by plaque forming technique (Cromeans et al., 1987).

Water samples for poliovirus were kept frozen until the monolayer of Buffalo Green Monkey Kidney (BGMK) cells were confluent. Assays were performed on BGMK cells in 6 well plates after growth for 5 days at 37° C in 5% CO_2.

Figure 1. Device diagram of a vortex flow filtration apparatus (Adapted from Membrex, Inc., Garfield, N.J.)



Reverse transcriptase - polymerase chain reaction (RT-PCR). Enteroviruses and HAV from the concentrated samples were detected using the RT-PCR method as described by De Leon et al. (1990). HAV strain HM 175 and poliovirus type I LSc (VR1001, American Type Culture Collection, Rockville, Md.) were used as positive controls. The amplification was performed with a GeneAmp RNA PCR kit (Perkin Elmer-Roche, Norwalk, Conn.) with slight modifications. Briefly, 2 μl of concentrated sample was added to 16 μl of RT reaction mixture containing 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 1 mM each of dGTP/dATP/dTTP/dCTP, and 2.5 μM random hexamers. Each sample mixture was heated at 99°C for 5 min and cooled at 4°C before the addition of 1 μl reverse transcriptase (50 U/μl) and 1 μl RNase inhibitor (20 U/μl). The reverse transcription was carried out in a thermocycler (GeneAmp PCR System 9600, Perkin Elmer Cetus, Norwalk, Conn.) using the following steps: 25°C for 10 min; 42°C for 30 min; 99°C for 5 min; and 4°C for 10 min. The finished RT mixture (20 μl) was added to PCR reaction mixture (80 μl) containing oligonucleotide primers (0.3 μM) and AmpliTaq polymerase (Perkin-Elmer) to obtain the optimal concentrations for PCR as suggested by the manufacturer. The pan-specific primers for enteroviruses (EV-R, 5'-ACCGGATGGCCAATCCAA-3'; EV-L, 5'-CCTCCGGCCCCTGAATG-3') and primers for HAV capsid (HAVC-R, 5'-CTCCAGAATCATCTCCAAC-3'; HAVC-L, 5'-CAGCACATCAGAAAGGTGAG-3') were used during PCR (Schwab et al., 1991) to amplify a 196 base-pair and a 192 base-pair cDNA fragments, respectively. The internal oligonucleotide probes for enteroviruses and HAV were EV-IN (5'-ACTACTTTGGGTGTCCGTGTTTC-3') and HAVC-IN (5'-TGCTCCTCTTTATCATGCTATG-3'), respectively (De Leon et al., 1990). Oligonucleotides were synthesized by a DNA/RNA synthesizer (Model 392, Applied Biosystems, Inc., Foster City, Calif.). The 3-temperature protocol (95°C, 1 min; 55°C, 1 min; 72°C, 1 min; 40 cycles) as described by De Leon et al. (1990) was adapted to amplify the target cDNA sequences of enteroviruses or HAV.

DNA hybridization. The PCR products were identified by electrophoresis on a 2% SeaKem GTG agarose (FMC BioProducts, Rockland, Maine) gel stained with ethidium bromide (0.5 μg/ml). The Genius 5 nonradioactive DNA labeling kit (Boehringer Mannheim, Indianapolis, Ind.) was used to label EV-IN and HAVC-IN internal probes using the protocols described by the manufacturer. The dot-blot, Southern analysis and DNA hybridization were performed as previously described (Tsai et al., 1993). The low-salt washes (0.1 X SSC in 0.1% sodium dodecyl sulfate) were performed at 50°C for EV-IN and at 47°C for HAVC-IN.

For the microconcentration recovery and RT-PCR sensitivity test, 100 μl of poliovirus ranging from 10^3 to 10^{-4} pfu/100 μl were seeded into 2 ml of sterile distilled water and reconcentrated with Centricon-100 to a final volume of 100 μl. For the VFF and microconcentration recovery test, 5 x 10^6 pfu of poliovirus were seeded into 15 l of filter (0.22 μm pore size) sterilized ocean water and the concentrates after VFF and microconcentration were analyzed using RT-PCR and cell culture to determine the recovery efficiency and viability of poliovirus. In the seeding experiment, 600 μl of serially diluted HAV or polioviruses (10^1-10^3 pfu/100 μl) were inoculated into 12 ml of nonsterile HAV/enteroviruses negative ocean water and incubated at two different temperatures (4°C and 25°C) in the dark for 28 days. Two-ml aliquots from each temperature were collected at 0, 1, 7, 14, 21, and 28 days, concentrated into 100 μl as described previously, and amplified using RT-PCR.

RESULTS AND DISCUSSION

In the present study we have combined VFF and microconcentration to concentrate seawater 10^5-fold for RT-PCR detection of viruses. The total time for concentrating 15 l of seawater down to 100 μl was about 3 hours. No additional purification steps on the concentrates were required before the RT-PCR analysis. The technician handling time involved in the whole process was only 15 min.

RT-PCR amplified target DNA fragments of pan-specific enteroviruses (196 bp) from concentrated control poliovirus virions and some of the sewage and ocean samples were clearly visible in a ethidium bromide stained agarose gel. Southern hybridization analysis using EV-IN as an internal probe revealed that sensitivity of detection for polioviruses was 10^{-1} plaque forming units (pfu) per 100 μl. Because only 2 μl of concentrated retentate (100 μl) was used as a template for RT-PCR, the results suggest that PCR is capable of detecting 0.002 pfu per reaction, which implies the detection of the non-culturable polio viral particles. Thus, it is apparent that the RT-PCR is much more sensitive (500-fold) than cell culture for the detection of enteroviruses. The detection level was at the same order of magnitude (10^{-1} pfu/100 μl) when a serially diluted stock poliovirus was tested as RT-PCR control. This indicated that polioviruses were fully recovered in the retentate after microconcentration. In a separate recovery experiment, the VFF in combination with microconcentration recovered the same order of magnitude of polioviruses as was seeded into 15 l of sterile ocean water based on the observation of the RT-PCR results. However, the preliminary cell culture results indicate the total pfu counts in the VFF concentrate were less than but remained at the same log of the initial seeded counts. The discrepancy could be due to the inactivation of the viral particles by the accumulated inhibitory compounds, by the operating temperature or by the mechanical disturbance of VFF during the concentration process, but those inactivated virions were still detected by RT-PCR. Additional studies are underway to better define recovery efficiency. Due to rapid enzymatic amplification in the RT-PCR, the presence of viral RNA was detected within 5 hours, whereas 48 hours to 10 days were required to determine the presence of polioviruses by cell culture method.

Table 1 shows the occurrence of enteroviruses and hepatitis A virus in sewage and ocean water by using the rapid RT-PCR detection method. Because this is an ongoing study, the data shown in Table 1 represents the results up to the present time. Positive signals for enteroviruses were observed in 80% (4/5) of influent, 60% (3/5) of primary effluent and 40% (2/5) of secondary effluent. The denominator shown in the parenthesis indicates the numbers of different sampling dates from the same sites. The results suggest that the occurrence of enteroviruses in treated sewage was reduced during the treatment process. However, 25% (1/4) of raw and treated sewage samples were positive for HAV. In coastal ocean water, 17% (1/6) and 25% (1/4) of the samples were enteroviruses positive and HAV positive, respectively, in the surfzone samples (HP). The

positive enteroviruses and HAV were also found in brackish water of a river (SAR) and of a flood control channel (D2) (Table 1). The SAR and D2 sites are only 2 kilometers away from the HP site. The one occurrence of enteroviruses and HAV in surfzone water could be due to migration from the closeby D2 and SAR sites which collect water runoff from storm drainage or the nearby residential areas. No inhibitory effects on RT-PCR were observed when undiluted concentrates were used for testing. However, the enteroviruses and HAV plaque assay on selected PCR-positive concentrated sewage and ocean samples resulted in less than 1 pfu/100 μl (i.e., negative) in 3-4 weeks. This could be because the PCR-positive viral particles were not culturable by tissue culture methods used in this study. Further, either 100 ml of sewage or 15-liter of sea water might not be an appropriate volume for virus plaque assay because of the low sensitivity of detection using cell culture methods. It was, however, an adequate volume for RT-PCR detection of enteroviruses and HAV.

Table 1. Detection of enteroviruses and hepatitis A virus from sewage and ocean water using RT-PCR.

Samples	RT-PCR					
	EV[a]			HAV[b]		
	Total	Positive	% positive	Total	Positive	% positive
			Microconcentration (100 mL)			
1° Inf.[c]	5	4	80	4	1	25
1° Eff.[d]	5	3	60	4	1	25
2° Eff.[e]	5	2	40	4	1	25
			VFF[k] and microconcentration (15 L)			
HP[f]	6	1	17	4	1	25
SAR[g]	7	1	14	7	2	28
D2[h]	10	2	20	10	1	10
OS[i]	8	1	12	8	1	12
OD[j]	8	5	62	8	2	25

[a] EV, enteroviruses.
[b] HAV, hepatitis A virus.
[c] 1° Inf.; primary influent.
[d] 1° Eff., primary effluent.
[e] 2° Eff., secondary effluent.
[f] HP, surfzone water.
[g] SAR, a river brackish water.
[h] D2, brackish water from a flood control channel.
[i] OS, surface ocean water above a sewage discharge pipe.
[j] OD, deep ocean water (30 m depth) around sewage discharge pipe.
[k] VFF, vortex flow filtration.

The results of HAV seeding experiments in nonsterile ocean water show that after a 7-day incubation at 25°C, HAV viral RNA was not detected using RT-PCR and slot blot hybridization (Table 2). However, HAV

was detected at 4°C throughout the 28-day incubation period. Degradation of HAV occurred rapidly at 25°C, but it survived well at 4°C for the entire 28-day period. These experiments indicate temperature affects the survival rate of HAV in the marine environment. The higher degradation rate found at 25°C could be due to the virus-inactivating microorganisms (Fujioka et al., 1980).

In poliovirus seeding experiments, polioviruses were not detected after 7 days incubation at 25°C nor were they detected after 21 days at 4°C. These results suggest that ambient temperature promoted degradation of poliovirus viral RNA in seawater. Clearly, HAV is more resistant than poliovirus in seawater at 4°C. Based on the study of logarithm changes in virus titer, Sobsey et al. (1988) using the tissue culture technique, reported that HAV were more resistant than poliovirus in nonsterile environmental waters at 25°C. Our results using RT-PCR confirm this finding.

Table 2. RT-PCR and dot-blot analyses of poliovirus and hepatitis A virus survival at two different temperatures.

Incubation time (day)	RT-PCR			
	Poliovirus		Hepatitis A virus	
	25°C	4°C	25°C	4°C
0	+[a]	+	+	+
1	+	+	+	+
7	-[b]	+	-	+
14	-	+	-	+
21	-	-	-	+
28	-	-	-	+

[a] +, positive for RT-PCR and dot blot hybridization.
[b] -, negative for RT-PCR and dot blot hybridization.

In the present study, in order to prevent RNA degradation by RNase activity, the RNA virions remained intact until they were burst open by heat denaturation right before RT-PCR. Because RNA was easily degraded in the seawater (Pichard and Paul, 1991), amplification products resulting from the free viral RNA were unlikely.

CONCLUSIONS

Minimal equipment and labor were required to use the VFF and/or microconcentration method to concentrate viruses from ocean water or sewage. Because neither pH adjustment nor beef extracts was involved, the concentrated samples were immediately ready for RT-PCR. The sensitivity of detection using RT-PCR was much higher than the traditional cell culture method. The RT-PCR on VFF and/or microconcentration samples proved to be a powerful and sensitive method for virus detection in environmental waters.

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REFERENCES

Alexander, L.M., Heaven, A., Tennant, A., and Morris, R. (1992) Symptomatology of children in contact with sea water contaminated with sewage. J. Epid. Commun. Health, **46**, 340.

American Public Health Association. (1992) Standard methods for the examination of water and wastewater, 18th ed. American Public Health Association, Washington, D.C.

Atmar, R.T., Metcalf, T.G., Neill, F.H., and Estes, M.K. (1993) Detection of enteric viruses in oysters by using the polymerase chain reaction. Appl. Environ. Microbiol., **59**, 631.

Balarajan, R., Raleigh, V.S., Yuen, P., Wheeler, D., Machin, D., and Cartwright. (1991) Health risks associated with bathing in sea water. Brit. Med. J., **303**, 1444.

Berg, G., and Metcalf, T.G. (1978) Indicators of viruses in waters, p.267-296. In G. Berg (ed.), Indicators of viruses in water and food. Ann Arbor Science, Ann Arbor, Mich.

Cromeans, T., Sobsey, M.D., and Fields, H.A. (1987) Development of a plaque assay for a cytopathic, rapidly replicating isolate of hepatitis A virus. J. Med. Virol., **22**, 45.

De Leon, R., Matsui, S.M., Baric, R.S., Herrmann, J.E., Blacklow, N.R. Greenberg, H.B., and Sobsey, M.D.. (1992) Detection of Norwalk virus in stool specimens by reverse transcriptase-polymerase chain reaction and nonradioactive oligoprobes. J. Clin. Microbiol., **30**, 3151.

De Leon, R., Shieh, C., Baric, R.S., and Sobsey, M.D. (1990) Detection of enteroviruses and hepatitis A virus in environmental samples by gene probes and polymerase chain reaction, p.833-853. In Advances in water analysis and treatment. Proceedings of the Water Quality Technology Conference, San Diego, Calif. American Water Works Association, Denver.

Fujioka, R.S., Loh, P.C., and Lau, L.S. (1980) Survival of human enteroviruses in the Hawaiian ocean environment: Evidence for virus-inactivating microorganisms. Appl. Environ. Microbiol., **39**, 1105.

Gerba, C.P., Rose, J.B., and Singh, S.N. (1985) Waterborne gastroenteritis and viral hepatitis. CRC. Crit. Rev. Environ. Control, **15**, 213.

Gouvea, V., Glass, R.I., Woods, P., Taniguchi, K., Clark, H.F., Forrester, B., and Fang, Z.-Y. (1990) Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. J. Clin. Microbiol., **28**, 276.

Guy, M.D., McIver, J.D., and Lewis, M.J. (1977) The removal of virus by a pilot treatment plant. Water

Res., **11**, 421.

Jiang, X., Wang, J., Grahem, D.Y., and Estes, M.K. (1992) Detection of Norwalk virus in stool by polymerase chain reaction. J. Clinical Microbiol., **30**, 2529.

Kopecka, H., Dubrou, S., Prevot, J., Marechal, J., and Lopez-pila, J.M. (1993) Detection of naturally occurring enteroviruses in waters by reverse transcription, polymerase chain reaction, and hybridization. Appl. Environ. Microbiol., **59**, 1213.

Leong, L.Y.C. (1983) Removal and inactivation of viruses by treatment processes for potable water and wastewater: a review. Water Sci. Tech., **15**, 91.

Lewis, G.D. ,and Metcalf, T.G. (1988) Polyethylene glycol precipitation for recovery of pathogenic viruses, including hepatitis A virus and human rotavirus, from oyster, water, and sediment samples. Appl. Environ. Microbiol., **54**,1983.

Pichard, S.L., and Paul, J.H. (1991) Detection of gene expression in genetically engineered microorganisms and natural phytoplankton populations in the marine environment by mRNA analysis. Appl. Environ. Microbiol., **57**, 1721.

Rotbart, H.A. (1990) Enzymatic RNA amplification of the enteroviruses. J. Clin. Microbiol., **28**, 438.

Schwab, K.J., De Leon, R., Baric, R.S., and Sobsey, M.D. (1991) Detection of rotaviruses, enteroviruses and hepatitis A virus by reverse transcriptase-polymerase chain reaction, p.475-491. In Advances in water analysis and treatment. Proceedings of the Water Quality Technology Conference, Orlando, Florida. American Water Works Association, Denver.

Sobsey, M.D., Shields, P.A., Hauchman, F.S., Davis, A.L., Rullman, V.A., and Bosch, A. (1988) Survival and persistence of hepatitis A virus in environmental samples, p.121-124. In Viral Hepatitis and Liver Disease, Alan R. Liss, Inc., New York.

Steffan, R.J., Goksoyr, J., Bej, A.K., and Atlas, R.M. (1988) Recovery of DNA from soils and sediments. Appl. Environ. Microbiol., **54**, 2908.

Tsai, Y.-L., and Olson, B.H.. (1992a) Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. Appl. Environ. Microbiol., **58**, 754.

Tsai, Y.-L., and Olson, B.H. (1992b). Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. Appl. Environ. Microbiol., **58**, 2292.

Tsai, Y.-L., Palmer, C.J., and Sangermano, L.R. (1993) Detection of Escherichia coli in sewage and sludge by polymerase chain reaction. Appl. Environ. Microbiol., **59**, 353.

Wilde, J., Van, R., Pickering, L., Eiden, J., and Yolken, R. (1992). Detection of rotaviruses in the day care environment by reverse transcriptase polymerase chain reaction. J. Inf. Diseases, **166**, 507.

Zhou, Y.-J., Estes, M.K., Jiang, X., and Metcalf, T.G. (1991). Concentration and detection of hepatitis A virus and rotavirus from shellfish by hybridization tests. Appl. Environ. Microbiol., **57**, 2963.