Development and application of molecular techniques for the detection of human enteric viruses in environmental samples

Patrick Michael Regan

University of New Hampshire, Durham

Follow this and additional works at: https://scholars.unh.edu/dissertation

Recommended Citation


This Dissertation is brought to you for free and open access by the Student Scholarship at University of New Hampshire Scholars' Repository. It has been accepted for inclusion in Doctoral Dissertations by an authorized administrator of University of New Hampshire Scholars' Repository. For more information, please contact Scholarly.Communication@unh.edu.
INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6” x 9” black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
DEVELOPMENT AND APPLICATION OF MOLECULAR TECHNIQUES FOR THE DETECTION OF HUMAN ENTERIC VIRUSES IN ENVIRONMENTAL SAMPLES

BY

PATRICK M. REGAN

B. S. University of New Hampshire, 1982

DISSERTATION

Submitted to the University of New Hampshire in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Microbiology

May, 1997
This dissertation has been examined and approved.

Aaron B. Margolin  
Dissertation Director, Dr. Aaron B. Margolin  
Associate Professor of Microbiology

Dr. John Collins, Associate Professor of  
Biochemistry

Frank Rodgers  
Dr. Frank Rodgers, Professor of Microbiology

Thomas Pistole  
Dr. Thomas Pistole, Professor of Microbiology

Robert M. Zsigay  
Dr. Robert M. Zsigay, Professor of Microbiology

May 7, 1997  
Date
DEDICATION

To my wife Cathy for all her support, help and encouragement and to my children Michael and Kevin for their patience and understanding.
ACKNOWLEDGEMENTS

I would like to thank Dr. Aaron Margolin for his never ending support and encouragement throughout my graduate career. I would like to thank the members of my dissertation committee Dr. John Collins, Dr. Frank Rodgers, Dr. Thomas Pistole, and Dr. Robert Zsigray for always making time available to help me meet my goal. Additionally, I thank Dr. William Watkins and Jack Gaines of the U. S. Public Health Service for their assistance in the Narragansett Bay study.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xi</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xiii</td>
</tr>
<tr>
<td>I. CHAPTER ONE</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Viral Structure</td>
<td>2</td>
</tr>
<tr>
<td>Human Enteric Viruses</td>
<td>4</td>
</tr>
<tr>
<td>Viruses in the Environment</td>
<td>4</td>
</tr>
<tr>
<td>Wastewater</td>
<td>4</td>
</tr>
<tr>
<td>Seafood-borne Outbreaks</td>
<td>7</td>
</tr>
<tr>
<td>Waterborne Outbreaks</td>
<td>10</td>
</tr>
<tr>
<td>Bacterial Indicators</td>
<td>12</td>
</tr>
<tr>
<td>Viral Diagnostics</td>
<td>13</td>
</tr>
<tr>
<td>Molecular Techniques</td>
<td>14</td>
</tr>
<tr>
<td>Nucleic Acid Hybridization</td>
<td>16</td>
</tr>
<tr>
<td>Solid phase hybridization</td>
<td>17</td>
</tr>
<tr>
<td>Solution phase hybridization</td>
<td>17</td>
</tr>
<tr>
<td><em>In situ</em> hybridization</td>
<td>17</td>
</tr>
<tr>
<td>Nucleic Acid Hybridization Components</td>
<td>18</td>
</tr>
<tr>
<td>Target</td>
<td>18</td>
</tr>
<tr>
<td>Probe</td>
<td>19</td>
</tr>
<tr>
<td>Reporter molecule</td>
<td>20</td>
</tr>
<tr>
<td>Hybridization conditions</td>
<td>21</td>
</tr>
<tr>
<td>Summary of Nucleic Acid Hybridization</td>
<td>22</td>
</tr>
<tr>
<td>Target Amplification Systems</td>
<td>23</td>
</tr>
<tr>
<td>Polymerase chain reaction</td>
<td>23</td>
</tr>
<tr>
<td>Other Molecular Detection Techniques</td>
<td>26</td>
</tr>
<tr>
<td>Ligase chain reaction (LCR)</td>
<td>26</td>
</tr>
<tr>
<td>Self-sustained sequence replication (3SR)</td>
<td>27</td>
</tr>
<tr>
<td>Strand displacement amplification (SDA)</td>
<td>28</td>
</tr>
<tr>
<td>Qβ replicase</td>
<td>28</td>
</tr>
<tr>
<td>Research Goal and Objectives</td>
<td>29</td>
</tr>
</tbody>
</table>
II. CHAPTER TWO
ABSTRACT ......................................................................................................... 39
INTRODUCTION .................................................................................................. 41
MATERIALS AND METHODS ............................................................................. 45
  Shellfish and Water Collection and Handling ............................................... 45
  Clam Microbiological Analyses ....................................................................... 46
    Total and fecal coliforms ............................................................................. 46
    Enterococci ................................................................................................... 47
    C. perfringens ............................................................................................... 47
    Male specific bacteriophage .......................................................................... 48
  Water Analysis .................................................................................................. 49
    Total and fecal coliforms ............................................................................. 49
  Poliovirus Elution from Clam Meats ............................................................... 50
  Phenol Chloroform Extraction of Viral Nucleic Acid ..................................... 52
  Dot Blot Procedure .......................................................................................... 53
  Poliovirus Hybridization Probe Preparation .................................................. 53
    Poliovirus cDNA clone ............................................................................... 53
    Plasmid mini-preparation ............................................................................ 54
    Large scale plasmid preparation .................................................................. 54
    Dialysis ........................................................................................................ 56
    Ethanol precipitation .................................................................................... 56
    Restriction digest .......................................................................................... 57
    Probe labeling ............................................................................................... 58
    Probe purification .......................................................................................... 58
    Probe specific activity determination ........................................................... 59
    Prehybridization ............................................................................................. 59
    Hybridization ............................................................................................... 60
    Washing procedure ....................................................................................... 60
    Autoradiography ............................................................................................ 60
  Cell Culture Analysis ...................................................................................... 61
RESULTS ............................................................................................................. 63
DISCUSSION ....................................................................................................... 70
REFERENCES ...................................................................................................... 78

III. CHAPTER THREE
ABSTRACT ....................................................................................................... 82
INTRODUCTION .................................................................................................. 84
MATERIALS AND METHODS .......................................................................... 95
  Part I: Development of a T7 Amplification Sequence .................................... 95
LIST OF TABLES

I. CHAPTER ONE

Table 1. Shellfish-borne disease agents occurring in and transmitted by sewage and/or wastewater (1898-1990) .......... 9

Table 2. Outbreaks associated with water intended for drinking, by etiological agent and type of water system in the United States, 1991-1992 .................................................. 11

II. CHAPTER TWO

Table 1. Microbial levels in water and clams from the approved area .......................................................... 66

Table 2. Microbial levels in water and clams from the conditional approved area ................................................. 67

Table 3. Microbial levels in water and clams from the prohibited area ............................................................. 68

Table 4. Ratio of the number of positive dot blot wells over the total number of wells to which sample was applied. .......... 69

III. CHAPTER THREE

Table 1. First generation T7 amplification sequence synthetic oligonucleotide components ................................ 97

Table 2. Second generation T7 amplification sequence synthetic oligonucleotide components .......................... 104

Table 3. Third generation T7 amplification sequence synthetic oligonucleotide components ............................. 108

IV. CHAPTER FOUR

Table 1. Seeded environmental sample results and controls .......... 185

x

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
# LIST OF FIGURES

## I. CHAPTER ONE

<table>
<thead>
<tr>
<th>Figure 1.</th>
<th>Exponential amplification potential of PCR under ideal conditions</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24</td>
</tr>
</tbody>
</table>

## II. CHAPTER TWO

<table>
<thead>
<tr>
<th>Figure 1.</th>
<th>Clam preparation for cell culture and nucleic acid hybridization</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>51</td>
</tr>
</tbody>
</table>

## III. CHAPTER THREE

<table>
<thead>
<tr>
<th>Figure 1.</th>
<th>Schematic representation of the major components contained in the T7 amplification sequence</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>89</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Figure 2.</th>
<th>Schematic representation of the T7 reaction showing the biotinylated capture sequence and the T7 amplification sequence</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>90</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Figure 3.</th>
<th>Diagram of the capture sequence</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>92</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Figure 4.</th>
<th>Flow chart depicting how the completed T7 amplification sequence was constructed</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>98</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Figure 5.</th>
<th>Synthesized oligonucleotide components used in the T7 amplification sequence</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>99</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Figure 6.</th>
<th>First generation T7 amplification sequence components</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>129</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Figure 7.</th>
<th>First generation T7 amplification sequence components.</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>132</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Figure 8.</th>
<th>Second generation T7 amplification sequence components.</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>133</td>
</tr>
</tbody>
</table>
FIGURE 9. Third generation T7 amplification sequence components (re-synthesized) .......................................................... 137

FIGURE 10. Third generation T7 amplification sequence assembly reactions .......................................................... 138

FIGURE 11. Preparation of the synthetic T7 amplification sequence and the PCR generated detection sequence for cloning .... 139

FIGURE 12. Result of the cloning and transformation procedures. .... 142

FIGURE 13. Sequence of the complete T7 amplification sequence. .... 143

FIGURE 14. T7 amplification reaction to evaluate the fidelity of the T7 amplification sequence ........................................ 144

FIGURE 15. Reverse transcription and PCR amplification of poliovirus ........................................................................ 146

FIGURE 16. Effect of increasing amounts of the streptavidin magnetic beads on amplification of poliovirus RNA by RT-PCR .... 148

FIGURE 17. Schematic depicting one of the ways that the partially single and double stranded T7 amplification sequence could be prepared .......................................................... 156

IV. CHAPTER FOUR

FIGURE 1. Oligonucleotides used for magnetic capture, reverse transcription and PCR amplification ................................. 173

FIGURE 2. Flow chart of the nucleic acid capture/amplification procedure ........................................................................ 178

FIGURE 3. Ethidium bromide stained agarose gel electrophoresis of poliovirus RNA by RT-PCR ........................................ 182

FIGURE 4. Sequence of the 195-bp PCR amplicon .......................................................... 183
ABSTRACT

DEVELOPMENT AND APPLICATION OF MOLECULAR TECHNIQUES FOR THE DETECTION OF HUMAN ENTERIC VIRUSES IN ENVIRONMENTAL SAMPLES

by

Patrick M. Regan
University of New Hampshire, May, 1997

Shellfish and water have been implicated in the transmission of viral diseases. National surveillance data on waterborne and foodborne illnesses underestimate the public health significance of these viruses. The focus of this research was to evaluate, develop, and apply molecular techniques to detect human enteric viruses in environmental samples.

The initial study evaluated a nucleic acid hybridization assay using a $^{32}$P labeled cDNA probe to detect poliovirus in clams. Clams (*Mercenaria mercenaria*) were harvested from Narragansett Bay, Rhode Island. Poliovirus was detected in clams from the conditionally approved area in 3 of 9 collections and in the prohibited area in 4 of 9 collections. Once in the prohibited area, the coliform standards for water and shellfish were acceptable, although poliovirus was detected by the hybridization probe assay. This study demonstrated that nucleic acid hybridization can be used to detect...
enteric viruses, but was limited by its degree of sensitivity.

There was a need to increase the sensitivity of viral diagnostics. Two alternative molecular tools were developed. First was the T7 polymerase-based amplification system designed to indirectly amplify target RNA without reverse transcriptase. The T7 amplification system contained a viral recognition sequence, a T7 polymerase recognition sequence and a detection sequence to generate RNA transcripts. Efficacy of the amplification sequence was measured by $^{32}$P incorporation in the RNA transcripts. Results demonstrated that the T7 amplification sequence was functional by its ability to produce radiolabeled RNA transcripts.

The second system developed was a nucleic acid isolation procedure that used magnetic bead technology. A biotinylated oligonucleotide probe was hybridized to poliovirus-RNA in solution. Streptavidin-coated magnetic beads were used to isolate the RNA-oligonucleotide hybrid. This procedure could recover viral RNA suitable for amplification by reverse transcription-polymerase chain reaction (RT-PCR). This technique was used to recover viral RNA from concentrated groundwater samples. Results indicated that this capture system was effective in both concentrating, and purifying poliovirus RNA while removing environmental RT-PCR inhibitors. A detection sensitivity of one plaque-forming unit in 250 µl of a concentrated groundwater sample was routinely attained.
CHAPTER ONE

AN OVERVIEW OF ENVIRONMENTAL VIROLOGY AND MOLECULAR TECHNIQUES AVAILABLE FOR THE DETECTION OF HUMAN ENTERIC VIRUSES

INTRODUCTION

Presently, there is no routine surveillance procedure to monitor for the presence of enteric viruses. Outbreaks of viral gastroenteritis are not confined to any one segment of the population, and have occurred in day-care centers, schools, nursing homes and cruise ships (Hedberg and Osterholm, 1993). The number of cases and the economic impact of foodborne and waterborne illnesses are difficult to measure. Most cases are self-limiting and do not require medical attention. The Centers for Disease Control (CDC) is the principal repository for outbreak data. However, there are several deficiencies in the system: 1) it is a passive reporting system, 2) not all states report each of the major types of diseases, and 3) not all disease agents associated with waterborne or foodborne illnesses are identified as a nationally notifiable infectious disease. Consequently, national surveillance data on foodborne and waterborne illness in the United States underestimates the public health significance of these viruses.
The application of molecular techniques to confirm epidemiologic findings from an outbreak of nonbacterial gastroenteritis would be a tremendous step in developing an understanding of how pervasive the problem is. This along with consumer education about the potential health risks associated with high risk foods and water could help reduce the incidence of nonbacterial gastroenteritis and hepatitis A infections. Based on the acceptance of viral diagnostic procedures, these techniques could be applied to monitoring environmental resources. These tools will be beneficial in the development of guidelines and the enforcement of regulations designed to ensure the public health safety. Therefore, the development of sensitive, rapid and specific methods to detect human enteric viruses is necessary to ensure a safe food and drinking water supply.

**Viral Structure**

Viruses are obligate intracellular parasites that continue to evolve. They are composed of a DNA or an RNA core surrounded by a protein coat. Outside the host, they cannot replicate. For an infection to occur the virus must attach to a specific target cell receptor and liberate its genome into the cell. Viral infection can lead to several outcomes: 1) nonproductive infection where replication is blocked and the viral genome is either eliminated or integrated in the host’s genome, 2) productive infection where the host cell dies, or 3) persistent infection where a low level of the virus is continually
produced (chronic infection) (Knipe, 1996).

The virus structure is a transport mechanism that delivers viral nucleic acid to the host cell so it can replicate. The virus structure must be capable of maintaining integrity and infectivity of the virion while it is outside its host and then able to disassemble rapidly once it has encountered a susceptible host. Viruses come in a great array of shapes and sizes. Structural features are determined by requirements for assembly, exit, transmission, attachment, penetration, and uncoating (Harrison et al., 1996). The capsid is composed of protein subunits specified by the viral genome. Due to genetic economy the viral structures are composed of several copies of one or a few kinds of proteins. It is the repeated occurrence of the similar protein-protein interfaces that leads to a symmetrical arrangement of the subunits. Most viruses fall into two major types of symmetry, icosahedral and helical, the symmetry of the remainder being complex or unknown (Madeley and Field, 1988).

The capsid may be surrounded by a lipid bilayer known as an envelope. The envelope consists of a lipid bilayer, with proteins, usually glycoproteins embedded in it. The envelope is acquired from budding through the host cell membrane, such as the plasma membrane, endoplasmic reticulum or the nuclear membrane. There is a particular distinction between enveloped and nonenveloped viruses. The distinction corresponds to the difference in the way a virus enters and leaves a cell (Harrison et al., 1996).
Human enteric viruses. There are at least 140 types of human enteric viruses that include, 72 serotypes of enteroviruses, Norwalk virus, calicivirus, adenovirus and rotavirus (Hurst, 1991). The enteroviruses are among the most common and important viral pathogens of humans (Abraham et al., 1992). Additionally, enteric viruses continue to be discovered and the list continues to grow. The minimum human infectious dose for enteroviruses is on the order of a single virus particle (Rao, 1982).

An enteric viral infection is typically acquired by the consumption of a product contaminated with fecal material (fecal-oral route). These viruses produce a wide range of illnesses that include, diarrhea, myocarditis, vomiting, paralysis and hepatitis. Due to the potential of 140 enteric viruses, the concept of monitoring for a specific virus as an indicator to determine the adequacy of treatment may not be feasible (Regli et al., 1991). However, this should not preclude the development of a viral monitoring strategy. Regli et al. (1991) proposed that a combination of characteristics for different viruses be developed to determine acceptable levels of risk and to prescribe appropriate levels of treatment.

Viruses in the Environment

Wastewater. Methods to detect viruses in sewage and sewage-polluted waters were initiated over a half century ago. Early efforts prompted by public health concerns and epidemiological research on poliovirus distribution in
nature represented the beginning of environmental virology (Metcalf et al., 1995).

A primary way that human enteric viruses can enter the environment is through the discharge of wastewater into waterways. The construction of storm water or sewerage systems, which began in the mid to late 1800s in urban centers resulted in the collection of human-derived wastes and their release into nearby coastal waterways (Fair et al., 1966). This practice resulted in the progressive contamination of shellfish-growing areas and outbreaks of enteric disease associated with shellfish harvested from them (Rippey, 1994).

Many communities discharge their waste into estuaries, bays, harbors and other coastal waters. The oceans thus receive billions of gallons of treated and untreated wastewater daily (Rao and Melnick, 1986). Human pathogenic enteric viruses (e.g., poliovirus, echovirus, coxsackievirus, infectious hepatitis, etc.) usually occur in domestic sewage and survive in significant numbers even after conventional secondary treatment, including chlorination (Gerba et al., 1975).

The concentrations of enteric viruses in the fecal material of an infected human can be several million viral particles per gram. In raw sewage the levels of human enteric viruses has been shown to reach 10,000 plaque-forming units (PFU)/l and in treated sewage 100 PFU/l (Rao and Melnick, 1986). In the marine environment the microbial inactivation occurs by two processes, dilution by diffusion into waterways and biological
inactivation (Borrego et al., 1983).

The adequacy of sewage treatment facilities to effectively treat and inactivate human enteric viruses has been questioned. A major concern is the use of combined sewage overflows (CSO). CSOs are a design problem where storm drains and sanitary sewer systems combine. During a heavy rainfall, many treatment plants cannot handle the increased volume and sewage is discharged without treatment. In 1990, Connecticut had about 300 CSOs, Massachusetts and Rhode Island had 90 along Narragansett Bay and New York City had 670 (Van Patten, 1990). Population growth and economic restraints have been at the root of the problem. A good example is the Metropolitan Boston, Massachusetts, sewage treatment facilities that were too small and broken-down to handle the average sewage inflows of 500 million gallons per day (Levi 1990). The situation in Boston led to legal action and the eventual mandate of a multibillion-dollar cleanup plan.

Because of inadequate treatment, the discharge of partially or untreated effluent can result in cases of nonbacterial gastroenteritis. Enteric viruses can be expected to occur in any surface water exposed to contamination by human fecal wastes (Rao and Melnick. 1986). Once released into the environment the enteric viruses will eventually become inactivated; however, they may survive for a prolonged time (Rao and Melnick. 1986).
Seafood-borne Outbreaks

The responsibility for monitoring and control of seafood safety is divided among various agencies of the federal government, primarily the Food and Drug Administration (FDA), the National Marine Fisheries Service (NMFS), and the states. In recent years the effectiveness of their respective programs has been questioned (NAS, 1991).

All species of commercially important shellfish have been shown to bioaccumulate enteric viruses from environmental seawater during feeding (Metcalf et al., 1980). Due to bioaccumulation, shellfish may become contaminated even at a considerable distance from the point of sewage discharge (Gerba et al., 1988). There are several factors that contribute to the incidence of outbreaks associated with shellfish: 1) the shellfish-growing area is contaminated with sewage, 2) the shellfish are illegally harvested from a closed area, and 3) the harvest area is improperly classified.

Nonbacterial gastroenteritis comprises most of the foodborne illnesses associated with the consumption of contaminated shellfish (NAS, 1991). The first documented cases of hepatitis A virus occurred in Sweden in 1956, where 629 cases were associated with raw oyster consumption (Rippey, 1994). Shellfish (clams, cockles, mussels and oysters) harvested from fecally contaminated areas have been associated with more than 100 outbreaks of viral gastroenteritis and hepatitis A in the United States in the past 50 years (Richards, 1985). Until the 1980s, the number of shellfish-associated
infectious disease outbreaks was less than 50 outbreaks per decade. More than 45% of the historical cases were reported in the 1980s, although there are no obvious reasons for this dramatic increase (Rippey, 1994). Table 1 lists the etiological agents of outbreaks and cases associated with the consumption of raw or lightly cooked molluscan shellfish (Rippey, 1994).

It is clear that the greatest number of cases are of unknown etiology. However, the symptoms, onset and duration of the illness are typical of viral origin. Methods to identify many viral agents have only recently been developed; these viral pathogens have rarely been identified in shellfish-associated outbreaks (Rippey, 1994).

The National Academy of Sciences' (NAS) Report on seafood safety (1991) developed several conclusions:

♦ Most seafood-associated illness is reported from consumers of raw bivalve molluscs and is due to unknown etiologies, but is clinically suggestive of Norwalk-like gastroenteritis.

♦ Adequate and proper treatment and disposal of sewage must be implemented to avoid contamination of shellfish harvest areas.

♦ Valid indicators for human pathogen contamination of growing waters must be developed.

♦ New or improved methodologies (hybridization probe and the polymerase chain reaction (PCR), should be developed that provide for the rapid identification and quantitation of seafood-associated pathogens.
<table>
<thead>
<tr>
<th>Agent</th>
<th>No. of cases</th>
<th>No. of incidents</th>
<th>No. Of outbreaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7,978</td>
<td>277</td>
<td>256</td>
</tr>
<tr>
<td>Typhoid</td>
<td>3,270</td>
<td>93</td>
<td>78</td>
</tr>
<tr>
<td>Hepatitis A</td>
<td>1,798</td>
<td>51</td>
<td>42</td>
</tr>
<tr>
<td>Norwalk virus</td>
<td>311</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>130</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>Snow Mountain virus</td>
<td>116</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Shigella spp.</td>
<td>111</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>Hepatitis&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Campylobacter spp.</td>
<td>27</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>Plesiomonas spp.</td>
<td>18</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Aeromonas spp.</td>
<td>7</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Adapted from: Rippey, 1994.

<sup>a</sup> No agent isolated or identified

<sup>b</sup> Type unspecified
**Waterborne Outbreaks**

Public water systems are regulated under the Safe Water Drinking Water Act (SWDA) of 1974. The microbial content of drinking water is regulated by the Environmental Protection Agency (EPA) through the Total Coliform Rule and the Surface Water Treatment Requirements (SWTR) (CDC, 1993).

Classic cases of waterborne outbreaks have been associated with private wells, small water systems, and community water systems (Hedberg and Osterholm, 1993). Groundwater contamination of wells has occurred as a result of municipal sewage, leakage from septic tanks and flooding after heavy rainfalls. The potential for groundwater pollution from on-site septic systems has emerged as a serious concern in the United States (Cogger, 1988). High water tables are the main concern because it results in the incomplete removal of microbes from wastewater when the groundwater is near or at the depth of the adsorption trenches (Cogger, 1988). Additionally, the discharge of viruses into waterways through sewage outfalls poses a hazard to bathers and other recreational users of contaminated beaches (Fattal et al., 1983).

Outbreaks associated with drinking water were previously reported by the CDC (1993) for a two-year period, 1991-1992. Seventeen states and territories reported 34 outbreaks associated with drinking water and these are listed in Table 2. Thirty-one (91%) of the outbreaks caused gastroenteritis. In 23 (68%) of these the etiological agent was not identified.
<table>
<thead>
<tr>
<th>Agent</th>
<th>Communitya</th>
<th>Noncommunityb</th>
<th>Individualc</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Outbreaks</td>
<td>Cases</td>
<td>Outbreaks</td>
<td>Cases</td>
</tr>
<tr>
<td>AGId</td>
<td>3</td>
<td>10,077</td>
<td>19</td>
<td>3,252</td>
</tr>
<tr>
<td>Giardia</td>
<td>2</td>
<td>95</td>
<td>2</td>
<td>28</td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td>2</td>
<td>3,000</td>
<td>1</td>
<td>551</td>
</tr>
<tr>
<td>HAV</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S. sonnei</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>150</td>
</tr>
<tr>
<td>Nitrate</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fluoride</td>
<td>1</td>
<td>262</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>13,434</td>
<td>23</td>
<td>3,981</td>
</tr>
</tbody>
</table>

From - MMWR, (1993), Vol. 42

a = Community system defined as public or investor owned that serve large or small communities
b = Noncommunity system serves institutions, industry or businesses that may be used by the general public
c = Individual are usually wells or springs used by individuals traveling outside a populated area
d = AGI = acute gastrointestinal illness of unknown etiology.
Bacterial Indicators

In 1925 the National Shellfish Sanitation Program (NSSP) was developed as a result of an oyster-borne typhoid epidemic (Richards, 1985, Metcalf et al., 1980). The NSSP is a joint state and federal effort that oversees the Interstate Shellfish Sanitation Conference (ISSC). The purpose of the ISSC is to foster and improve shellfish sanitation by providing participating states a more direct and organized forum to exert leadership.

Shellfishing is one of the most heavily regulated food industries (Klauber, 1991). State and federal regulatory agencies monitor for the sanitary quality of shellfish-growing waters and shellfish using bacterial indicators (Richards, 1985). Current standards rely on bacterial indicators to determine the sanitary quality of shellfish and their growing waters (Richards, 1985). Presently the microbiological standard in the United States for shellfish-harvesting waters is a median of 70 coliforms per 100 ml with no more than 10% of the samples exceeding a value of 230 (Gerba et al., 1979).

One of the shortcomings of the bacterial indicator group is that it does not reliably index the presence or absence of enteric viruses in shellfish or their growing waters (Cabelli, 1982). The reason may be in part that fecal coliforms are not as resistant to chlorine disinfection and environmental stresses of salinity and sunlight as some enteric viruses. Therefore, the viral quality of recreational and shellfish waters cannot be determined adequately by reference to fecal coliform indexes (Metcalf et al., 1995). A report by the
CDC (1990) stated that shellfish harvested from shellfish-growing waters that meet the bacteriological standards of hygiene may contain viral agents. Additionally, it was noted that depuration practices are less effective in the elimination of viral contamination than bacterial contamination.

According to Cabelli (1982), the ideal indicator: 1) should be consistently and exclusively associated with the source of the pathogens, 2) must be present in sufficient numbers to provide an “accurate” density estimate whenever the level of each of the pathogens is such that the risk of illness is unacceptable, 3) should approach the resistance to disinfectants and environmental stress of the most resistant pathogen potentially present at significant levels in the source, and 4) must be quantifiable by inexpensive, specific and sensitive methods.

**Viral Diagnostics**

In part, the ability to effectively evaluate the safety of a product for consumption is based on the availability of adequate testing procedures. The traditional approach to viral diagnosis relied on cell culture techniques. Although this approach is very sensitive, there are several inherent problems that preclude its use: 1) the sample can be toxicity to the cells, 2) the procedure is time consuming, 3) there is no universal cell line, and 4) some viruses cannot be propagated by cell culture (Enriquez et al., 1993).

Even with the availability of electron microscopy and enhanced
serologic methods for detection, there was a need to increase the specificity and sensitivity while providing answers quickly. The development of enzyme-linked immunosorbent assays (ELISA) has proven to be an important diagnostic tool in the clinical field and has been applied to environmental samples. There are commercially available antigen detection kits for the detection of rotavirus, calicivirus, Norwalk, snow-mountain agent and astrovirus (CDC, 1990). However, direct identification of viral pathogens in incriminated food and water has been extremely rare due to the lack of a sensitive assay (Gouvea et al., 1994). These assays are best suited for the clinical diagnostic field where there is a higher titer of the infectious agent in the sample.

**Molecular Techniques**

The development of molecular genetics has had a dramatic influence on many scientific disciplines. It has given us the tools to identify both cultivatable and noncultivatable viruses. Technical milestones such as the discovery of restriction endonucleases, ligases, polymerases and improved sequencing techniques have made significant improvements in the field of viral diagnostics (Eisenstein, 1990). On the other hand, molecular techniques are not able to differentiate between an infectious and noninfectious virus, and some sample matrixes can inhibit enzymatic amplification.

More recently, a variety of techniques such as nucleic acid
hybridization, PCR, and antibody-capture have been studied and applied to a variety of microbiological settings (Spencer et al., 1994). The development of more rapid and improved diagnostic techniques is continually being sought.

Rapid methods and automation are a dynamic area in applied microbiology (Fung, 1995). They cover the isolation, enumeration and characterization of microorganisms. A principal area that molecular techniques is being applied is in the field of food and water microbiology. The development of nucleic acid hybridization techniques and PCR have provided sensitive and specific assays to detect low levels of human enteric viruses in environmental samples. These assays are based on the detection of an organism’s genome.

In food microbiology, there is no universally accepted method for the detection of human enteric viruses in foods. There are several factors that make molecular techniques promising for the analysis of foodborne viruses: 1) viral contamination is low in environmental samples, 2) there is no universal cell line to cultivate all of the viruses of public health importance, and 3) some viruses cannot be propagated by cell culture. Sensitive, automated methods need to be developed to provide a means to survey high-risk food products, especially in identifying the cause of an outbreak. It is for these reasons that molecular techniques could play an important role in surveying incriminated foods for viruses such as hepatitis A, rotavirus Norwalk, and Norwalk-like viruses.
Scientific literature describes several molecular techniques used to detect human enteric viruses (Eisenstein et al., 1990; McIntosh, 1996; Podzorski and Persing, 1995). Additionally, many of these techniques are evolving and their widespread application is questionable. The acceptance of any one of these methods will be based on method collaboration to ensure the sensitivity and specificity of the assay. A closer look at molecular techniques follows.

**Nucleic Acid Hybridization**

A microorganism can be identified based on the presence of a particular nucleic acid sequence. This is in contrast to most biochemical and immunological tests that detect gene products such as an antigen or chemical end products of a metabolic pathway (Hill et al., 1995). The basis for a nucleic acid hybridization assay results from the structure of the nucleic acid molecule. Here a specific region within an organism’s genome is selected for detection. Nucleic acid hybridization centers on the formation of a double helix formed from two complementary strands. One of the nucleic acid strands is from the organism (target) and the other strand is the probe. Typically, the probe is prepared by an automated chemical process or by recombinant techniques. In a hybridization reaction, the association of the target and probe can take on several forms, DNA:DNA, DNA:RNA, and RNA:RNA.
There are three primary formats for hybridization, solid-phase, solution-phase and *in situ* hybridization. Both solid-phase and solution phase-hybridization require the target genome to be liberated from the protein coat prior to hybridization. *In situ* hybridization is the application of the probe to tissue sections in a natural state (Kandolf et al., 1987).

**Solid phase hybridization.** In solid-phase hybridization the target is applied to a solid support (nylon or nitrocellulose membrane). A labeled complementary probe is added and hybridized to the target. The membrane is washed to remove the excess probe and the reporter signal is measured.

Solid phase hybridization results in a first-order kinetic reaction since the target is immobilized on a membrane. A first-order kinetic reaction can affect the rate and presentation of the target in the hybridization step.

**Solution phase hybridization.** Solution phase hybridization is where both the probe and target can freely interact in solution. Following hybridization the unbound probe is removed and the reporter signal is measured. Solution hybridization follows a second-order kinetic reaction. The ability of the probe and target interaction to interact in solution increases the hybridization rate.

**In situ hybridization.** *In situ* hybridization is similar to both solid and
solution phase hybridization. In this procedure the probe diffuses through the organism's cell membrane where it then hybridizes to the target. An important application of in situ hybridization is in diagnostic pathology. This method allows the evaluation of the histology and also the localization of the diagnostic probe-target complex within a specific tissue. Among many other applications, its uniqueness comes from the fact that it can be used to characterize latent and persistent viral infections.

The following discussion covers information about the target, probe, reporter moiety and hybridization conditions used in the nucleic acid hybridization assay. The sensitivity and specificity of an assay are dependent on each of these components.

**Nucleic Acid Hybridization Components**

**Target.** For viruses, the nucleic acid target is packaged within the viral protein coat. The nucleic acid must be liberated first to make it available for hybridization. Release of the nucleic acid can be by heat, or use of a protein denaturant such as guanidine thiocyanate or phenol-chloroform. When working with RNA viruses the control of RNase activity is important. Lysis solutions containing guanidine thiocyanate, guanidine hydrochloride, sodium dodecyl sulfate (SDS), and phenol:chloroform will all inhibit RNase activity. The method used to liberate the viral nucleic acid is dependent on the product being assayed, for example, poliovirus in water would not be
processed the same as poliovirus in shellfish.

**Probe.** A probe is a segment of nucleic acid with a specified nucleic acid sequence that is used to determine the presence of a complementary sequence. Any form of nucleic acid can be used as a probe for hybridization, provided it can be suitably labeled (Arrand, 1988).

The choice of a probe depends on three factors: 1) the hybridization strategy, 2) the availability of material for use as a probe, and 3) the degree to which it can be labeled (Arrand, 1988). Furthermore, the selection of a probe must be based on whether it will be used to identify a particular genotype or if it will detect an entire species. Therefore, both the conserved and variable regions of the viral genome needs to be evaluated. Multiple probes can be used in the detection of organisms with significant sequence heterogeneity.

Some of the first probes were created by recombinant DNA techniques, where specific fragments of nucleic acid were inserted into a plasmid vector. The plasmid is transformed into a bacterium that is then grown in culture to increase the concentration of the plasmid / probe component. The probe is isolated, purified and sometimes excised from the plasmid. The availability of a transcription vector to generate single stranded RNA probes proved beneficial for RNA targets for several reasons: 1) RNA-RNA hybrids have a greater affinity than DNA-RNA hybrids, 2) there are no vector sequences present that could permit nonspecific hybridization, and 3) there is no self
annealing of the probe (Rotbart, 1991). With the availability of automated nucleic acid synthesis, nucleic acid probes (up to approximately 150 bases) can be rapidly and efficiently generated.

The purity of a probe is extremely important to prevent background hybridization. The diagnostic usefulness of a probe is dependent on its specificity and sensitivity, sensitivity being the minimal amount of the target the probe can detect and specificity, the ability of the probe to distinguish the target from a background of other cells, nucleic acid and proteins. Background hybridization from nonspecific binding can obscure the results and prevent their interpretation.

**Reporter molecule.** Attached to the probe is a reporter molecule that will produce a signal if the target is present. The choice of a reporter molecule is based on the desired sensitivity of the assay. In the early 1980s probes were primarily labeled with a radioactive isotope. This was a result of the sensitivity advantage of a radioactive probe over a nonradioactive probe. The radioactive label is the “Gold Standard” of probe technology (Wetmur, 1991). The isotope of choice for filter and solution hybridizations is $^{32}$P since its high energy results in short scintillation counting times and short autoradiographic exposures. Due to the concern over safety and convenience, much effort has been placed in the development of nonradioactive probes. Nonradioactive detection systems incorporate labels such as biotin,
digoxigenin, fluorescein, and horseradish peroxidase (HRP). Nonradioactive probes, such as chemiluminescent detection, reportedly have a sensitivity that is equal to or greater than isotopic probes.

The type and length of the probe dictate the method of labeling and the number of reporter molecules that can be incorporated. Probes that consist of a cloned segment of DNA can be labeled by nick translation, random priming, or PCR and typically can accommodate several reporter molecules. Synthetic oligonucleotides are usually labeled during synthesis, or after synthesis with a terminal transferase (bacteriophage T4 polynucleotide kinase) or photobiotin.

To enhance the sensitivity further, signal amplification schemes have been developed. Most notable is a method developed by Chiron Corporation known as branched DNA (bDNA) probe system (Nolte et al., 1994). This system uses multiple probes along with multiple reporters. Here following hybridization of the target and probe there is a second hybridization of an “amplification multimer” that can bind up to 3000 reporter molecules.

**Hybridization conditions.** The most critical step is the determination of the hybridization conditions. Two factors that affect the stringency of the assay are the temperature used in the hybridization and washing steps, and the ionic strength of the hybridization and washing fluids (Britten and Davidson, 1988). The hybridization conditions are based on the type of probe used and the desired results.
Summary of Nucleic Acid Hybridization

Nucleic acid hybridization techniques have been used to detect poliovirus (Margolin et al., 1993 and Regan et al., 1993), hepatitis A virus (Le Guyader et al., 1993 and Ticehurst et al., 1987), and rotavirus (Fernandez et al., 1992). Hybridization techniques are reported to be more sensitive than immunoassays, but not as sensitive as cell culture. Comparing the sensitivities is difficult, since there are no universal standards to measure this.

The success of hybridization assays is dependent on the level of the virus in the sample and the efficiency at which they can be recovered. It was determined that the assay sensitivity limit, using the most sensitive of probe reagents (RNA labeled with $^{32}\text{P}$), is approximately 10 pg of a target nucleic acid (Rotbart, 1991). The 10 pg of target translates to $10^6$ RNA molecules. Considering a particle-to-infectivity ratio of 100:1, the sensitivity of the hybridization assay would be as low as $10^4$ infectious viruses (Rotbart, 1991). Furthermore, this was done in saline, which in theory would be an ideal condition. The application of hybridization techniques without target amplification is limited. Therefore, standard hybridization techniques are best applied to systems where target amplification is included.
**Target Amplification Systems**

The next generation of molecular assays centered on amplification of the target. As previously mentioned, there was a need to increase the assay sensitivity, especially in those samples that inherently have a low level of contamination or where there was a low recovery efficiency. Several amplification techniques have been developed that focus on different reaction components: 1) target amplification, 2) probe amplification, and 3) signal amplification.

**Polymerase chain reaction.** The development of a nucleic acid amplification system known as PCR has been a technological milestone for the field of molecular biology (Podzorski and Persing, 1995). In theory, under optimal conditions this technique will amplify a single copy of a target to millionfold levels in several hours (Figure 1). This technique has been used extensively in environmental, and clinical diagnostic assays for the detection of viruses that includes hepatitis A virus (Le Guyader et al, 1994; Jansen et al, 1990; and Monceyron and Grinde, 1994), poliovirus (Chapman et al., 1990; Rotbart, 1990; and Takeda et al., 1994), and rotavirus (Gouvea et al., 1994 and Ushijima et al., 1994). However, at this time these assays are for research purposes and are not applied to evaluate and support regulatory action.

The advantage of PCR for viral diagnostics is that it does not require propagation of the virus. Here, the presence of a targeted segment of an
Figure 1. Exponential amplification potential of PCR under ideal conditions.
organism’s genome is detected. On the other hand, viability / infectivity is not known.

The principle of the system requires the generation of a set of complementary primers (oligonucleotides) that flank a target region of the organism’s genome. Temperature changes are then applied, typically using an automatic thermal cycler to heat the sample to denature the target nucleic acid, cool the reaction mix to allow the hybridization of the single stranded target and primers, and a thermostable polymerase then extends the primers along the target. This procedure is repeated several times. In theory each complete cycle doubles the amount of target DNA. The major product generated is a segment of double stranded DNA whose termini are defined by the primers used and whose length is defined by the distance between the primers (Maniatis et al., 1989).

The assay was developed to amplify DNA; however, RNA genome viruses can be detected with a procedural modification. There are two ways to analyze microorganisms with an RNA genome. Each uses a different enzyme to convert the RNA to cDNA. The original procedure used a heat-labile retrovirus reverse transcriptase (M-MLV). Following the production of cDNA, the reverse transcriptase enzyme is inactivated and additional components, including the thermostable polymerase (Taq), are added to the reaction. The recent discovery of a thermostable DNA polymerase (Tth) that has both reverse transcription and polymerase activity has allowed the assay
to be done under a single set of reaction conditions (Podzorski and Persing, 1995). The benefits of using this enzyme are that reverse transcription can be done at a higher temperature that will reduce secondary genomic structures and that the reaction tube does not have to be opened after reverse transcription, thus reducing the possibility of contamination.

There are several drawbacks with PCR: 1) the presence of inhibitors in environmental and clinical samples that could prevent amplification (false negative), 2) the amplification of contaminating nucleic acid (false positive), and 3) the cross-contamination of an amplified product with new sample (false positive). Therefore, the use of a positive control to ensure the conditions would support amplification and negative controls should be run concurrently with each assay.

**Other Molecular Detection Techniques**

There are several other amplification systems that have been reported. These systems have seen limited application. Although many were thought to rival PCR, it appears as though PCR has taken most of the interest from any other amplification technique. An overview of four techniques, ligase chain reaction (LCR), self-sustained sequence replication (3SR), strand displacement amplification (SDA), and Qβ replicase is as follows.

**Ligase chain reaction (LCR).** This is a probe amplification procedure,
here the target is not amplified. The assay is based on the ligation of adjacent primers hybridized to the target nucleic acid (Barany, 1991). This forms a "long" product that is later detected by agarose gel electrophoresis. This system has been used principally to detect mutations in specific genes. If there was a deletion the primers could not hybridize. Therefore, the absence of the ligated product would suggest a mutation.

There are two approaches to LCR, linear amplification where there are only two primers in the reaction or exponential amplification where there are two pairs of primers, one pair complementary to the target and the other pair complementary to the first pair. The first step is for the two primers complementary to the target to hybridize and they are ligated with a thermostable ligase. The reaction is then heated to denature the hybrid and then cooled to repeat the process.

**Self-sustained sequence replication (3SR).** The 3SR reaction utilizes a transcription-based approach that was developed in 1989 (Kwoh et al., 1989). This system was designed to amplify single-stranded RNA. Three enzymes are used in the amplification process, reverse transcriptase, RNase H and bacteriophage T7 polymerase and a primer containing a T7 polymerase binding site. Ultimately double-stranded cDNA is produced that contains a T7 promoter region; from this RNA transcripts are generated. Additional cDNA is generated from the newly formed transcripts and the process repeats
itself. Theoretical amplification of ten-millionfold has been reported (Kwoh et al., 1989).

**Strand displacement amplification (SDA).** SDA was reported in 1992 (Walker et al., 1992). This procedure requires a DNA template, specific primers, a restriction endonuclease and a DNA polymerase to amplify the target. The principle behind this reaction is the incorporation of α-thio-substituted nucleotides (dATP α S). This produces a hemiphosphorothioated DNA template that is resistant to enzyme cleavage. The initial rounds of the reaction transform the original target sequence into the hemiphosphorothioate form with nickable Hinc II sites at each end. The second part of the reaction involves exponential amplification of the transformed target sequence (Podzorski and Persing, 1995).

**Qβ replicase.** The last system is the Qβ replicase probe amplification procedure reported in 1988 (Lizardi et al, 1988). This system is based on the amplification of a target specific substrate using Qβ replicase. Either a single-stranded RNA or DNA template can be used. The system is prone to false-positive results and therefore uses a double capture method for purification. The capture procedure incorporates a target-specific area with a poly-G tail and a target-specific probe with a poly-A tail. Following hybridization, the tails are hybridized to poly-(dC) and poly-(dT) magnetic beads. The bead
complex is immobilized with a magnetic particle concentrator, the supernate is then removed and the beads are washed.

**Research Goal and Objectives**

The focus of this research was to evaluate, develop and apply molecular techniques for the detection of human enteric viruses in shellfish and water. Currently, there is an analytical void in the detection of human enteric viruses in environmental samples. Techniques generated from this research will be of interest to the environmental scientist in the field of viral diagnostics. The application of these molecular techniques to the field of environmental virology will allow us to evaluate the magnitude of environmental viral contamination.

**Phase 1.** The first objective was to evaluate microbial indicators for the detection of the sanitary quality of shellfish harvested from approved, conditionally approved and prohibited shellfish-growing areas. The viral quality of recreational and shellfish growing waters cannot be determined adequately by reference to fecal coliform indexes. Furthermore, the numbers of viruses and fecal coliforms in water and shellfish do not seem to correlate (Metcalf et al., 1995).

This study determined and evaluated the levels of total coliforms, fecal coliforms, *Clostridium perfringens*, enterococci, male-specific bacteriophage, and poliovirus in hardshell clams. The results of this study further support
the need to develop a multifaceted approach to better evaluate the sanitary quality of shellfish and their growing waters. This approach will require the development of more sensitive molecular techniques used for virus detection. The impetus here is that no one indicator can adequately ensure that a public health hazard does not exist.

**Phase 2.** Even with a radiolabeled probe, the use of nucleic acid hybridization to detect viruses in environmental samples has demonstrated a limited sensitivity (Metcalf et al., 1995). Also, the use of radioactivity made this assay available to only those laboratories licensed to handle radioactivity. Nonradioactive reporter molecules include enzymes such as alkaline phosphatase, fluorescent molecules, and chemiluminescent moieties. They have the advantage of being nonhazardous, but the level of sensitivity using these molecules in hybridization assays is generally lower than with a radioactive label. The ability to amplify the target nucleic acid would circumvent the problems associated with the hybridization assay. Nucleic acid amplification techniques would increase the reaction's sensitivity, thereby eliminating the requirement of a radiolabeled probe.

The second objective explored the development of two molecular tools to detect viruses in environmental samples. The first technique was the development of a T7 amplification sequence. The use of this sequence would serve to indirectly amplify a captured viral target. Indirect amplification
would increase the assay's sensitivity and permit the use of a nonradioactive reporter molecule. This T7 amplification sequence was designed and constructed from individual components to contain: 1) a viral recognition site, 2) a T7 polymerase recognition site, and 3) a segment of DNA downstream from the T7 promoter to produce RNA transcripts. The T7 amplification sequence is hybridized with the target, washed, and a T7 polymerase reaction is employed to generate RNA transcripts that could be detected by a nonisotopic procedure.

The second technique was the development of a nucleic acid capture sequence. Here a nucleic acid-based capture system was developed to isolate target viral nucleic acid from an environmental sample. The use of nucleic acid amplification techniques can be hindered by the presence of enzymatic inhibitors. These inhibitors cover a wide range of compounds that are not well-defined. The presence of an inhibitor in a sample may yield a false-negative result. The ability to remove these inhibitors is paramount to an effective assay. The capture technique was based on magnetic bead technology. A biotinylated capture probe is hybridized to the target and subsequently removed with the use of streptavidin-coated superparamagnetic beads and a magnetic particle concentrator.

The capture sequence methodology has several beneficial attributes: 1) the sample volume is reduced, 2) the isolated nucleic acid can be easily washed to reduce or eliminate enzymatic inhibitors, and 3) the resulting
product can be used directly in PCR and RT-PCR protocols.

**Phase 3.** In this phase, the previously developed nucleic acid-based magnetic capture system was used to isolate poliovirus from seeded, concentrated groundwater samples. The success with this technique for groundwater was suggestive of its further application to other environmental samples. This nucleic acid capture system could selectively isolate target nucleic acid, remove enzymatic inhibitors, and could be used directly in RT-PCR. The capture technique described in this research will be beneficial to the field of environmental diagnostics.
REFERENCES


33

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Le Guyader, F., E. Dubois, D. Menard, and M. Pommepuy. 1994. Detection of hepatitis A virus, rotavirus, and enterovirus in naturally contaminated...


CHAPTER TWO

EVALUATION OF POLIOVIRUS AND OTHER MICROBIAL INDICATORS
FOR THE DETERMINATION OF THE SANITARY QUALITY AND SAFETY
OF SHELLFISH

ABSTRACT

Shellfish consumed either raw or partially cooked have been implicated in
the transmission of viral gastroenteritis and hepatitis A infections. The
effectiveness of bacterial indicators to signal the presence of human
pathogenic viruses has been questioned. Traditional viral assays, such as cell
culture, made it impractical to monitor shellfish for viral contaminants.
There exists a need to develop rapid and sensitive assays to detect human
enteric viruses to ensure the sanitary quality of shellfish. The primary
objective of this study was to evaluate a hybridization assay to detect
poliovirus in hardshell clams. Sample collections of hardshell clams
(Mercenaria mercenaria) were taken from approved, conditionally approved
and prohibited shellfishing areas in Narragansett Bay, Rhode Island, between
July 1989 and May 1990. Clams were assayed for poliovirus by nucleic acid
hybridization using a $^{32}$P labeled cDNA probe. Other microbial indicators
(total and fecal coliforms, Clostridium perfringens, enterococci and
male-specific bacteriophage) were evaluated for comparative purposes. Of these indicators, the bacteriophage were most consistently recovered from each of the three collection areas at each collection time. The enterococci were recovered with the least frequency. Poliovirus was detected in clams from the conditionally approved area in 3 of 9 collections and in the prohibited area in 4 of 9 collections. On one occasion in the prohibited area, the coliform standard for water and shellfish were not exceeded, although poliovirus was detected by the hybridization probe assay. This study demonstrates that nucleic acid hybridization can be used to detect a human enteric viruses. New advances in nucleic acid technology may soon enable routine monitoring of shellfish for enteric viruses.
INTRODUCTION

Shellfish have been widely recognized as a means of transmission of foodborne enteric disease since early this century, when a number of serious shellfish-associated typhoid fever outbreaks were reported (Guzewich and Morse, 1985). Edible bivalve molluscs of the class Pelecypoda (oysters, clams, and mussels) are the only molluscan shellfish of commercial importance for which sanitary controls are currently required (Metcalf, 1975).

Although the National Shellfish Sanitation Program (NSSP) bacterial indicator system has decreased the incidence of shellfish-associated enteric disease, its efficacy as a reliable indicator for protecting against the presence of human enteric viruses is questionable (Wait et al., 1983). One of the principal concerns with the present indicators and standards are that coliform bacteria are much more sensitive to chlorine than are a number of human enteric viruses, such as hepatitis A virus (Engelbrecht and Greening, 1978). Also, the survival of certain human enteric viruses in environmental water, during the winter months, is substantially greater than that of coliforms.

During the last several decades, viral infections appear to account for the majority of foodborne illnesses in the United States. During 1982 there were 103 well-documented cases of gastroenteritis associated with the consumption of raw shellfish involving 1,017 individuals in New York. The
predominant etiological agent was determined to be Norwalk virus (Guzewich and Morse, 1985, Morse et al., 1986). Other outbreaks of viral gastroenteritis and hepatitis A related to the consumption of raw or partially cooked shellfish have been reported (Gill et al., 1983, Portnoy et al. 1975, Richards, 1985) as well. A study by Digirolamo et al., (1970) indicated that poliovirus would survive in refrigerated oysters for a period varying from 30 to 90 days, depending on the storage temperature. There is a good likelihood that the incidence of individual cases and isolated outbreaks of shellfish-associated viral illnesses are significantly under-reported.

All viruses known to be normally transmissible through foods are derived from the human intestine (Blackwell et al., 1985). The discharge of both treated and untreated sewage into waterways being utilized as sources of seafood has gained much attention in regard to viral contaminated shellfish (Gerba and Goyal, 1978, Landry et al, 1983). Metcalf et al. (1995) indicated that an average of about 50 plaque-forming units (PFU) per liter can be expected in effluent from wastewater treatment plants. Solids-associated virus in wastewater effluent discharged into an aquatic environment settles through water columns onto bottom sediments, where the concentration may be 10 to 10,000 times greater than those found in water (Metcalf et al., 1995).

All species of commercially important shellfish have been shown to accumulate enteric viruses from environmental seawater during routine feeding activities (Metcalf et al., 1980). Since ordinary wastewater treatment
does not always completely remove or disinfect such viruses, there is a need to be able to assess the efficacy of current indicators and standards.

Currently, there is no one organism that is considered to be the ideal indicator. Since it is impractical, indeed impossible to test for each individual bacterial or viral pathogen, a multi-faceted approach would be desirable. This approach would best correlate with the survivability and occurrence of the most resistant human pathogens, both viral and bacterial. The die-off rate of coliforms in the marine environment typically shows an initial lag phase followed by a mortality rate of 90% in 3-5 days (Gerba and McLeod, 1976). The die-off rate is controlled by factors such as salt concentration, predation by native flora, heavy metals, and a limited nutrient supply (Fujioka et al., 1981). The feasibility of using other indicator organisms such as fecal streptococci (Berg and Metcalf, 1978) *Clostridium perfringens* (Emerson and Cabelli, 1982) and bacteriophage (Havelaar et al., 1986) has been discussed. Assays involving the detection of enteroviruses in shellfish by cell culture (Bemiss et al. 1989, Idema et al. 1991) and by the use of hybridization probes (Bruce et al., 1989, Jiang et al., 1986, Margolin et al., 1986) have been evaluated.

The objective of this study was to determine the levels of and compare the relationships between bacterial indicators, male-specific bacteriophage, and poliovirus found in shellfish collected from approved, conditionally approved, and prohibited waters. According to the NSSP an approved area is a shellfish-growing area that has been approved by the state shellfish control
authority for growing or harvesting shellfish for direct marketing. A conditionally approved area is one that meets the approved area criteria for a predictable period. The area is closed when it does not meet the approved growing area criteria, such as after a heavy rainfall. A prohibited area is closed at all times to shellfish harvesting.

Paramount to this study was the use of nucleic acid hybridization for virus detection. The probe used in this study was specific for poliovirus type 1. Additionally, cell culture was used to evaluate the presence of virus cultivatable using Buffalo Green Monkey Kidney cells (BGM). Due to the ability to cultivate non-poliovirus by cell culture, the two systems were not used for comparative reasons.
MATERIALS AND METHODS

Shellfish and Water Collection and Handling

Hardshell clams (M. mercenaria) for this study were harvested from Narragansett Bay, Rhode Island. Samples were collected at approximately one month intervals. Clams were obtained with a long handled shellfish-rake ("bull rake") from approved, conditionally approved, and prohibited waters and held in polypropylene bags on ice. Samples from each of the three collection areas were taken from approximately the same sites over the course of the study. Clams were not segregated by size prior to analyses; therefore, large sized and also those typically eaten raw ("little necks") were analyzed together. The clams were divided into two equal portions, one assayed for poliovirus, and the other assayed for male-specific bacteriophage (MSB) and other bacterial indicators. Surface water samples were obtained at each site when shellfish were harvested. Water samples were collected in sterile, 500 ml, polypropylene screw cap bottles (Nalgene Laboratories Inc., Rochester NY), that were held approximately 6 centimeters below the water surface. Following collection, bottles were capped and held on ice until examined in the laboratory. Water samples were analyzed for total coliforms and fecal coliforms.
Clam Microbiological Analyses

Approximately 10 clams were used in each analysis. Clams were scrubbed with a sterile brush, opened, and the entire contents (meat and liquor) were placed in sterile blender jars (Waring Corp., Corning, NY). Samples were blended at high speed for 2 min and held on ice (up to 60 min) until assayed.

Total and fecal coliforms. Total and fecal coliform densities in shellfish were determined by a most-probable-number (MPN) procedure, using lauryl tryptose broth (LST) (Difco) as the selective enrichment medium prescribed in Recommended Procedures (American Public Health Association 1970). Tubes were incubated for 48 hr at 35°C and checked at both 24 and 48 hr for the presence of growth in the fermentation tube.

All positive LST tubes were confirmed for coliforms by transferring a loopful of the gas positive tube to brilliant green lactose bile broth (BGLB). Tubes were incubated for 48 hr at 35°C, and examined for the presence of gas in the fermentation tube. The presence of gas was a confirmed test for the presence of coliforms.

All positive fecal coliforms were confirmed in EC-MUG medium (Difco) (Rippey et al. 1987). All tubes exhibiting gas in the LST tubes were transferred to EC-MUG tubes (EC medium containing 0.05 g MUG/l). Tubes
were incubated at 44.5°C for 24 hr. Positive and negative control cultures, *E. coli* and *K. pneumoniae* respectively were included. Following incubation, an ultraviolet light (UV) source (4 watt long wave lamp) was used to determine fluorescence. The control tubes were used to judge if a tube was positive or negative for fluorescence.

**Enterococci.** Enterococci densities were determined by a 5-tube MPN procedure, utilizing azide dextrose broth (Difco) as the selective enrichment medium. Confirmation of tubes exhibiting growth was carried out at 24 and 48 hr; all positive tubes were streaked onto membrane filters (HC filters; Millipore Corp., Bedford, MA) placed onto mE agar (Levin et al., 1975) as previously described (Dufour, 1980) with indoxyl-β-D-glucoside (Sigma, St. Louis, MO). The modified mE plates were incubated for 24 hr at 41°C, and tubes positive for enterococci were confirmed by the presence of blue growth along the agar streaks.

**C. perfringens.** The levels of *C. perfringens* in shellfish were determined by an iron milk MPN procedure (Abeyta, 1983). The iron milk assay was done as a 5 tube MPN procedure. Following inoculation the tubes were incubated for 16 to 18 hr at 45°C. The presence of classical stormy fermentation signifies a positive tube.
Male specific bacteriophage. Male specific bacteriophage levels were determined using a modified double-agar-overlay procedure previously described by Cabelli (1988). Approximately 35 g of homogenate was removed from the initial blend and centrifuged (9,000 \( \times \) g for 15 min at 3°C). Supernatants were immediately decanted into sterile, tared beakers, weighed and then warmed to 20 to 25°C. Briefly, the enumeration of the male-specific bacteriophage was performed as follows: The host culture used was streptomycin-resistant and harbors a conjugative plasmid mediating both ampicillin resistance and temperature-dependent pilus production (\( E. \) coli strain (HS[pFamp]RR). The host was grown at 35°C for approximately 4 hr in 10 ml of tryptone broth (tryptone, 10.0 g; NaCl, 5.0 g; and 1.0 liter of deionized water). Following the 4 hr incubation of the host \( E. \) coli, 0.3 ml was inoculated into 5 ml of sterile tempered (46°C) double strength (soft) top agar. Top agar was prepared as follows: tryptone, 20.0 g; dextrose, 2.0 g; NaCl, 10.0 g; Bacto-agar, 14.0 g; 1.0 ml of \( 1 \) M CaCl\(_2\) solution, and deionized water to 1 liter. Approximately 4 to 6 ml of the warmed supernate was then added and gently mixed, the mixture was then immediately poured onto the base (bottom) agar, and distributed evenly over the surface of the plate. The bottom agar was prepared as follows: tryptone, 10.0 g; dextrose, 1.0 g; NaCl, 5.0 g; Bacto-agar, 15.0 g; and 1 liter of deionized water, the medium was then sterilized, tempered to 46°C, and 50 mg each of streptomycin sulfate and
ampicillin were dissolved in the medium. The medium was dispensed into 150 x 15 mm petri dishes. Plaques were counted after 18 to 24 hr of incubation at 35°C. MSB densities were calculated per 100 g of shellfish determined by the number of plaques per volume of supernate assayed times the total volume of supernate obtained times 100 g, divided by the number of grams of homogenate examined.

Water Analysis

**Total and fecal coliforms.** Samples were analyzed utilizing a multiple-tube fermentation technique with lauryl tryptose broth as the selective enrichment medium (Difco), according to the Recommended Procedures (American Public Health Association, 1970). A 5-tube MPN procedure was used. Following inoculation, the tubes were incubated at 35°C and examined for the production of gas (gas in the fermentation tube) at both 24 and 48 hr. All tubes exhibiting gas production at the end of 24 and 48 hr were inoculated into confirmation media. The coliforms confirmed test utilized brilliant green lactose bile broth (BGLB) (Difco) and the fecal coliform confirmed test used EC broth (Difco). Each of the presumptive tubes exhibiting gas was gently shaken and a loopful of the broth was transferred to each of the two confirmation media. The BGLB tubes (coliform test) were then incubated 48 hr at 35°C. The EC tubes (fecal coliform test) were incubated for 24 hr at
44.5°C. At the end of the incubation periods all of the tubes were observed for the presence of gas in the fermentation tubes. The presence of gas indicates a confirmed positive and the absence of gas a confirmed negative. Results were reported using the Most Probable Number (MPN) index. Positive and negative controls were included in the testing procedure.

**Poliovirus Elution from Clam Meats**

A flow chart of the clam processing procedure for viral analysis is shown in Figure 1. Clams were scrubbed with a sterile brush, opened, and 200 g of meat was transferred to a stainless steel canister (Omni Corporation, Waterbury, CT). Two hundred ml of elution medium, consisting of 3% beef extract (Type V, BBL, Cockeysville, MD), 3.2% NaCl (Sigma) and 90 mM glycine (Sigma), at pH 9.5, was added to the sample (DeLeon et al. 1986). The sample was homogenized using a Omni-Gen homogenizer (Omni-Gen), the pH checked and adjusted to 9.5 with 1 N NaOH, and the samples were centrifuged (10,000 x g for 10 min) (Beckman model J2-21M, Fullerton, CA). The supernatant was decanted, pH adjusted to 7.0 with 1 N HCl, and the sample was divided into two aliquots. One aliquot (non-flocculated) was used for direct analysis by a hybridization probe and cell culture. The second aliquot was concentrated by acid precipitation (flocculated) (Katzenelson et al., 1976) prior to analysis by probe and cell culture techniques. The pellet
Hybridization Methodology

Clean and Shuck Clams

Homogenize in Beef Extract Eluate

Centrifuge and Retain Supernate

Divide Sample

Eluate

Organic Extraction

Assay by probe

Eluate

Flocculate

Cell Culture

Figure 1. Clam preparation for cell culture and nucleic acid hybridization
generated by flocculation was resuspended in 0.1 M Na$_2$PO$_4$ buffer at pH 9.5. The sample pH was checked and adjusted to 9.5 with 1 N NaOH, mixed for 5 min, and then centrifuged (10,000 x g for 10 min). The supernate was adjusted to pH 7.0 and the final volume adjusted to 30 ml.

**Phenol Chloroform Extraction of Viral Nucleic Acid**

Viral nucleic acid was liberated from both the flocculated and non-flocculated samples as follows; approximately 50 ml of the non-flocculated and 10 ml of the flocculated sample were individually mixed with phenol:chloroform:isoamyl alcohol (25:24:1). Samples were vortexed for 2 min, centrifuged (10,000 x g for 10 min), and the aqueous phase was removed and transferred to fresh phenol:chloroform:isoamyl alcohol. The original tube was extracted two more times by the addition of diethyl-pyrocarbonate (DEPC) treated water, and each time the aqueous phase was removed and transferred to new phenol:chloroform:isoamyl alcohol. The aqueous phases from the original tubes were extracted until there was a minimal amount of protein present. Residual phenol was removed by chloroform extractions followed by an ether extraction to remove the residual chloroform. Filtered air was passed through the sample using DEPC-treated pipet tips to evaporate off the remaining ether. The complete removal of the organics was important due to their incompatibility with the dot blot apparatus. The final sample volume was determined and a nylon membrane
was prepared for the dot blot procedure.

**Dot Blot Procedure**

The samples were applied to a positive charged nylon hybridization membrane, Genescreen Plus™ (DuPont, Boston, MA), using a vacuum manifold dot blot apparatus (Bio-Rad, Richmond CA). The membrane was wet with DEPC-treated water prior to securing it in the dot blot apparatus. One to three ml of extracted sample was applied per well, using DEPC-treated micropipets and a micropipetter. The membranes were baked in an 80°C incubator for 2 hr.

**Poliovirus Hybridization Probe Preparation**

*Poliovirus cDNA clone.* Fragments of poliovirus cDNA, from poliovirus cDNA (bp 115 - 7440) (kindly supplied by David Baltimore) cloned into the *Pst* 1 site of pBR322 and transformed in *E. coli* HB-101 were used as the probe.

The plasmid was amplified and isolated as follows. Initially, the *E. coli* HB-101 was streaked out onto Luria-Bertaini (LB) agar containing 12.5 μg/ml of tetracycline. Plates were incubated at 37°C for 24 hr. The plates were observed for colony morphology. Several colonies were then picked and transferred to another fresh LB agar plate with tetracycline and incubated until growth was observed. A plasmid mini-prep was then done on several
isolates to ensure the plasmid's presence.

**Plasmid mini-preparation.** The mini-prep was done as follows: 1) a colony was transferred to a sterile tube containing 10 µl of Tris-EDTA (TE), 2) 20 µl of cold lysozyme (50 mg/ml) was added and the sample was placed on ice for 10 min, 3) 20 µl of 10% sodium dodecyl sulphate (SDS) was added and the sample was gently mixed and held at 37°C until the cells lysed, and 4) the sample was centrifuged at 10,000 x g for 10 min, and the supernate was then ready for electrophoresis. A 0.7% agarose gel was prepared and loaded with the sample preparations, and pBR 322 without an insert. Following electrophoresis the gel was stained with ethidium bromide and viewed under UV light. The migration of the plasmid with and without the insert was used to identify the clone.

**Large scale plasmid preparation.** Using an isolate previously determined to contain the vector/insert, a large scale preparation was done. Two-hundred ml of LB broth containing 12.5 µg/ml of tetracycline was inoculated with a 24 hr culture of *E. coli* HB-101 that harbors the plasmid and incubated at 37°C for 24 hr with constant shaking. One-hundred ml of the overnight culture was inoculated into each of two flasks containing 900 ml of LB broth with tetracycline and incubated at 37°C for 18 hr. Following
incubation, the culture was transferred to 250 ml polypropylene bottles and the cells were harvested by centrifugation at 10,000 x g for 10 min. The supernates were decanted and each pellet was suspended in 10 ml of TE and transferred to a 50 ml centrifuge tube. The sample was centrifuged at 10,000 x g for 10 min and the supernate was decanted. The cells were resuspended in 6 ml of TE, added 0.6 ml of lysozyme (10 mg/ml), and held the sample on ice for 10 min. To each tube 1.5 ml of 0.25 M EDTA was added, the tube was gently mixed, and placed on ice for 10 min. Then 5 ml of 10% SDS was added to the tube and it was placed at 37°C until the cell "whiteness" disappeared. The sample was centrifuged at 15,000 x g for 15 min and the supernate was decanted into a clean tube. The supernate contains the plasmid that was further purified by ultracentrifugation.

Ultracentrifugation was done as follows: 1) to each ml of the plasmid supernate 1 g of cesium chloride was added and the sample was mixed by inversion and placed at 37°C until the cesium chloride dissolved, 2) the sample was transferred to a clean oakridge tube and centrifuged at 10,000 x g for 10 min, 3) the supernate was poured into a Beckman Ti 50 ultracentrifuge tube, heat sealed, and processes in a Beckman ultracentrifuge using a Beckman 80 ti rotor at 50k rpm for 24 hr.

After the sample was centrifuged, a UV light was used to locate the plasmid band. A 20-gauge needle was inserted into the top of the tube to allow air to enter, then a 20-gauge needle with a syringe attached was used to
collect the plasmid band. The band was transferred to a clean tube. Ethidium bromide was removed by mixing the sample with water saturated n-butanol. The mixture was mixed and then centrifuged at 3,000 x g for 5 min. The extraction procedure was repeated until there were no traces of ethidium bromide (pink color disappears from aqueous and organic phases).

**Dialysis.** The plasmid solution was then dialyzed according to Maniatis et al (1989). The dialysis tubing was placed in a beaker of 2% (w/v) sodium bicarbonate and 1 mM EDTA (pH 8.0) and boiled for 10 min. The tubing was then rinsed in distilled water and then boiled in 1 mM EDTA (pH 8.0). Prior to use, the tubing was rinsed in distilled water. The plasmid mixture was added to the treated dialysis tubing and was placed in TE buffer and continually mixed at 4°C for 24 hr. The TE buffer was changed twice over a 24 hr period.

**Ethanol precipitation.** After the sample was dialyzed, the plasmid was removed and placed in a sterile polypropylene tube (USA Plastics) and 3.0 M sodium acetate (pH 5.2) was added at 0.1 times the total plasmid volume and 95% ethanol was added at 2.5 times the volume of the plasmid mixture. The mixture was placed in a -20°C freezer for approximately 1 hr. For further processing the plasmid was recovered by transferring an aliquot to a microcentrifuge tube and centrifuged it at 12,000 x g (Beckman) for 15 min at
4°C. The supernate was carefully decanted and the pellet was washed with 70% ethanol followed by centrifugation at 12,000 x g for 10 min at 4°C. The supernate was decanted and the tube was kept open until all visible traces of ethanol were evaporated. The pellet was resuspended in sterile water (Milli-Q) for use in the restriction digest procedure.

**Restriction digest.** The insert was excised from the vector by restriction endonuclease cleavage using *Pst* 1 (Boehringer Mannheim Corp., Indianapolis, IN). There are three *Pst* 1 recognition internal recognition sites in addition to the sites where the plasmid and insert are ligated. Digestion with *Pst* 1 results in four poliovirus cDNA fragments of 4028, 1689, 1174, and 434 base pairs. Following digestion (according to the manufacturer’s instructions) the digest was electrophoresed in a 0.7% agarose gel, stained in a solution of ethidium bromide (0.5 μg/ml) and visualized by UV light. Two bands corresponding to poliovirus cDNA, the 1174 base pair and the 1689 base pair bands, were excised from the gel and placed in a sterile polypropylene tube. The bands were purified by electro-separation (Schleicher and Shuell, Keene, NH) according to the manufactures protocol. The eluate was removed from the electro-separation trap and concentrated by ethanol precipitation as previously described. These fragments were used as the probe in this study.
**Probe labeling.** The cDNA fragments were labeled with $^{32}$P dCTP using a random primer extension labeling kit (New England Nuclear, Boston, MA). Approximately 25 to 50 ng of the isolated poliovirus cDNA was used in the reaction. The poliovirus cDNA concentration was estimated by preparing dilutions of the purified cDNA and by electrophoresis, running these dilutions adjacent to the kit control DNA that was provided at a concentration of 10 ng/μl. A visual comparison of the control and poliovirus cDNA was then used to determine the approximate poliovirus cDNA concentration. The poliovirus cDNA was denatured by boiling for 5 min in a sterile polypropylene microfuge tube and then placed in an ice bath. The cDNA was combined with 6 μl of the 5X deoxynucleotide triphosphate mixture, 5 μl of $^{32}$P labeled dCTP, and water to bring the final reaction volume to 30 μl. The reaction was initiated by the addition of 1 μl of DNA polymerase I (klenow fragment) and incubated at room temperature for 1 hr. The reaction was terminated by the addition of 400 μl of reagent A (0.1 M Tris-HCl, 10 mM triethylamine, 1mM disodium EDTA, pH 7.7).

**Probe purification.** Nensorb™ cartridges (DuPont) were used to separate the labeled cDNA from the unincorporated nucleotides and other low molecular-weight materials. The column was activated by pushing through 2 ml of methanol followed by 2 ml of reagent A, using a 5 cc disposable plastic syringe. The sample / reagent A mix was then added and
pushed through the column. The sample was washed by pushing 3 ml of reagent A through the column, followed by 3 ml of sterile water. The probe, that was bound to the column matrix was then eluted off with Reagent B (50% propanol). The effluent was then collected in sterile microcentrifuge tubes.

_Probe specific activity determination_. Probe activity was determined by a scintillation counter. A 2 µl portion of the probe was placed in a scintillation vial and containing 5 ml of Scinti-Verse (Fisher) and counted with a scintillation counter. The specific probe activity (counts per minute (cpm)/ µg), was calculated by:

\[
\text{cpm/µg} = \frac{\text{cpm} \times \text{probe eluted volume}}{\text{volume counted} / \text{µg of DNA used}}
\]

Specific activities of \(1 \times 10^8\) to \(1 \times 10^9\) cpm/µg of DNA were obtained.

_Prehybridization_. Membranes containing the extracted nucleic acid samples were prehybridized in heat-sealed poly bags (Sears, Chicago, IL) as described in the Gene Screen Plus™ protocol (New England Nuclear, Boston MA). Prehybridization solution was prepared by combining 2 ml water, 5 ml deionized formamide, 2 ml of 50% dextran sulfate and 1 ml of 10% SDS in a 10-ml tube and heated to 42°C. Added 0.58 g of NaCl to the tube and the sample was mixed by inversion. Enough prehybridization solution was
added to cover the membrane and all air bubbles were removed prior to sealing the bag. The membranes were prehybridized at 42°C for 2 to 4 hr in a reciprocating water bath.

**Hybridization.** The upper corner of the prehybridization bag was cut and approximately $10^6$ to $10^7$ cpm of the heat-denatured radiolabeled probe was added. Excess air bubbles were removed and the bag was resealed. Hybridization was carried out under the same conditions for 36 to 48 hr. All of the samples to be assessed by dot blot analysis were prepared in this manner.

**Washing procedure.** The membranes were removed from the hybridization solution and washed twice in a solution of 2X sodium chloride / sodium citrate (SSC) 1% sodium dodecyl sulfate (SDS) for 15 min with constant agitation at room temperature, and once in a solution of 2X SSC / 0.1% SDS with constant agitation at 52°C for 30 min. The membranes were removed from the wash solution, air dried, and wrapped in saran wrap™.

**Autoradiography.** The membranes were carefully taped, sample side up, to a solid support and placed in a cassette with Dupont Cronex intensifying screens. In a darkroom, a sheet of Kodak XAR-5 film was placed
over the membranes. The cassette was incubated at -70°C for 36 hr. The cassette was removed and held at room temperature to equilibrate. The film was removed and developed in a darkroom under safelights according to the manufacturer's instructions (Kodak).

**Cell Culture Analysis.**

The viral assay was performed using a continuous cell line of Buffalo Monkey Green Kidney (BGM) cells (supplied by C. P. Gerba, University of Arizona) Cells were grown in minimal essential media (MEM) (Sigma) supplemented with 8% fetal calf serum, 292 mg/l glutamine, 0.075% sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml kanamycin and 25 U/ml mycostatin.

Prior to each viral assay the cells were prepared by splitting confluent flasks and seeding new flasks. The cells were split by: 1) decanting the growth medium, 2) washing the cell monolayer with phosphate-buffered saline (PBS) (Sigma), 3) adding 1X trypsin (Sigma) solution to the monolayer and placing it in a 37°C incubator for 2 min, and 4) removing the trypsin by aspiration and the cells were viewed with an inverted scope. If necessary, the cells were placed back into the incubator until cells rounded-up and dissociated from the flask. Once the cells were dissociated, fresh medium was added and the cells were diluted 1:4. Once the cells reached confluency, they were used in the shellfish assay.
Three 75 cm² tissue culture flasks with confluent monolayers of BGM cells were each inoculated with 3 ml of the flocculated sample. The sample was prepared for cell culture by adding an equal volume of ethyl ether. The mixture was shaken and left to settle. The aqueous phase was removed and placed into a sterile petri plate. The samples were placed in a laminar flow hood and the ether was given time to evaporate. Adsorption of virus was allowed to proceed for 2 hr at 37°C, with rocking every 15 min. Following adsorption, the cells were washed with PBS and overlaid with maintenance medium containing 2% fetal calf serum. Flasks were incubated at 37°C and examined periodically for the presence of cytopathic effects (CPE), up to 14 days following inoculation. Flasks that exhibited CPE were confirmed by passage to new monolayers of BGM cells and observing for CPE.
RESULTS

Samples of surface waters and clams from Narragansett Bay were obtained during the period of July 1989 to May 1990. A total of 9 collection trips were made for each of the three different shellfish classification areas. The microbiological quality of clams and their overlying waters from the approved area are presented in Table 1 from the conditionally approved area in Table 2 from the prohibited area in Table 3.

Water from the approved area exceeded the total coliform standard (70/100 ml) once (April), and the fecal coliform standard (14/100 ml) was exceeded in another collection (May). Water quality for the majority of the conditionally approved area samples exceeded the coliform and fecal coliform levels found in the approved area (note: this area was conditionally closed during all but the 8/30/89 sample collection). From the conditionally approved area, 6 of 9 water samples exceeded the total coliform standard for approved areas, whereas 5 of 9 samples exceeded the fecal coliform standard. Coliform and fecal coliform MPNs from the prohibited area were greater than levels found for both the approved and conditionally approved areas. In the prohibited area 8 of 9 of water samples exceeded the coliform standard, and 7 of 9 samples exceeded the fecal coliform standard. One of 8 clam samples obtained from the approved area and the conditionally approved area

63
exceeded the fecal coliform market guideline (230/100 g), whereas 2 of 8 prohibited area samples exceeded the guideline.

*Clostridium perfringens* was detected in the prohibited area with the greatest frequency, and levels there remained detectable throughout all the collection times. Overall, levels of *C. perfringens* were seen to decrease from the prohibited to conditionally approved to the approved area, and the last exhibited the most samples with levels in clams below the detectable limits. Enterococci densities were generally below detectable levels throughout all the collection times in all the areas. The highest levels of enterococci were detected in clams from the prohibited area, and densities appeared to decrease in clams from the conditionally approved area. No enterococci were detected in any clam samples from the approved area.

The occurrence of MSB and results obtained for poliovirus in the approved area are shown in Table 1, Table 2 shows the results for the conditionally approved area, and those obtained for the prohibited area given in Table 3. Phage levels detected were greatest in the prohibited area, and these were notably higher than those detected in the conditionally approved area; the lowest levels detected were found in the approved area. MSB in clams (per 100 g) were detected in 7 of 8 samples examined from the approved area. In the conditionally approved and prohibited area MSB (per 100 g) were detected in all samples analyzed.

In the approved area, hybridization probe results for poliovirus for
both the non-flocculated and flocculated sample portions, were negative at all times. In the conditionally approved area, 3 of 9 non-flocculated clam samples were positive for viral nucleic acid, by probe analysis, whereas none of the flocculated samples were found to be positive. In the prohibited area, 4 of 9 non-flocculated samples were found positive by the probe assay, but only 2 of these 4 were positive using the flocculated samples. Table 4 lists the number of wells to which sample was applied for the hybridization assay and the number of wells that were positive.

Cell culture analyses of clam samples for poliovirus were performed using the flocculated portions only, since the non-flocculated portions were toxic to the BGM cells. Cell culture results of the clams from the approved and conditionally approved area failed to detect cytopathic effects (CPE) with any of the samples. One of the 9 samples from the prohibited area was found to cause CPE on the BGM cells.
TABLE 1. Microbial levels in water and clams from the approved area. Water was tested for coliforms only. Clams were tested for bacterial indicators, male specific bacteriophage (MSB) and poliovirus.

<table>
<thead>
<tr>
<th>Date</th>
<th>coliforms&lt;sup&gt;a&lt;/sup&gt;</th>
<th>coliforms&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Clostridium perfringens&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Enterococci&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MSB&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Probe Eluate</th>
<th>Floc</th>
<th>Cell Culture Eluate</th>
<th>Floc</th>
</tr>
</thead>
<tbody>
<tr>
<td>7/31/89</td>
<td>&lt;1.8</td>
<td>&lt;1.8</td>
<td>nd&lt;sup&gt;d&lt;/sup&gt;</td>
<td>nd</td>
<td>nd</td>
<td>-</td>
<td>-</td>
<td>na&lt;sup&gt;e&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>8/30/89</td>
<td>&lt;1.8</td>
<td>&lt;1.8</td>
<td>20</td>
<td>20</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>na</td>
<td>-</td>
</tr>
<tr>
<td>10/3/89</td>
<td>&lt;1.8</td>
<td>&lt;1.8</td>
<td>110</td>
<td>110</td>
<td>200</td>
<td>&lt;20</td>
<td>15</td>
<td>na</td>
<td>-</td>
</tr>
<tr>
<td>11/7/89</td>
<td>49</td>
<td>7.8</td>
<td>68</td>
<td>68</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>127</td>
<td>na</td>
<td>-</td>
</tr>
<tr>
<td>1/10/90</td>
<td>2</td>
<td>2</td>
<td>20</td>
<td>20</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>7</td>
<td>na</td>
<td>-</td>
</tr>
<tr>
<td>2/12/90</td>
<td>9.3</td>
<td>2</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>14</td>
<td>na</td>
<td>-</td>
</tr>
<tr>
<td>3/13/90</td>
<td>&lt;1.8</td>
<td>&lt;1.8</td>
<td>&lt;20</td>
<td>20</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>15</td>
<td>na</td>
<td>-</td>
</tr>
<tr>
<td>4/16/90</td>
<td>130</td>
<td>2</td>
<td>78</td>
<td>20</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>&lt;16</td>
<td>na</td>
<td>-</td>
</tr>
<tr>
<td>5/31/90</td>
<td>39</td>
<td>17</td>
<td>1,300</td>
<td>140</td>
<td>2,200</td>
<td>&lt;20</td>
<td>83</td>
<td>na</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>MPN/100 ml water.
<sup>b</sup>MPN/100 g clam meat.
<sup>c</sup>Densities per 100 g calculated from plaque counts.
<sup>d</sup>Not determined
<sup>e</sup>Not analyzed due to toxicity.
TABLE 2. Microbial levels in water and clams from the conditional approved area. Water was tested for coliforms only. Clams were tested for bacterial indicators, male specific bacteriophage (MSB) and poliovirus.

<table>
<thead>
<tr>
<th>Date</th>
<th>Water</th>
<th>Clams</th>
<th>Poliovirus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Fecal</td>
<td>Total</td>
</tr>
<tr>
<td>7/31/89</td>
<td>4.5</td>
<td>&lt;1.8</td>
<td>nd</td>
</tr>
<tr>
<td>8/30/89</td>
<td>240</td>
<td>49</td>
<td>78</td>
</tr>
<tr>
<td>10/3/89</td>
<td>33,000</td>
<td>2,300</td>
<td>5,400</td>
</tr>
<tr>
<td>11/7/89</td>
<td>79</td>
<td>11</td>
<td>490</td>
</tr>
<tr>
<td>1/10/90</td>
<td>2</td>
<td>2</td>
<td>&lt;20</td>
</tr>
<tr>
<td>2/12/90</td>
<td>920</td>
<td>220</td>
<td>&lt;20</td>
</tr>
<tr>
<td>3/13/90</td>
<td>&lt;1.8</td>
<td>&lt;1.8</td>
<td>&lt;20</td>
</tr>
<tr>
<td>4/16/90</td>
<td>2,400</td>
<td>790</td>
<td>68</td>
</tr>
<tr>
<td>5/31/90</td>
<td>79</td>
<td>33</td>
<td>790</td>
</tr>
</tbody>
</table>

*MPN/100 ml water  
*MPN/100 g clam meat  
*Densities per 100 g calculated from plaque counts  
*Not determined  
*Not analyzed due to toxicity
TABLE 3. Microbial levels in water and clams from the prohibited area. Water was tested for coliforms only. Clams were tested for bacterial indicators, male specific bacteriophage (MSB) and poliovirus.

<table>
<thead>
<tr>
<th>Date</th>
<th>Water coliforms*</th>
<th>Water coliforms(b)</th>
<th>Clams</th>
<th>Clams</th>
<th>Clams</th>
<th>Clams</th>
<th>Poliovirus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Fecal</td>
<td>Total</td>
<td>Fecal</td>
<td>Total</td>
<td>Fecal</td>
<td>Probe</td>
</tr>
<tr>
<td>7/31/89</td>
<td>&gt;1,600</td>
<td>1,600</td>
<td>5,400</td>
<td>nd(d)</td>
<td>5,400</td>
<td>460</td>
<td>3,042</td>
</tr>
<tr>
<td>8/30/89</td>
<td>&gt;1,600</td>
<td>&gt;1,600</td>
<td>9,200</td>
<td>1,100</td>
<td>2,400</td>
<td>230</td>
<td>465</td>
</tr>
<tr>
<td>10/3/89</td>
<td>49,000</td>
<td>2,300</td>
<td>&gt;16,000</td>
<td>3,500</td>
<td>3,500</td>
<td>3,500</td>
<td>5,078</td>
</tr>
<tr>
<td>11/7/89</td>
<td>1,100</td>
<td>170</td>
<td>790</td>
<td>220</td>
<td>490</td>
<td>230</td>
<td>1,027</td>
</tr>
<tr>
<td>1/10/90</td>
<td>49</td>
<td>6.8</td>
<td>20</td>
<td>&lt;20</td>
<td>140</td>
<td>&lt;20</td>
<td>1,036</td>
</tr>
<tr>
<td>2/12/90</td>
<td>130</td>
<td>2</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>110</td>
<td>&lt;20</td>
<td>994</td>
</tr>
<tr>
<td>3/13/90</td>
<td>490</td>
<td>130</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>78</td>
<td>&lt;20</td>
<td>2,700</td>
</tr>
<tr>
<td>4/16/90</td>
<td>22,000</td>
<td>2,300</td>
<td>1,700</td>
<td>45</td>
<td>330</td>
<td>140</td>
<td>1,180</td>
</tr>
<tr>
<td>5/31/90</td>
<td>350</td>
<td>49</td>
<td>2,200</td>
<td>93</td>
<td>3,500</td>
<td>&lt;20</td>
<td>124</td>
</tr>
</tbody>
</table>

*MPN/100 ml water  
\(b\)MPN/100 g clam meat  
\(c\)Densities per 100 g calculated from plaque counts.  
\(d\)Not determined  
\(e\)Not analyzed due to toxicity
<table>
<thead>
<tr>
<th>Month</th>
<th>Approved Area</th>
<th>Conditional Approved Area</th>
<th>Prohibited Area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eluate</td>
<td>Floc</td>
<td>Eluate</td>
</tr>
<tr>
<td>7/89</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8/89</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10/89</td>
<td>0</td>
<td>0</td>
<td>2 of 30</td>
</tr>
<tr>
<td>11/89</td>
<td>0</td>
<td>0</td>
<td>1 of 24</td>
</tr>
<tr>
<td>1/90</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2/90</td>
<td>0</td>
<td>0</td>
<td>1 of 33</td>
</tr>
<tr>
<td>3/90</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4/90</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5/90</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 4.** Ratio of the number of positive dot blot wells over the total number of wells to which sample was applied.
DISCUSSION

Periodic outbreaks of non-bacterial gastroenteritis and hepatitis A infections have indicated that the current means of evaluating the sanitary quality of shellfish and their harvesting waters requires reevaluation. This study compared several bacterial indicators and MSB to the occurrence of poliovirus in shellfish collected from approved, conditionally approved, and prohibited areas over about a one-year period. Poliovirus was used because of a recently developed nucleic acid probe technique, its ease of detection by cell culture techniques, and the generally higher degree of poliovirus prevalence in sewage, relative to that expected for other enteric viruses. Earlier studies, (Margolin, unpublished results), demonstrated that this hybridization probe assay was able to detect virus with a sensitivity comparable to cell culture.

The clam eluate portion could be assayed directly for poliovirus by nucleic acid hybridization. This could not be done with cell culture due to the toxic effects of the non-flocculated portion on BGM cells. Consequently, all samples evaluated by cell culture were further processed by flocculation of the sample. Although flocculation adequately reduced the sample toxicity, it also provided results with a reduced level of detectable poliovirus, indicating the procedure was not 100% efficient for poliovirus recovery. Water samples were analyzed for coliforms only, as this currently is a monitoring tool in
determining the sanitary quality of shellfish-harvesting areas.

As expected, the approved area exceeded the coliform standard the least number of times. Results for the conditionally approved area show a wide variation in coliform levels, likely due to the effects from rainfall events that occurred prior to many of the collection periods. During most of the sample collections (all collection times except 8/89), the area was temporarily (conditionally) closed due to excess rainfall. Samples taken from the prohibited area usually exceeded the coliform standard. However, in one instance (January), coliform levels detected in waters from the prohibited area were found to be acceptable, while the shellfish non-flocculated portion was positive for poliovirus by the hybridization probe. The advent of molecular detection techniques has greatly enhanced our ability to detect specific types of bacteria and viruses. Future modifications for probe procedures should focus on minimizing virus loss during sample processing. Since viruses appear to have a longer survivability time at lower temperatures than do vegetative bacteria, and since standards based on levels of bacteriophage do not exist, it seems advisable to verify the sanitary conditions of shellfish, particularly those in conditionally managed areas, using a multifaceted approach, which includes assays for viruses.

*Clostridium perfringens* is a spore-forming, obligate anaerobe. This organism is widespread in the environment and is not solely of fecal origin, although its presence has been associated with the presence of feces and
wastewater (Bisson and Cabelli, 1980). Compared to the coliform bacterial indicators, *C. perfringens* spores have a significantly longer survival time in the estuarine environment and are less susceptible to environmental stresses. The levels of *C. perfringens* spores in the prohibited area were markedly higher than those found in the conditionally approved and approved areas. Compared to the coliforms and enterococci, *C. perfringens* levels are less severely affected during the colder months in the conditionally approved and prohibited areas. In the approved area *C. perfringens* levels were similar to the coliform indicators, in that levels were undetectable during the colder months (November - March). While levels of *C. perfringens* spores were detectable more often than the coliforms or enterococci, there is no reliable relationship between *C. perfringens* and the occurrence of human enteric viruses.

Matches and Liston (1974) and Smith (1975) further expressed doubt that it could be used as an effective indicator because 1) it is so persistent that *C. perfringens* may be difficult to index to current pollution conditions, 2) it is widely distributed in soils and sediments, and 3) it is carried into shellfish-growing waters by stormwater runoff. Lastly, *C. perfringens* spores survive too well for application to shellfish-growing and recreational waters where the benthic location of the resource or the potential for resuspension of benthic sediments is a factor (Cabelli, 1982).

Enterococci levels were significantly lower than those found for
coliforms, *C. perfringens* and male-specific bacteriophage. In the approved area, enterococci densities were below the assay detection limit at all times. The lower levels of enterococci determined in this study are likely reflective of the lower numbers found in wastewater effluent. Cabelli et al. (1983) indicated that enterococci levels were a better indicator than fecal coliforms or *E. coli*. The data obtained from this study suggest that enterococci levels would no further ensure the sanitary quality of shellfish than the present coliform system. There are several items that need to be addressed to better determine the validity of enterococci as an indicator: 1) improved methods for stressed cells, 2) improved specificity for various enterococci spp. and streptococci spp., 3) reduce the incidence of false positives, which can vary with temperature, season and geographic location, and 4) minimize background growth (NAS, 1991).

The hybridization probe assay to detect poliovirus was performed with both the non-flocculated and flocculated sample portions. Non-flocculated portions were found to be positive more often than the flocculated samples, and in no instances were flocculated samples found positive where non-flocculated portions were negative. This indicates that the flocculation procedure is not 100% efficient in recovery of virus particles, resulting in loss of sensitivity. Considering the low level of viral contamination expected in the clams, it appears preferable to rely on results from non-flocculated portions to yield the greatest degree of sensitivity.

73
All clam samples were assayed for cultivatable virus by cell culture using BGM cells. Comparison of the hybridization probe and cell culture results for the non-flocculated portion was not possible, due to sample toxicity on BGM cell monolayers. Therefore, only the flocculated portions of the clam samples were examined by cell culture. Flocculation followed by resuspension in phosphate buffer was sufficient in detoxifying the samples for cell culture analysis. In the approved area none of the flocculated samples were positive by cell culture; this correlates with the hybridization probe results for this area. Similar results were obtained for flocculated samples from the conditionally approved area, in that all cell culture and hybridization probe assay results for poliovirus were negative, although three non-flocculated probe samples from this area were positive. In the prohibited area, there was only one sample (January) that showed CPE in cell culture; this sample was probe-positive with the non-flocculated sample portion, but not with the flocculated portion.

One explanation for the discrepancies between tissue culture and probe analysis would be that the lower virus concentrations in the flocculated sample were below the limits of detection for the nucleic acid hybridization assay. Another possibility was that a poliovirus-specific probe was used in the hybridization technique and the cell culture assay could detect other cultivatable viruses (other than just poliovirus).

MSB have received consideration as an indicator of enteric viral
pathogens. Though not regularly detected in fresh human fecal material, this group of bacterial viruses is consistently present in sewage and sewage-polluted waters (Debartolomeis and Cabelli 1991). Also, some members of the MSB group have been shown to be as resistant to disinfection by chlorination, such as Norwalk virus (Keswick et al. 1985).

Bacteriophage are appealing as an indicator due to the ease of their detection, low analytical cost, and short assay period as compared to direct enteroviral methods. On the other hand, Gerba (1987) expressed concern over the lack of basic data describing ratios of specific coliphages to viral pathogens and the occurrence, persistence, and seasonal stability of coliphage in shellfish-growing waters. Another possible limitation concerns their comparative occurrence in feces and sewage. MSB are an infrequent component of human (and some animal) feces and occur at densities significantly larger in sewage (Furuse, 1987 and Havelaar, 1987). Their abundance in sewage treatment plants may be a consequence of phage multiplication at environmental temperatures on bacterial hosts that form pili at temperatures above 30°C (Havelaar, 1987). Therefore, although the phage may serve as an indicator of wastewater and sewage contamination, its use as an indicator of fecal contamination or a predictor of health risk in shellfish-growing waters requires careful analysis.

MSB were the only indicators in this study consistently detected at all of the collection sites. However, MSB were present in all waters, including
approved waters. Their wide-spread presence would prevent their use in a simple presence/absence test, since this would probably result in the unnecessary closure of safe shellfish beds. Further data are needed to determine if there is a correlation between certain levels of MSB and the presence of human enteric viruses, and to establish a predictive index and protective MSB standards. Such studies are essential before MSB can be considered as an indicator organism without excessively restricting shellfish waters.

In summary, the indicators in this study exhibit widely ranging results. Enterococci exhibited the lowest numbers throughout all of the collection sites and periods, followed by the fecal coliforms and total coliforms. These vegetative bacterial indicators are greatly affected by environmental stresses, such as water temperature, low nutrients, and salinity. *C. perfringens* and the MSB were present at greater frequencies than the coliform and enterococci groups, with MSB being detected more often and at greater levels than any of the other indicators.

The use of hybridization probes in this study demonstrated an alternative technique to detect the presence of enteric viruses in clams. Even so, the direct detection of viral nucleic acid by probe analysis appears to require improvements in its detection limits. Detection of low levels of viruses in the shellfish was compounded by the low efficiency of viral recovery from clam meats. Additionally, the hybridization procedure used in
this study was, time-consuming and used harmful substances.

The results of this study support those reported by others (Berg et al., 1978, Portnoy et al., 1975, and Metcalf and Stiles, 1968) which suggest that bacterial indicators and standards while serving to adequately protect against bacterial pathogens in shellfish, do not reliably predict the presence of human enteric viruses. The development of better extraction procedures and rapid, inexpensive, automated molecular techniques may soon allow for the direct detection of most if not all pathogens potentially present in shellfish. Consequently, a more complete approach in evaluating shellfish sanitary quality and the safety of shellfish is currently needed. This would include indicator organisms along with analyses to detect certain viral pathogens directly using a molecular based assay.
REFERENCES


Cabelli, V. J. 1988. Microbial indicator levels in shellfish, water, and sediments from the upper Narragansett Bay conditional shellfish growing area. Report to the Narragansett Bay Project, Providence, RI.

78


81
CHAPTER THREE

DEVELOPMENT OF A T7 POLYMERASE-BASED AMPLIFICATION SYSTEM AND A NUCLEIC ACID CAPTURE TECHNIQUE TO MONITOR ENVIRONMENTAL SAMPLES FOR HUMAN ENTERIC VIRUSES

ABSTRACT

The development of a rapid and sensitive method to isolate and detect human enteric viruses is essential to identify the etiological agent of foodborne and waterborne outbreaks of viral origin. Additionally, the application of a molecular technique as a surveillance tool could help circumvent these outbreaks. The level of human enteric viruses in the environment is generally regarded as low. Nucleic acid hybridization techniques have been used, but drawbacks such as the use of a radioactive isotope, organic solvents, and sensitivity have limited its acceptance. Reverse transcription - polymerase chain reaction (RT-PCR) has been employed in the detection of enteroviruses in water and shellfish. Two obstacles in using RT-PCR directly are enzymatic inhibitors inherent in environmental samples and the amount of a sample that can be analyzed. Reported here is the development of two alternative molecular tools for virus detection. The first was the construction of a T7 polymerase-based amplification sequence to
indirectly amplify target RNA without the use of reverse transcriptase. The T7 amplification system was designed with a viral recognition sequence, a T7 polymerase recognition sequence and a detection sequence for the generation of RNA transcripts. The T7 amplification sequence proved to be effective in generating radiolabeled RNA transcripts. The second technique was the development of a nucleic acid capture technique that would selectively isolate target nucleic acid from an aqueous mixture. A biotinylated capture sequence was used to hybridize to the target viral nucleic acid. Streptavidin-coated magnetic beads were then added to isolate the RNA-oligonucleotide hybrid. This procedure allowed for the recovery of viral RNA suitable for amplification by RT-PCR. Known quantities of poliovirus type 1 were seeded into purified water, recovered and amplified using RT-PCR. The techniques outlined here, although developed using poliovirus, should be equally applicable to detection of other human enteric viruses.
INTRODUCTION

By 1952 the chemical structures of both classes of nucleic acids, RNA and DNA had been established. In 1953 Watson and Crick proposed a double helix model for the structure of DNA. This model was the beginning of an explosion of knowledge concerning gene structures, functions, regulations, and evolution at the molecular level (Stansfield et al., 1996). Molecular techniques have become the focus of many virus detection assays. Many of these assays are designed to detect a virus based on the presence of a specific nucleic acid sequence.

The remarkable chemical stability of nucleic acids relative to protein targets contributes to the efficacy of these nucleic acid detection systems. Temperatures as high as 100°C, mixtures of organic solvents, chaotropic salts, and concentrated base solutions are commonly employed in probe assays or in the sample preparation steps used to liberate and make the nucleic acids available to assay (Parsons, 1988).

Hybridization assays have permitted the detection of human enteric viruses in environmental samples (Shieh et al., 1991, Speirs et al., 1987, and Jiang et al., 1987). However, to attain the required sensitivity, probes were usually labeled with a radioactive isotope ($^{32}$P). The half-life of $^{32}$P is two
weeks and therefore, it must be continually produced to maintain the desired sensitivity. Moreover, to attain optimal sensitivity, the probe had to be labeled so heavily with the isotope, that it had to be used on the day it was prepared for optimal results. Fragmentation of the probe occurs as the constituent $^{32}$P atoms undergo their decay. This probe fragmentation can lead to higher backgrounds and decreased sensitivity (Parson, 1988). Furthermore, the use of $^{32}$P and other isotopes has limited the assay to laboratories licensed to work with radioactive elements.

Even with the use of a radioactive reporter molecule, hybridization assays have a reported detection limit in water of $10^4$ virus particles / ml, which is not sensitive enough for low level virus contamination (Bej, 1995). Due to the requirement of a more sensitive assay, nucleic acid hybridization was replaced with nucleic acid amplification techniques.

The best known nucleic acid amplification system is the polymerase chain reaction (PCR) developed by scientists at the Cetus Corporation (Saikai et al., 1985). PCR involves the direct amplification of selected target molecules using complementary primers and a polymerase that catalyzes the formation of DNA. Under optimal conditions, the theoretical yield from a polymerase chain reaction would be a millionfold amplification with 20 cycles. PCR has been used to detect viruses in shellfish (Atmar et al., 1993, and Lees et al., 1994) and in water (Abbaszadegan et al., 1993, and Ma et al.,
On the other hand, a disadvantage of PCR is that it was designed to amplify DNA and not RNA. The nucleic acid of enteric viruses is RNA. The protocol for the detection of an RNA virus by PCR first requires the conversion of RNA to cDNA. The production of cDNA is a separate reaction that uses reverse transcriptase. Conventional reverse transcriptase (RT) reactions are fastidious and use enzymes that cannot tolerate temperatures above 42°C, which is a nonstringent hybridization temperature for most primers (Podzorski and Persing, 1995). The presence of secondary and tertiary structures in single stranded RNA that are not disrupted at the RT incubation temperature can result in widely varying efficiencies of conversion.

Additionally, the sample size that can be accommodated in a reverse transcription reaction is small. In a standard RT reaction, only 3 μl of a sample can be used. The reaction can be scaled-up, but there is also a risk of increasing the level of enzymatic inhibitors inherent in many environmental samples.

PCR is not the only amplification system available. Several techniques have been described that use other amplification approaches, such as the ligase chain reaction (LCR) (Barany, 1991), transcriptional-based amplification system (Kwoh et al., 1989), and strand displacement amplification (SDA) (Walker et al., 1992).

The first non-PCR nucleic acid amplification technique was the
transcription-based amplification system (TAS) developed by Kwoh et al. (1989). This was a multi-enzyme system that incorporated T7 polymerase. This technique was successfully applied to the detection of human papilloma virus (Brown and Wortman, 1993), human immunodeficiency virus (HIV) (Gingeras et al., 1991), and C. trachomatis, (Haydock and Kochik, 1990). Milligan et al, (1987) demonstrated a 1500 times increase in a 93-base single-stranded template that contained a T7 polymerase recognition sequence.

There is much room for improvement in RNA detection by molecular techniques. Several areas that need to be explored are: 1) use of a nucleic acid-based amplification system for RNA targets that is not dependent on reverse transcriptase and is amenable to non-isotopic reporter molecules, 2) an improved method to isolate and concentrate the viral target in an environmental sample, and 3) a method to remove the enzymatic inhibitors inherent in many environmental samples.

What is reported here is the development of two molecular tools to enhance the recovery and detection of human enteric viruses in environmental samples. The first tool was the development of a T7 amplification sequence. This system is based on the ability of T7 polymerase to produce numerous RNA transcripts from a single template, hence, amplifying a specific segment of nucleic acid (Gross et al., 1992 and Jorgensen et al., 1991). This system uses an indirect amplification approach. Here, the target viral nucleic acid is not amplified; instead there is amplification of a
specifically designed sequence that is part of the overall amplification machinery. For this to occur, three distinct nucleic acid sequences were brought together in the following manner. The first nucleic acid sequence was a single strand of DNA that is complementary to the target viral nucleic acid and is known as the viral recognition sequence. The second section of nucleic acid is double stranded and contains the T7 polymerase promoter sequence. The third segment of nucleic acid is the detection sequence that serves as the template for the production of RNA transcripts. A diagram of the T7 amplification sequence components is shown in Figure 1, and a diagram of the way it would appear in a reaction is shown in Figure 2.

The premise of the T7 amplification sequence was based on the ability to assemble segments of nucleic acid end-to-end into a multi-functional unit. There are many benefits to this assay system: 1) indirect amplification of the viral genome would circumvent the need to produce cDNA by the reverse transcriptase reaction, 2) increased hybridization temperature using a longer capture sequence would increase specificity and disrupt secondary genomic structures, 3) multiple amplification sequences could be prepared within a single genome, and 4) non-isotopic labels (biotin, branched biotin, or alkaline phosphatase) can be directly incorporated into the RNA transcripts to further enhance the sensitivity, without the problems associated with radioactive isotopes.
Figure 1. Schematic representation of the major components contained in the T7 amplification sequence. Outer most extremes show vector component.
CAPTURE AND AMPLIFICATION OF VIRAL NUCLEIC ACID

Figure 2. Schematic representation of the T7 reaction showing the biotinylated capture sequence and the T7 amplification sequence. Note, the amplification procedure was also done using RT-PCR following capture of the target nucleic acid.
Lastly, the design of the T7 amplification sequence made it amenable to change. Restriction endonuclease recognition sites were included so segments could be easily replaced. Therefore, the production of various virus recognition sequences within a single genome would serve to increase the sensitivity of an assay.

The second part of this investigation was the development of a nucleic acid capture sequence. The capture sequence was designed to: 1) facilitate the recovery of a targeted nucleic acid from a sample matrix, 2) permit the analysis of a larger sample volume, 3) provide an environment for optimal hybridization and, 4) permit a washing step to remove any enzymatic inhibitors.

A novel means of recovering a target nucleic acid is by biomagnetic technology. Magnetic capture of viral nucleic acid has been used in clinical diagnostics (Muir et al., 1993, Nicholson et al., 1994, and Van Doom et al., 1994) but has not been developed for environmental assays. For this component, a nucleic acid capture sequence was determined from a complementary region of the poliovirus genome (Racaniello and Baltimore, 1981, and DeLeon et al., 1990). This procedure incorporates three main components: 1) synthetic biotinylated oligonucleotide complementary to the viral target sequence, 2) streptavidin-coated magnetic beads, and 3) a magnetic particle concentrator (MPC).

A schematic diagram of the capture reaction is shown in Figure 3. A
The target RNA is hybridized in solution with a biotinylated capture sequence and then mixed with the streptavidin (SA) magnetic beads. The product can then be concentrated and washed by using a magnetic particle concentrator (MPC).
brief description of the reaction procedure is as follows. The target nucleic acid is liberated (using heat, proteinase K, chaotropic salt, organic extraction etc.) so it is available for hybridization. The target nucleic acid is captured with a complementary sequence biotinylated at the 5' end. The biotin is separated from the DNA backbone by a linker arm to minimize steric hindrance during the subsequent hybridization procedure. Following hybridization, streptavidin-coated superparamagnetic beads are added to the reaction mixture (biotinylated capture sequence and target). The streptavidin beads and the hybridized complex are mixed to permit the formation of the streptavidin-biotin bond. The resulting complex is immobilized by placing the reaction tube in a MPC rack. The rack contains a magnet that draws the complex to the sidewall (Figure 3) and immobilizes it. The supernate is removed while the microfuge tube is held in the rack. The beads can readily be washed by: 1) adding a wash buffer, 2) removing the tube from the rack and resuspending the beads, and 3) placing the tube back into the MPC rack to immobilize the beads and remove the supernate. After the final wash, the beads are resuspended in the desired buffer for subsequent testing.

In summary, the magnetic isolation procedure is based on the principle that the target nucleic acid to be isolated is specifically bound to the streptavidin superparamagnetic beads. The bound nucleic acid can easily and specifically be removed from a heterogeneous sample mixture by inserting the test tubes containing the target material and the beads into an MPC rack.
This research describes the development of a T7 polymerase-based amplification sequence and a biotin-streptavidin based nucleic acid capture technique. These techniques should be beneficial in the detection of enteric viruses in environmental samples.
MATERIALS AND METHODS

There are two separate components described in the materials and methods section that follows. First is the development of a T7 amplification sequence and is identified as in this section as Part I. The second component is the development of a nucleic acid capture technique, and is identified in this section as Part II.

Part I: Development of a T7 Amplification Sequence

In the development of the T7 amplification sequence, several modifications were incorporated into its design over time. The modifications were implemented to facilitate its development and to circumvent some of the inherent difficulties of synthetic DNA. What follows in this section is the chronological progression of the T7 amplification sequence development. To facilitate the discussion of the T7 sequence, the three major modifications that resulted in overall changes in the assay system are referred to as the, first, second, and third generation T7 amplification sequence. The first generation being the initial attempt and the third generation represents the final product.

There were two primary phases in development of the completed T7 amplification sequence. Phase-I was the construction of the viral recognition sequence and the T7 polymerase recognition sequence. The nucleic acid in
phase-I was produced by a DNA synthesizer. Phase-2 was the production of the detection sequence (template used to generate the RNA transcripts). The detection sequence was generated by PCR using poliovirus cDNA as the template for PCR. Figure 4 illustrates the final construction procedure.

**First Generation T7 Amplification Sequence**

As mentioned previously, synthetic oligonucleotides were used to produce the viral recognition and T7 polymerase recognition sequence. The oligonucleotides were provided by Mary Trucksess, FDA, Washington, DC. The sequence of the four primers is outlined in Table 1. The complementary sequences were of different lengths and once annealed would have single stranded cohesive termini. Figure 5 shows the orientation of the synthesized oligonucleotides. Oligo-1 and oligo-2 are complementary and when they are annealed they have single-stranded cohesive termini (Sal I sequence) adjacent to the viral recognition sequence. Oligo-3 and oligo-4 are complementary and when they were annealed, two single-stranded cohesive termini were produced (Sal I and Pst I). The Sal I site would be ligated to the oligo-1 / oligo-2 generated Sal I site and the Pst I site was for the ligation to the detection sequence. The sequence of each oligonucleotide is shown in Table 1.
Table 1. First generation T7 amplification sequence synthetic oligonucleotide components

<table>
<thead>
<tr>
<th>Identification</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligo-1</td>
<td>5' AAA AAA AAA AAA CAT ACT ACG TAG AGA GTC GGG ACG 3'</td>
</tr>
<tr>
<td>Oligo-2</td>
<td>5' TCG ACG TCC CGA CTC TCT ACG TAG TAT GTT TTT TTT TTT TTT T 3'</td>
</tr>
<tr>
<td>Oligo-3</td>
<td>5' TCG ACA ATT TAA TAC GAC TCA CTA TAG GGA CTG CA 3'</td>
</tr>
<tr>
<td>Oligo-4</td>
<td>5' GTC CCT ATA GTG ACT CCT ATT AAA TTG 3'</td>
</tr>
</tbody>
</table>

Oligonucleotide preparation. Single-stranded oligonucleotides were received lyophilized and were rehydrated with 1 ml of TE (10 mM Tris-HCl, 1 mM EDTA). A spectrophotometric reading was taken on each strand using a Beckman DU model 2400 spectrophotometer. Absorbance readings at A$_{260}$ were recorded. The oligonucleotides were aliquotted into cryo-microfuge tubes (USA Plastics) and stored at -70°C.

Oligonucleotide annealing procedure. Here the complementary oligonucleotides were annealed. Oligo-1 and oligo-2 that make up the viral recognition sequences, and oligo-3 and oligo-4 that make up the T7 polymerase recognition site were separately annealed. Annealing temperatures were set at 25°C below the T$_m$ (temperature at which 50% of the strands are hybridized), which would allow for the maximum rate of association. The T$_m$ was determined using the following equation:

$$T_m = 16.6 \left( \log [Na^+] \right) + 0.41 \left( \% \text{ GC} \right) + 81.5 - (675/N),$$

where N= probe length.
Figure 4. Flow chart depicting how the completed T7 amplification sequence was constructed using the synthesized oligonucleotides and the PCR amplified detection sequence from poliovirus cDNA.
Figure 5. Synthesized oligonucleotide components used in the T7 amplification sequence. Schematic A is the first generation T7 amplification sequence, here cohesive termini were generated upon annealing the individual oligonucleotides. Schematic B is the second generation T7 amplification sequence where the cohesive termini were generated after a restriction digest of the annealed oligos. Schematic C is the third generation T7 amplification sequence, where the cohesive termini were generated after a restriction digest of the annealed oligos.
The annealing temperature of the recognition sequence was calculated to be $40.2^\circ C$ and the T7 polymerase recognition sequence was $34.8^\circ C$. The ionic strength used in the hybridization reaction was defined by using a 0.18 M concentration of Na$^+$ (1X SSC). This Na$^+$ concentration has been described in the literature as the minimum concentration that should be utilized under standard conditions (Maniatis et al, 1989). The annealing reaction was done by combining 5 µg of each of the complementary fragments in separate reaction tubes to which 5 µl of 20x SSC was added and the final reaction volume was brought to 100 µl with sterile water (Sigma). The samples were heated to $65^\circ C$ for 10 min to disrupt any random intrastrand hydrogen bonds that exist. The temperature was reduced to that previously calculated and held for 2 hr.

**Phosphorylation.** To ligate the cohesive termini of the previously annealed oligonucleotides, the 5’ end needed to be phosphorylated. When an oligonucleotide is synthesized, it is generated from 3’ to 5’ and the terminal 5’ nucleotide is not phosphorylated. Without the 5’ phosphate, a 5’ to 3’ phosphodiester bond cannot form between two oligonucleotides. Therefore, the 5’ ends of the annealed fragments involved in ligation must be phosphorylated.

T-4 polynucleotide kinase was used to catalyze the transfer of the γ-
phosphate of ATP to the 5' terminus of the oligonucleotide. Transfer reactions were set up according to the manufacturers protocol (BRL, Gaithersburg, MD). The standard reaction consisted of a 25 μl reaction volume containing 2.5 μg of nucleic acid, 5 μl of 5x forward reaction buffer, 2.5 μl 10 mM ATP and water to bring the final reaction volume to 25 μl. The reactions were incubated at 37°C for 1 hr. The phosphorylation reaction was done before the annealing reaction.

**Ligation of the hybridized fragments.** The next step in the construction of the T7 amplification sequence was the ligation of the annealed and phosphorylated oligonucleotides. The annealed fragments each had a protruding cohesive 5' terminus. Sticky ends of the two fragments were annealed and the single stranded breaks covalently sealed with T4 DNA ligase (BRL). The ligase reaction contained equal molar concentrations of the two annealed fragments, 1 μl of T4 DNA ligase, 5 μl of 5x ligase buffer, and water to bring the final reaction volume to 25 μl. The reaction was incubated at 16°C for 2 to 12 hr.

**Ligation control.** A ligation control was included to monitor the efficiency of ligation. This control would show if the reaction was properly setup by observing the ligation of digested DNA fragments. For this procedure, lambda DNA, Eco R1, Hind III digested (Sigma) were used as the
Electrophoresis. A non-denaturing polyacrylamide gel was used for separation and identification of the single-stranded, annealed and ligated oligonucleotides. A 29:1 acrylamide to bisacrylamide ratio used. The gel was prepared according to Maniatis (1989) by adding 29 g of acrylamide (Sigma), 1 g of N,N'-methylenebisacrylamide (Sigma) and water to 100 ml. The percent acrylamide used in casting the gels was 20% and was prepared by combining 13.32 ml of 30% acrylamide, 2.54 ml of water, 4 ml of 5x TBE, 140 µl of 10% ammonium persulfate, and 7 µl of TEMED (Sigma). Other gel concentrations were prepared according to Maniatis et al., (1989). The components were mixed, degassed and expelled between the glass forming plates using a syringe with a needle. The gel was left to polymerize for 1 hr, and then placed in the electrophoresis apparatus (Owl Scientific, Cambridge, MA). Sample wells were rinsed with TBE before loading the sample. Gels were run in 1x TBE buffer and stained with ethidium bromide (0.5 µg/ml ethidium bromide in 1x TBE). A UV light box (Fotodyne, New Berlin, WI) was used to visualize the nucleic acid bands.

Second Generation T7 Amplification Sequence

Work with the first generation T7 amplification sequence was terminated due to the difficulty in producing a single ligated product as
determined by the polyacrylamide gel results. It was theorized that there was phosphorylation occurring on "short products" generated during oligonucleotide synthesis. Short products are defined here as those oligonucleotides that are not complete due to inefficiencies in the DNA synthesis process. This resulted in several species of ligated products. It was not practical to determine the sequence of each ligation product to determine which one was complete. Therefore, the second generation T7 amplification sequence was designed to produce the cohesive termini after a restriction digest. In this design, phosphorylation of the 5' end would not be necessary. Any synthesized products that were not of the desired length would not be phosphorylated, hence they were not ligatable. This method incorporates a means to "cleanup" the ends of the annealed product by a restriction endonuclease digest that consequently would generate a phosphorylated 5' end.

**Modified oligonucleotides.** The oligonucleotide sequences were modified to contain a *Sac* 1 restriction site in the adjoining region of the viral recognition and T7 polymerase recognition regions. All four of the fragments were of the same length (50 bases). Figure 5 shows the orientation of the synthetic oligonucleotides. The restriction site also contained additional bases beyond the enzyme recognition area, to ensure an efficient digest. After the annealed oligonucleotides were *Sac* I digested, a ligatable cohesive end that
had a 5' phosphate was generated. The sequence of the four primers is outlined in Table 2.

**Table 2. Second generation T7 amplification sequence synthetic oligonucleotide components**

<table>
<thead>
<tr>
<th>Identification</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligo-1</td>
<td>5' AAA AAA AAA AAA AAA CAT ACT ACG TAG AGA GTC GGG ACG AGC TCA AGC TT 3'</td>
</tr>
<tr>
<td>Oligo-2</td>
<td>5' AAG CTT GAG CTC GTC CCG ACT CTC TAC GTA GTA TGT TTT TTT TTT TTT TT 3'</td>
</tr>
<tr>
<td>Oligo-3</td>
<td>5' AAG CTT GAG CTC CAA TTT AAT ACG ACT CAC TAT AGG GAC TGC AGA AGC TT 3'</td>
</tr>
<tr>
<td>Oligo-4</td>
<td>5' AAG CTT CTG CAG TCC CTA TAG TGA GTC GTA TTA AAT TGG AGC TCA AGC TT 3'</td>
</tr>
</tbody>
</table>

**Oligonucleotide preparation.** The oligonucleotides were obtained lyophilized and were resuspended in 1 ml of Milli-Q water (Millipore, Bedford, MA). The oligonucleotides were analyzed by polyacrylamide gel electrophoresis to determine their purity and to estimate their concentration.

**Annealing.** Assembly of the T7 amplification sequence was initiated by annealing the complementary single-stranded oligonucleotides. The viral recognition sequence was represented by oligo-1 and oligo-2. The T7 polymerase recognition sequence was represented by oligo-3 and oligo-4.

Two sets of reactions were done, each with one pair of the complementary oligonucleotides (oligo-1 with oligo-2 and oligo-3 with oligo-4). Combined in a microfuge tube were equal concentrations of each of the
two complementary oligonucleotides and 20x SSC to give a final concentration of 0.5x SSC. The reaction was heated to 95°C for 5 min to disrupt any secondary structures and then reduced to 50°C for 2 hr for annealing.

The outcome of the annealing reaction was visualized by polyacrylamide gel electrophoresis as previously described. Each single-stranded oligonucleotide was electrophoresed with the annealed products to compare the electrophoretic mobility of the single-stranded and double-stranded species.

**Restriction digest.** As previously stated, cohesive termini were produced by a restriction digest of each of the two annealed oligonucleotides. Each of the two annealed products was Sac 1 restriction digested separately according to the manufacturers instructions, (Boehringer Mannheim, UK). Combined in a microfuge tube were 10 μl of the annealed product, 6.5 μl of water, 2 μl of 10x restriction digest buffer, and 1.5 μl of Sac 1. The mixture was incubated at 37°C for 1.5 hr, then heated to 65°C to inactivate the enzyme and allowed to equilibrate to room temperature. A polyacrylamide gel containing both the digested and undigested product was run as previously described to assess the results.
**Ethanol precipitation of the digests.** Added to each of the restriction digest reaction tubes was 0.5x the digest volume of 3.0 M sodium acetate (pH 5.2), and 2.5x the volume of 95% ethanol. The mixtures were stored at -20°C for 30 min then centrifuged at 12,000 x g for 15 min at 4°C in a microcentrifuge (Beckman). The supernate was decanted and the tube was filled half way with 70% ethanol and centrifuged at 12,000 x g for 5 min at 4°C in a microcentrifuge (Beckman). The supernate was again decanted and the tube was left open so the remaining ethanol would evaporate. The pellets were each suspended in 15 μl of 1x T4 DNA ligase reaction buffer (Boehringer Mannheim).

**Ligation.** The viral recognition and T7 recognition sequences were ligated at their complementary cohesive termini. Combined in a microfuge tube were 15 μl of each of the two previously digested ligation components (oligo-1 with oligo-2 and oligo-3 with oligo-4) and 1 μl of T4 DNA ligase (Boehringer Mannheim). The reaction was mixed and incubated at 20°C for 2 hr. A polyacrylamide gel containing ligated and non-ligated products was run to determine the outcome of the reaction.

**Third Generation T7 Amplification Sequence**

As happened with the first generation sequence, the second generation ligation reaction also generated multiple products. The purity of the
oligonucleotides was questioned; however, there were no single-stranded molecular weight markers to conclusively determine if the desired product was present. As a result, all further work with the second generation sequence was ended. A change in the supplier of the oligonucleotides along with a design change was incorporated.

**Modified oligonucleotides.** The first and second generation sequences were produced in four sections (oligo-1 through oligo-4). The oligos needed to be annealed and then ligated to produce the desired product. The third generation sequence was designed to have two-95 base pieces of single-stranded DNA. This would circumvent the need for the restriction digest and ligation reaction to join the viral recognition and T7 polymerase recognition sequences. A restriction digest would still be needed to join the detection sequence downstream from the T7 polymerase recognition sequence. Figure 5 shows the orientation of the synthetic oligonucleotides. Additionally, a restriction endonuclease site was included at the 3' end of the viral recognition sequence to facilitate cloning. The oligonucleotides were obtained from a Bio-Synthesis, (Lewisville, Texas). The labeled quantity of each oligonucleotide was 5 optical density (OD) units $A_{260}$. The sequences of the two primers are shown in Table 3.
**Table 3. Third generation T7 amplification sequence synthetic oligonucleotide components**

<table>
<thead>
<tr>
<th>Identification</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligo-1</td>
<td>5' GCA GAT CCT CTA GAA AAA AAA AAA AAC ATA CTA CGT AGA GAG TCG GGA CGT CGA CAA TTT AAT ACG ACT CAC TAT AGG GAC TGC AGA AGC TT 3'</td>
</tr>
<tr>
<td>Oligo-2</td>
<td>5' AAG CTT CTG CAG TCC CTA TAG TGA GTC GTA TTA AAT TGT CGA CGT CCC GAC TCT CTA CGT AGT ATG TTT TTT TTT TTT TCT AGA GGA TCC GC 3'</td>
</tr>
</tbody>
</table>

**Oligonucleotide preparation.** Each of the two fragments was resuspended in 500 µl of sterile water (Sigma) and the resulting concentration was calculated to be 330 ng/µl. The concentration was based on the manufacturer's labeled concentration and that 1 OD unit $A_{260}$ is equal to 33 µg of single stranded DNA. The oligonucleotides were visualized by polyacrylamide gel electrophoresis as previously described to determine their purity and to estimate their concentration.

**Annealing conditions.** Oligo-1 and oligo-2 were annealed by combining the following in a microcentrifuge tube, 2 µl of oligo-1, 2 µl of oligo-2, 18.5 µl of water, and 2.5 µl of 10x restriction endonuclease reaction buffer (Tris-HCl, 500 mM; NaCl, 1 M; MgCl$_2$ 100 mM; dithioerythritol, 10 mM; pH 7.5) (Boehringer Mannheim). The reaction was heated to 95°C for 5 min to disrupt any secondary structures and then reduced for annealing to 50°C for 2 hr.
**Restriction digest.** The next phase was the ligation of the annealed product to the detection sequence. The detection sequence was previously described as the template for the production of RNA transcripts. The generation of the detection sequence is outlined in the next section of the materials and methods.

This section covers the restriction digest of the previously annealed oligo-1 and oligo-2. The termini of the annealed product contained restriction endonuclease recognition sites (see Figure 1). A *Pst I* site was adjacent to the T7 polymerase recognition sequence and was used to ligate the detection sequence. A *Xba I* site was adjacent to the viral recognition sequence and was used to ligate the complete T7 amplification sequence into pUC18.

The ability to cut at each of these two restriction endonuclease recognition sites was determined. Two restriction digests were prepared, a) *Pst I* and *Xba I* combined and b) *Pst I* only. For the double digest, 1 µl each of the *Pst I* and *Xba I* enzymes were added to 18 µl of the previously annealed fragments (annealed in restriction endonuclease buffer). In the *Pst I* digest, 1 µl of the enzyme was added to 19 µl of the previously annealed fragments (annealed in restriction endonuclease buffer). Both reactions were incubated at 37°C for 3 hr. The digest outcome was visualized by polyacrylamide gel electrophoresis as previously described; non-digested product was included in the gel as a control.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Preparation of the Detection Sequence

In the T7 amplification sequence design (see Figure 1) the detection sequence was incorporated downstream from the T7 polymerase recognition site and was the template for the production of RNA transcripts. The detection sequence was derived from an area in the poliovirus genome using poliovirus cDNA as the source (poliovirus cDNA [bp 115 - 7440]) cloned into the Pst 1 site of pBR322 and transformed in E. coli HB-101). The sequence could be obtained by either a plasmid prep or PCR technique. The PCR procedure was chosen since primers for the reaction were available and the size of the product could be defined by the primers selected. The PCR generated detection sequence was ligated to the synthetic oligonucleotide portion of the T7 amplification sequence. The next section describes the process used to generate and ligate the detection sequence to the annealed synthetic oligonucleotides.

PCR production of the detection sequence. Primers used in the amplification reaction were synthesized and polyacrylamide gel purified by Bio-Synthesis, Inc. Primers used for PCR were upstream, nucleotides 1821 through 1802 (5'-TAT CTT ACT GCA GAC AAC TT-3') and downstream, nucleotides 2203 through 2184 (5'-CCA GAT CAC ATG TGT TCC CA-3'). Use of these primers generated a 402-bp amplicon by PCR.

The optimal PCR amplification procedure was determined empirically
by varying concentrations of the MgCl₂, primers and target. Components contained within the GeneAmp RNA PCR kit (Perkin-Elmer by Roche Molecular Systems, Branchburg, NJ) were used in all the reactions. The final PCR concentrations were 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM MgCl₂, 1.0 µM upstream primer, 1.0 µM of the downstream primer and 2.5 U of Taq polymerase. Amplification was conducted for 40 cycles with a temperature cycling profile of 95°C for 1 min, 55°C for 2 min, and 72°C for 2 min, in a Thermal Cycler 480 (Perkin-Elmer).

The PCR 402-bp amplicon was electrophoresed in a 1.5% agarose gel (Sigma) using 1X TBE, stained with ethidium bromide (0.5 µg/ml and viewed with a UV light source. The band representing the amplified product (amplicon) was compared to a DNA molecular weight marker (pBR322 Hae III, Sigma) and the amplicon was excised from the gel with a sterile surgical blade and placed in a clean polypropylene tube. The DNA was isolated from the gel by electro-separation using a Schleicher & Schuell (Keene, NH) electro-separation system. The electroeluted DNA was harvested from the “trap” and concentrated by ethanol precipitation (Maniatis et al 1989), as previously described.

**Restriction digest of the detection sequence.** The detection sequence was digested with Pst I to produce the cohesive termini necessary to ligate the
detection sequence to the third-generation oligonucleotide component. The reaction was done by combining 10 μl of the PCR amplified / electroeluted ampicon (approx. 1 μg DNA); 6.5 μl water; 2 μl of 10x restriction buffer, and 1 μl of Pst 1 (Boehringer Mannheim). The mixture was incubated at 37°C for 1.5 hr. The resulting product was separated using a 2.0% agarose gel (Sigma) prepared and electrophoresed in 1X TBE buffer. The digested product was excised from the gel, purified by electro-separation, and concentrated by ethanol precipitation as previously described.

**Ligation of the T7 Amplification Sequence Components**

The next sequence of events in the construction of the complete T7 amplification sequence was to ligate the Pst 1 digested synthetic fragments (viral detection sequence and T7 polymerase portion) with the Pst 1 digested detection sequence (PCR amplified portion). The ligation reaction contained 8 μl of the synthetic fragment (approx. 80 ng of DNA); 8 μl of the detection sequence (approx. 40 ng of DNA); 2 μl 10X ligase buffer and 1 μl of ligase (Boehringer Mannheim). The mixture was incubated at 16°C for 3 hr. The ligation outcome was visualized by agarose gel electrophoresis using a molecular weight marker as previously described. The remaining portion of the ligation mix was concentrated by ethanol precipitation as previously described.
**Production of the T7 amplification sequence by PCR.** To ensure that an ample amount of a product was available for cloning, the entire T7 amplification sequence was amplified by PCR. The primers used for PCR were complementary to the 5' termini of the T7 amplification sequence. The upstream primer was complementary to the first 15 bases (5'-GCG GAT CCT CTA GAA-3') and the downstream primer was complementary to the last 20 bases (5'-CCA GAT CAC ATG TGT TCC CA-3').

Components contained within the GeneAmp RNA PCR kit (Perkin-Elmer) were used in the procedure. The final PCR reaction mixture contained 50 mM of KCl, 10 mM of Tris-HCl (pH 8.3), 2 mM of MgCl₂, 1.0 µM of the upstream primer, 1.0 µM of the downstream primer, 2.5 U of Taq polymerase, and 3 µl of the target. Amplification was conducted for 40 cycles with a temperature cycling profile of 95°C for 1 min, 55°C for 2 min, and 72°C for 2 min, in a Perkin-Elmer Thermal Cycler 480. The amplified product was visualized by polyacrylamide gel electrophoresis as previously described.

**Preparation of the T7 Amplification Sequence for Cloning**

**Restriction digest.** The PCR (consisting of the T7 amplification sequence) product contained restriction sites at both termini (see Figure 1). One end had a *Bam* H1 site and the other end had a *Xba* 1 site. The two sites were incorporated into the T7 amplification sequence to directionally...
orientate it into the vector (pUC 18). Prior to cloning, both the T7 amplification sequence and pUC 18 were cut with restriction endonuclease enzymes (Bam HI and Xba 1). The vector, pUC 18, was double digested by first combining 8 μl of pUC 18 (2 μg); 8 μl of water, and 2 μl of 10X buffer. The restriction enzymes were added sequentially; first, 1 μl of Bam HI (Boehringer Mannheim) was added and the reaction mix was incubated at 37°C for 2 hr, followed by 1 μl of Xba 1, and the reaction mix was incubated at 37°C for 5 hr. The T7 amplification sequence was digested with Bam HI and Xba 1 as previously described for the pUC 18 procedure.

**Dephosphorylation.** The pUC 18 was dephosphorylated using calf intestinal alkaline phosphatase (CIP)(Boehringer Mannheim). The ethanol precipitated pUC 18 digest previously described was resuspended in 90 μl of H₂O, 10 μl of CIP 10X buffer, and 1.2 μl of CIP enzyme. The reaction mix was incubated at 37°C for 15 min then an additional 1.2 μl of the CIP enzyme was added and the reaction was incubated at 37°C for an additional 45 min. After incubation, EDTA (pH 8.0) was added to 5 mM and the mixture was heated at 65°C for 1 hr.

**Phenol:chloroform extraction.** Both the insert and the pUC 18 vector were phenol:chloroform extracted. An equal volume of phenol:chloroform
(Sigma) was added to each reaction, contained in a polypropylene microfuge tube (USA Plastics). The contents were mixed by inversion and then centrifuged at 12,000 rpm (Sorval) for 15 sec at room temperature. The aqueous phase was transferred to a clean tube and the original tube was back-extracted by adding an equal volume of TE (pH 7.8). The sample was mixed, centrifuged, and the aqueous layer transferred to the first aqueous phase. To the aqueous phase an equal volume of chloroform (Sigma) was added and the tube was mixed and centrifuged as previously described. The aqueous phase was recovered and placed in a clean tube and the nucleic acid was ethanol precipitated as previously described.

**Ligation.** The insert (T7 amplification sequence) was ligated into the vector pUC 18) by the following reaction. The concentration of both the vector and insert were approximately 20 ng/μl as determined by visual estimation of the electrophoresed products. The ligation reaction was set up by adding 2 μl of pUC 18, 1 μl of the insert, 2 μl of the 10X ligation buffer, 14 μl of water, and 1 μl of T4 ligase. The reaction mix was incubated at 12°C for 18 hr.

**Transformation.** Competent *E. coli, INV αF* cells were commercially obtained (Invitrogen Corporation, San Diego, CA). The transformation
protocol was: 1) equilibrated water bath to 42°C, 2) warmed one vial of SOC medium (Invitrogen, San Diego, CA) to room temperature, 3) thawed 50 μl of frozen competent *E. coli* cells and held on ice, 3) added 2 μl of β-mercaptoethanol to the competent cells and mix by gently tapping the tube, 4) added 1 μl of the ligation reaction mixture directly to the competent cells and mixed by tapping, incubated on ice for 30 min, followed by 42°C for 1 min and finally back on ice for 2 min, and 5) added 450 μl of pre-warmed (42°C) SOC and placed the tubes on a gyratory shaker (225 rpm) (Lab-Line) contained in a 37°C incubator for 1 hr. With a sterile, bent glass rod, 25 μl and 100 μl of the transformation reaction was spread onto separate LB agar plates that contained ampicillin (50 μg / ml)(Sigma), and 25 μl of X-Gal (40 mg/ml stock solution)(Sigma). Plates were inverted and incubated at 37°C until growth was observed (12 to 40 hr). White colonies were selected for further analysis.

**Selection of transformants.** The vector, pUC 18, carries an ampicillin-resistance gene and the insertion of a foreign fragment of DNA into the polycloning site of the plasmid usually results in the absence of β-galactosidase production by α-complementation. In the presence of X-gal the Lac⁺ bacteria resulting from the uptake of a plasmid without the insert
will form blue colonies. Bacteria carrying a recombinant vector (Lac') will produce white colonies. Following plating of the transformation reaction a total of 10 white colonies was picked from the LB agar plates. Isolates were restreaked onto LB agar (containing ampicillin and x-gal as previously described) for confirmation and pure culture determination.

**Mini plasmid prep.** Each isolate was grown in LB broth with 50 μg / ml ampicillin, overnight at 37°C with shaking. After incubation an aliquot of the culture was transferred to a sterile microfuge tube (USA Plastics) and the cells were harvested by centrifugation at 10,000 x g for 30 sec (Sorval). The supernate was decanted and the pellet was resuspended in 0.5 ml of STE (0.1 M NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) and recentrifuged.

The plasmid DNA was isolated from the cells by an alkaline lysis procedure (Maniatis et al., 1989). Briefly, the procedure was to: 1) resuspend the bacterial pellet in 100 μl of ice-cold Solution I (50 mM glucose, 25 mM Tris-HCl [pH 8.0], 10 mM EDTA [pH 8.0]) by vortexing, 2) added 200 μl of Solution II (0.2 N NaOH, 1% SDS), mixed by inversion and placed the tube on ice, 3) added 150 μl of ice-cold Solution III ( 60 ml, 5 M potassium acetate; 11.5 ml, glacial acetic acid; 28.5 ml H2O), vortexed for 10 sec, placed on ice for 5 min. The solution was centrifuged at 12,000 x g for 5 min at 4°C in a microcentrifuge (Sorval). The supernate was transferred to a fresh tube and

117
the sample was phenol:chloroform extracted and ethanol precipitated as previously described.

**Restriction digest of the recombinants.** The recombinants were then screened to make sure that the plasmid contained the insert. The isolated plasmid was double digested with *Xba* 1 and *Bam* H1 as previously described. The double digest would cut the insert out of the vector. The outcome was determined by agarose gel electrophoresis as previously described. Each recombinant shown to have the vector / insert was frozen at -70°C in glycerol for future use.

**Sequence Determination of the Complete T7 Amplification Sequence**

The T7 amplification sequence was excised from the clone by a double digest as previously described and purified by an electro-separation system as previously described. The sequence was determined using an ABI PRISM™ Dye Terminator Cycle Sequencing Kit with AmpliTaq® DNA polymerase, FS (Perkin Elmer) and an ABI 373A automated sequencer (Perkin Elmer) by a sequencing service provider (DNA Sequencing Facility, Durham NH). The T7 amplification sequence was sequenced in both the forward and reverse direction.
Generation of RNA Transcripts

The T7 amplification sequence was evaluated to: 1) measure its ability to produce RNA transcripts, 2) determine the end point of RNA production, and 3) determine the benefit of adding a second aliquot of the T7 enzyme after the initial reaction. The template consisted of the excised T7 amplification sequence purified by electro-separation and visually quantitated by electrophoresing several concentrations of the purified material.

RNA transcripts were prepared according to the manufacturer’s instructions (BRL). The reaction mixture contained 2 μl of 5X T7 RNA polymerase reaction buffer, 1 μl of NTP stock (10 mM each, ATP, GTP, and CTP), 1 μl of 50 mM DTT, 1 μl of 3000 Ci/mmol 32P UTP, up to 4 μl of template (100 to 500 ng), and water to bring the reaction volume to 9 μl. The reaction was equilibrated at 37°C for 5 min, then added 1 μl (50 units) of T7 RNA polymerase. The reaction was incubated at 37°C for 1 hr. Samples were removed at various times and used to determine the incorporated radioactivity.

Analysis of RNA yield. Trichloroacetic acid (TCA) precipitation was used to measure the incorporation of the radiolabel. The procedure was done according to BRL Technical Bulletin 8033-1. A pencil was used to label the Whatman GF/C glass fiber filters before sample application. The TCA
procedure was as follows: 1) 1 or 2 µl of the reaction mix was transferred to a tube containing 5 µl of yeast tRNA (1 mg/ml) and sterile water to bring the final volume to 100 µl, 2) 5 µl of the mixture was transferred to the filter and allowed the filter to dry, 3) the filter was transferred to 10 ml of ice-cold 10% TCA-1% NaPPi, swirled several times for 5 min, 4) washed the filter 4 times, 5 min each time in 10 ml of ice-cold 5% TCA, swirling several times, 5) transferred the washed filter to a beaker of 70% ethanol for 2 min, removed it and air dried it. An unwashed control was prepared by spotting the sample onto the filter and allowed it to air dry (filter was not washed). Each filter was placed in a vial that contained scintillation fluid (Scinti-Verse I, Fisher). The sample was counted and the $^{32}$P incorporated was calculated by:

Specific activity = \(rac{\text{Total cpm reaction}}{\text{Reaction volume}} \times \frac{\text{reaction volume}}{\text{Total pmole labeled NTP}}\) = cpm/pmole labeled NTP

Yield = \(\frac{\text{Precipitable cpm}}{\text{Reaction volume}} \times \frac{\text{Reaction volume}}{\text{pmol labeled NTP}} \times \frac{\text{pmol labeled NTP}}{1 \mu g} = \mu g \text{RNA}\)

\[
\text{Yield} = \frac{\text{Precipitable cpm}}{\text{Reaction volume}} \times \frac{\text{pmol labeled NTP}}{\text{pmol labeled NTP}} \times \frac{3225 \text{ pmol}}{1 \mu g} = \mu g \text{RNA}
\]

**Part 2: Capture Sequence Development**

The second part of this investigation was the development of a nucleic acid capture sequence (Figure 3). The capture sequence was designed to: 1) facilitate the recovery of a targeted nucleic acid from a sample matrix, 2) permit analysis of larger sample volumes, 3) provide an environment for optimal hybridization, and 4) permit a washing step to remove any enzymatic inhibitors.
The following description covers the experiments done to develop a technique to directly isolate viral nucleic acid. Briefly, the capture sequence described here was biotinylated at the 5' end. Following hybridization, the hybrid was mixed with streptavidin coated magnetic beads and immobilized using a magnetic rack.

**Poliovirus Preparation**

Poliovirus type 1 (LSc strain) was propagated by infecting Buffalo Green Monkey Kidney (BGM) cells. Flasks were incubated at 37°C until a generalized cytopathic effect was apparent. Flasks were then freeze-thawed to release cell-bound viruses, and the solution clarified by centrifugation.

Virus was titered by the plaque-forming unit (PFU) method (Dulbecco and Vogt, 1954). Twenty-five cm² flasks of confluent BGM cells were inoculated in duplicate with 0.1 ml of each viral dilution. Adsorption of the virus was allowed to continue for 80 min at 37°C, flasks were rocked every 15 min. Following adsorption, monolayers were washed with phosphate-buffered saline (PBS) and overlaid with Medium 199 (Sigma, ST. Louis, MO) supplemented with 2% serum (Sigma), 2% agar (Difco, Detroit, MI), and neutral red (Sigma). Flasks were incubated at 37°C, until plaques were formed. Then the flasks were counted and the results averaged. Poliovirus was used as the target throughout the study.
Capture Sequence and Primers

The poliovirus primer and capture sequences were selected from a highly conserved area of the 5' noncoding region. The oligonucleotides were synthesized and polyacrylamide gel purified by Bio-Synthesis, Inc. The capture sequence consisted of the complement to nucleotides 644 through 620 (5'-ACT TTC ACC GGA TGG CCA ATC CAA T-3'). The capture sequence was biotinylated at the 5' end by the manufacturer during synthesis. Primers used for PCR are as follows: upstream, nucleotides 444 through 460 (5'-CCT CCG GCC CCT GAA TG-3') and downstream, nucleotides 638 through 621 (5'-ACC GGA TGG CCA ATC CAA-3'). These primers generated a 195-bp amplicon.

Reverse Transcription and PCR

For both reverse transcription and PCR amplification procedures, optimal conditions were determined empirically. Components contained within the GeneAmp RNA PCR kit (Perkin-Elmer by Roche Molecular Systems, Branchburg, NJ) were used in all the reactions. The first test was to determine the sensitivity and specificity of the primers used to amplify poliovirus.

Sensitivity was determined by preparing tenfold dilutions of poliovirus from $10^2$ through $10^{-2}$ PFU, and each dilution was assayed by RT-PCR. Gel electrophoresis was used to determine the presence of an expected 195-bp amplification product. Specificity was determined by
examination of the agarose gel for bands indicative of nonspecific amplification.

Each RT reaction was done in a 20 μl final volume, with a final concentration of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 5 mM MgCl₂, 1 mM each of the deoxynucleoside triphosphates, and 0.75 μM downstream primer. The RNA was liberated from the viral capsid by heating the mixture to 99°C for 5 min. Mineral oil (Sigma) was added to control evaporation. Lastly, 20 U of RNase inhibitor and 50 U of M-MLV reverse transcriptase were added. The RT temperature profile was 42°C for 60 min, 99°C for 5 min, and 5°C for 5 min. The reaction was done in a Perkin-Elmer Thermal Cycler 480.

Following reverse transcription, 80 μl of the PCR reaction mixture was added to each RT tube. The final PCR concentrations were, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM MgCl₂, 0.15 μM upstream primer, 0.15 μM downstream primer and 2.5 U of Taq polymerase. Amplification was conducted for 35 cycles with a temperature cycling profile of 95°C for 1 min, and 55°C for 1 min, followed by a final extension at 55°C for 7 min in a Perkin-Elmer Thermal Cycler 480.

**Streptavidin Coated Beads**

Superparamagnetic polystyrene beads (2.8 μm ± 0.2 μm diameter), with streptavidin covalently attached, were purchased from Dynal (Lake Success,
NY). The beads were washed before use as follows: 1) beads were immobilized with a Dynal magnetic particle concentrator (MPC) (Dynal), 2) supernate was aspirated from the tube while it was in the MPC rack, and 3) the beads were resuspended in buffer (5 mM Tris-HCl, pH 8.0 and 1.0 M NaCl).

**Magnetic bead effect on RT-PCR.** The effect of the streptavidin-coated magnetic beads on the RT-PCR assay was investigated to establish if their presence would affect the RT-PCR process. A series of RT-PCR reactions was done, all with a concentration of $10^1$ PFU of poliovirus. Several concentrations of streptavidin beads were added to each RT-PCR tube (20 μl to 60 μl in 20 μl increments) which represented 200 μg to 600 μg of beads. RT-PCR was done and the results were visualized by agarose gel electrophoresis.

**Effect of NaCl concentration.** A critical component of the capture reaction is the NaCl concentration. According to the magnetic bead manufacturer's protocol, the NaCl concentration should be increased above the recommended 1 M concentration when the size of the target is greater than 1-kb. In these experiments the poliovirus nucleic acid target was greater than 7-kb. A series of capture reactions was prepared in purified water using
10¹ PFU of poliovirus. The reactions were the same except the concentration of NaCl, which varied from 0.0 M to 3.0 M in 1 M increments. Following capture, RT-PCR was performed as previously described and the results were viewed by agarose gel electrophoresis.

**Nucleic acid recovery with the capture sequence.** The nucleic acid capture technique was evaluated by determining the efficacy of recovering poliovirus nucleic acid from seeded purified water (Sigma). Each reaction contained 250 μl of purified water with a NaCl concentration of 2.5 M, 2.5 μl of 100 mM Tris-HCl (pH 8.0), 0.75 μm of the biotinylated capture sequence,, and 10 μl of a poliovirus serial dilution (10¹ through 10⁻² PFU/μl).

The components were combined in a microcentrifuge tube, and heated to 99°C for 10 min to liberate the poliovirus RNA from the viral capsid. The temperature was then reduced to 42°C for 20 min to permit hybridization. The supernate was transferred to another 0.5 ml siliconized tube containing approximately 200 μg of previously washed streptavidin magnetic beads. The sample was gently mixed to resuspend the beads and then incubated at 45°C for 45 min by placing the tubes in a sample mixer that rotated at one revolution per minute. Continuous rotation was used to prevent the beads from settling and maximize the bead / target interaction rate.
The beads were then concentrated by placing the tubes in the MPC rack which immobilized the bead complexes against the microfuge tube side wall. For all of the immobilization steps the beads were held in the magnetic rack for at least 1 min before aspirating the supernate. The supernate was removed while the tube was in the MPC rack.

The beads were washed three times by adding 200 µl of washing buffer consisting of 2.5 M NaCl and 10 mM Tris-HCl [pH 8.0], and the beads were gently resuspended by removing the tube from the magnetic rack. After the final wash, the supernate was removed and the tube was centrifuged briefly to facilitate the removal of any remaining wash buffer. The bead / target complex was then directly used in the reverse transcription and PCR steps previously described with the exception that the reverse primer was not added to the reaction. In this reaction the capture sequence would function as the primer for reverse transcription.

**Evaluation of the Capture RT-PCR System**

The ability of the magnetic beads to bind to the target \ capture sequence hybrid and effectively immobilize the complex was examined. Due to the sensitivity of PCR, a possibility existed that amplification could result from the carry-over of the target viral nucleic acid that was not removed during the washing steps.

Three reactions were done in purified water to evaluate the recovery
process. Each reaction used $10^1$ PFU of poliovirus and was done in a 250 μl reaction volume as previously described with the following modifications: one reaction contained the biotinylated capture sequence, and streptavidin-coated magnetic beads, one reaction contained the biotinylated capture sequence and no superparamagnetic beads, and the last reaction contained a non-biotinylated primer and the superparamagnetic beads. RT-PCR was done as previously described.
RESULTS

T7 Amplification Sequence

First generation T7 amplification sequence. Figure 6 shows the polyacrylamide gel electrophoresis results of each of the four oligonucleotides. The concentration of each oligonucleotide was determined by a spectrophotometric reading at $A_{260}$ and were: 1) oligo-1 = 275.5 ng/μl, 2) oligo-2 = 294.5 ng/μl, 3) oligo-3 = 381.4 ng/μl, and 4) oligo-4 = 166.4 ng/μl. No molecular weight markers were commercially available to compare to the oligonucleotides. Each lane (Figure 6) showed a prominent band and numerous other species.

The annealing reaction of oligo-1 with oligo-2 was compared to the band migration of the individual single stranded components (Figure 6), the same comparison was done for oligo-3 and oligo-4 (Figure 6). Results from the annealing reaction showed that the electrophoretic mobility of the annealed strands was less than the mobility of either of the individual components that comprise the double-stranded species. The decrease in mobility of the band seen in the annealing reaction was an indication that hybridization had occurred, but a molecular weight marker was not available to confirm this supposition. However, the absence of a detectable single-
Figure 6. First generation T7 amplification sequence components. Lane 1, 2, 3, and 4 are the single stranded synthetic oligonucleotides 1 - 4 respectively. Lane 6 is the annealing reaction of oligo-1 and oligo-2. Lane 7 is the annealing reaction of oligo-3 and oligo-4. Lane 9 is the ligation reaction between the product shown in lanes 6 and 7, the 5' ends were not phosphorylated before ligation.
stranded species in the annealed reaction further supported this conclusion. The annealing of the complementary pairs of single-stranded oligos produced complementary cohesive termini that would be used to ligate the two double-stranded fragments. Initial attempts at ligating the two fragments were not successful (Figure 6). Polyacrylamide gel electrophoresis was used to determine the presence or absence of a ligated product. It was apparent in the ligation reaction that no additional band resulted from the ligation of the two double-stranded oligonucleotides. The only distinct bands present were those from the non-ligated components (Figure 6). A control reaction that used digested lambda DNA indicated that the reaction was properly working. Many attempts at ligation were made. Through further study it was determined that the 5' terminus of each synthetic oligonucleotide was not phosphorylated, hence, ligation would be prohibited.

Subsequently, phosphorylation of the 5' ends of the two oligos that comprise the ligation between the viral recognition sequence and a T7 polymerase recognition site was done. Phosphorylated and non-phosphorylated oligos were prepared and electrophoresed. Results had shown that there was a change in mobility that indicated the phosphorylation was successful.

A ligation reaction that incorporated the phosphorylated oligonucleotides revealed the presence of two additional bands (Figure 7). Also seen was a decrease in the intensity of the two individual
phosphorylated double stranded fragments. This further supported the belief that the new bands were not a random occurrence.

Experimentation with these oligos was ended due to the high degree of other species obtained. It was determined that since the quality of the oligos was so poor, the oligonucleotide sequences may not be 100% complete. Therefore, a second generation T7 amplification sequence was designed.

**Second generation T7 amplification sequence.** Briefly, the second generation amplification sequence was designed to have an internal restriction endonuclease recognition site incorporated into each of the two pairs of complementary oligonucleotides (viral recognition sequence and T7 polymerase recognition site). After annealing, a restriction endonuclease digestion would generate the complementary cohesive termini. This configuration would preclude the need for phosphorylation.

The quality of the 50-base single-stranded synthetic DNA was determined by polyacrylamide gel electrophoresis. All four fragments were detected. However, their purity was questionable due to a large amount of smearing shown in the gel lanes (Figure 8). Additional gels were prepared and loaded with a smaller sample size. The results still showed the presence of smearing.
Figure 7. First generation T7 amplification sequence components. Lane 1 is the annealing reaction of oligo-1 and oligo-2. Lane 2 is the annealing reaction of oligo-3 and oligo-4. Lanes 3 and 4 are the ligation reaction between the product shown in lanes 1 and 2, the 5' ends were phosphorylated. Lane 5 is the annealing reaction of oligo-1 and oligo-2 that had been previously excised from a gel and purified by electro-separation. Lane 6 is the annealing reaction of oligo-3 and oligo-4 that had been previously excised from a gel and purified by electro-separation.
Figure 8. Second generation T7 amplification sequence components. Lane 1 is oligo-1 and oligo-2 annealed. Lane 2 is the Sac I restriction endonuclease digestion of product shown in lane 1. Lane 3 is oligo-3 and oligo-4 annealed. Lane 4 is the Sac I restriction endonuclease digestion of product shown in lane 3. Lanes 5 and 6 are the ligation reaction results between the Sac I digested product shown in lanes 2 and 4.

133
The complementary fragments were annealed and cut with Sac 1 restriction endonuclease and visualized by polyacrylamide gel electrophoresis. The gel in Figure 8 showed that there was a difference in the electrophoretic mobility of the cut and uncut fragments. This demonstrated that the fragments were cut and the cohesive termini would be present. However, again a molecular weight marker was not available for confirmation of product size.

The two Sac 1 digested products were ligated. The ligation (Figure 8) showed that there were several bands generated, and a significant amount of smearing. Apparently, many ligation products were generated. There were no molecular weight markers available for comparison.

Further experimentation with these oligos was ended. The inability to obtain a single ligated species and the presence of a high degree of other species was the deciding factor. The source of the synthetic oligonucleotides was postulated to play a key role in the quality of the oligonucleotides and the success of the T7 amplification sequence development. It was known that the efficiency of the synthesizer was poor and this would directly affect the quality of the oligonucleotides. The next generation of oligos was obtained from another source.

**Third generation T7 amplification sequence.** The third generation T7 amplification sequence incorporated some additional design changes.
Restriction endonuclease sites (*Bam* H1 and *Xba* 1) were added at the 5' end (viral recognition sequence end). Also, the oligonucleotides were synthesized as two complementary 95-base fragments, instead of four shorter fragments.

Oligonucleotide purity was determined by polyacrylamide gel electrophoresis. Results of the first pair of oligos was poor there were no distinct bands present. There was a high degree of smearing indicating a low efficiency synthesis. No further work was done with these two primers. Another pair of oligos was obtained from the same source (Bio-Synthesis) and was visualized by polyacrylamide gel electrophoresis. Results of the new oligos showed that each had a distinct band with the same gel migration rate (Figure 9). Although each oligo was purified by polyacrylamide gel electrophoresis by the manufacturer (Bio-Synthesis), additional bands were detected in both of the oligo gel results (Figure 9). Since this was a non-denaturing gel, some products may be due to intrastrand annealing.

The two oligos were annealed and the results determined by polyacrylamide gel electrophoresis (Figure 10). The gel shows that the mobility of the annealed product was greater than the single-stranded species. This was an indication that annealing had occurred. Here, an increase in mobility indicated that a double stranded species was formed. A comparison of the single-stranded and double-stranded DNA with a molecular weight marker in Figure 10 supports this conclusion.

The annealed product was double digested with restriction
endonuclease *Pst* 1 and *Xba* 1. The digestion result is shown in (Figure 10). The gel shows a change in the mobility when compared to the non-digested fragment which indicates that the oligonucleotide was cut. Additional experiments using each of the two restriction enzymes in separate reactions demonstrated that both of the restriction endonuclease sites were recognized and cut.

These positive results led to the next phase in the T7 amplification sequence development. This was the generation of the detection sequence that functioned as the template for the production of RNA transcripts. Adjacent to the T7 polymerase recognition region was a *Pst* 1 restriction site where the sequence would be ligated.

**Detection sequence.** The detection sequence was produced by PCR amplification (using poliovirus cDNA). Polyacrylamide gel electrophoresis was used to compare the PCR product with a molecular weight marker. The result had shown the expected 402-bp amplicon (Figure 11).

The 402-bp amplicon was *Pst* 1 digested. Polyacrylamide gel electrophoresis was used to compare the *Pst* I digest with a molecular weight marker. The result had shown the expected 394-bp product (Figure 11). The *Pst* 1 digest reduced the detection sequence length several bases since the cut site was not at the end of the recognition sequence. The result was the generation of a fragment with one *Pst* 1 cohesive end to ligate to the synthetic

136

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Figure 9. Third generation T7 amplification sequence components that were re-synthesized. Lanes 1, 2, 3, and 4 are various dilutions of oligo-1. Lanes 5, 6, 7, and 8 are various dilutions of oligo-2. Lane 11 is a double stranded molecular weight marker pBR322, Hae III digest.
Figure 10. Third generation T7 amplification sequence assembly reactions. Lane 1 is oligo 1. Lane 2 is oligo 2. Lane 3 is a double stranded molecular weight marker pBR322, Hae III digest. Lane 4 and 5 are the result of an Xba I and Pst I digest of the annealed oligos 1 and 2. Lane 7, 8, and 9 are various concentrations of oligo-1 and oligo-2 annealed.
Figure 11. Preparation of the synthetic T7 amplification sequence components and the PCR generated detection sequence for cloning. Lane 1 is a double stranded molecular weight marker pBR322, Hae III digest. Lane 2 is oligo-2. Lane 3 is the PCR amplified T7 amplification sequence (464-bp), before it was digested with Bam HI and Xba I for cloning. Lane 4 is the PCR amplified detection sequence (402-bp). Lane 5 is the PCR amplified detection sequence Pst I digested for ligation to the synthetic, annealed fragments (394-bp). Lane 6 is the complete T7 amplification sequence (354-bp) digested with Bam HI and Xba I ready for cloning.
sequence and one blunt end.

**Ligation of the synthetic fragment and the detection sequence.** Initial results had shown that the annealed synthetic oligonucleotide that contained the viral recognition and T7 promoter sequence was ligated to the detection sequence. A faint but a distinct band of 464-bp was visualized and was compared to the molecular weight marker. Although the entire T7 amplification sequence was ligated, there was not a sufficient amount of the product for cloning. Therefore, the 464-bp T7 amplification sequence was amplified by PCR.

The T7 amplification sequence was successfully amplified by PCR; results are shown in Figure 11. Restriction endonuclease digest of the PCR product also showed that the sequence could be cut with Bam HI and Xba 1 (Figure 11). The Bam HI and Xba 1 digest of the 464-bp PCR amplicon generated the expected 354-bp T7 amplification sequence for cloning.

The T7 amplification product (354-bp) was successfully ligated and transformed into E. coli. The plasmid was isolated from transformants and digested with Bam HI and Xba 1. Results had shown that the T7 amplification sequence was removed from the plasmid, this clearly demonstrated that the cloning was successful (Figure 12).

**Sequence determination of the T7 amplification sequence.** The
sequence of the T7 amplification sequence is shown in Figure 13. The sequence was determined with an ABI PRISM™ Dye Terminator Cycle Sequencing Kit and an ABI 373A automated sequencer. The result of the forward and reverse sequence reactions were compared with the expected sequence (Racaniello and Baltimore, 1981). The sequence was found to match the known sequences.

**T7 reaction.** The T7 promoter region was shown to be functional. The production of RNA transcripts was demonstrated by measuring the incorporation of a radiolabeled nucleotide into TCA-precipitable material. The optimal incubation period was determined for RNA production. TCA precipitable counts were obtained over a period of 90 min. Results showed that the maximum amount of transcripts was at 60 min and after that the counts leveled off. The detectable level of RNA transcripts leveled off after 60 min. This was expected since the activity of the T7 enzyme diminishes after 1 hr. The benefit of adding an additional aliquot of the T7 RNA polymerase was determined. It was shown that by adding an additional aliquot of the enzyme, there was a 33% increase in the amount of transcripts generated (Figure 14).

**Capture Sequence**

**Poliovirus RT-PCR.** Initial experiments evaluated the primers and
Figure 12. Result of the cloning and transformation procedures. Lanes 1 and 6 are a double stranded molecular weight marker pBR322, \textit{Hae} III digest. Lane 2 is the PCR amplified detection sequence. Lane 3 is the PCR amplified, T7 amplification sequence digested with \textit{Xba} I and \textit{Bam} HI. Lanes 4 and 5 are transformation isolates restriction digested with \textit{Xba} I and \textit{Bam} HI.
<table>
<thead>
<tr>
<th>Sequence</th>
<th>Description</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGAAAAAAA AAAAAAAACA TACTACGTAG</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>AGAGTCGGGA CGTCGACAA T TTAATACGA C</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>TCACTATAG G GACTGCAGA C AACTTCCAG T</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>CACCGTGTGC TCGTCCTGA A TTTGATGTG A</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>CCCCCACCTAT TGACATACCC GGTGAAGTAA</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>AGAACATGAT GGAATGGGCA GAAATCGACA</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>CCATGATTCC CTTTGACTTA AGTGCCACAA</td>
<td>210</td>
<td></td>
</tr>
<tr>
<td>AAAAGAACAC CATGGAAATG TATAGGGTTC</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>GGTTAAGTGA CAAAACCACAT ACAGACGATC</td>
<td>270</td>
<td></td>
</tr>
<tr>
<td>CCATACTCTG CTTGTCACTC TCTCCAGCTT</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>CAGATCCTAG GTTGTCACAT ACTATGCTTG</td>
<td>330</td>
<td></td>
</tr>
<tr>
<td>GAGAAATCCT AAATTACTAC ACACACTGGG</td>
<td>360</td>
<td></td>
</tr>
<tr>
<td>CAGGATCC</td>
<td>390</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 13.** Sequence of the complete T7 amplification sequence as determined by ABI PRISM™ Dye Terminator Cycle Sequencing Kit with AmpliTaq® DNA polymerase, FS (Perkin Elmer) and an ABI 373A automated sequencer (Perkin Elmer).
**T7 REACTION**

ADDITIONAL POLYMERASE ADDED

![Graph](image)

Figure 14. T7 amplification reaction to evaluate the fidelity of the T7 amplification sequence. Results of a two-step reaction where T7 polymerase was added to initiate the reaction and a second aliquot was added at 60 min.
biotinylated capture sequence to ensure RT-PCR would result in the generation of the 195-bp target. The two step PCR procedures, consisting in part of a 55°C anneal and extension temperature resulted in a single band. Nonspecific products were not detectable by agarose gel electrophoresis (Figure 15). This data showed that the primers and capture sequence would consistently and specifically amplify the poliovirus RNA target region.

**Detection of poliovirus RNA in purified water.** In order to evaluate the efficacy of nucleic acid capture, poliovirus-seeded purified water was initially incorporated. A minimal detection level of $10^0$ PFU of poliovirus could be detected in a 250 µl sample size.

**Target capture.** The magnetic capture system was evaluated to ensure that amplification was a result of target capture. It was necessary to show that amplification was not solely the result of carryover targets remaining after the washing steps. It was demonstrated that amplification was detected in the reaction that contained a biotinylated capture sequence and the streptavidin-coated superparamagnetic beads. Amplification was not detected in the reaction that contained the streptavidin-coated superparamagnetic beads and a non-biotinylated capture sequence. The reaction without the streptavidin coated superparamagnetic beads and with the biotinylated capture sequence
Figure 15. Reverse transcription and PCR amplification of poliovirus. Amplification was done with the upstream and downstream primers. Lane 1 is the 195-bp product and M is the molecular weight marker, pBR322 Hae III, the arrow indicates the location of the 195-bp band.
also did not exhibit amplification. In the two modified reactions (without the beads and without the biotinylated capture sequence) the removal of a critical capture component confirmed that the RNA target was captured.

**Effect of magnetic beads on RT-PCR.** The incorporation of the magnetic beads in the RT-PCR assays was investigated to establish if there was an inhibitory effect on amplification. The control was compared with each of the three bead concentrations and there was minimal detectable decrease in the intensity of the amplified target (Figure 16).

**Sodium chloride concentration.** Varying the NaCl concentration was shown to affect the capture efficiency. A reaction without NaCl exhibited the least degree of amplification. Both the 2 M and 3 M NaCl concentrations gave comparable results and the band intensity was shown to be greater than the 1 M NaCl concentration. Because of these data, the NaCl concentration for subsequent environmental capture reactions was 2.5 M NaCl.
Figure 16. Effect of increasing amounts of the streptavidin magnetic beads on amplification of poliovirus RNA by RT-PCR. Lane 1 is a control with $10^1$ PFU of poliovirus and no beads. Lanes 2 through 4 are reactions that each contain $10^1$ PFU of poliovirus and 200, 400 and 600 μg of beads, respectively. Lane 5 is the negative control (no target). Lane M is the molecular weight marker. The arrow indicates the position of the 195-bp band.
DISCUSSION

Use of Synthetic Oligonucleotides

Many problems with the assembly of the T7 amplification sequence centered on the quality of the synthesized oligonucleotides. The production of synthetic nucleotides must take place under highly efficient conditions. The expected yield can be determined with the formula $Y^x$, where $Y$ = number of cycles and $x$ = the number of bases. As an example using this equation a synthesizer operating at 98% efficiency would have a 55% yield for a 30-mer, 36% yield for a 50-mer, and a 15% yield for a 95-mer. Whereas a synthesizer operating at 95% efficiency would have a 21% yield for a 30-mer, 8% yield for a 50-mer, and a 0.8% yield for a 95-mer. For these reasons it is important to understand that in a given oligonucleotide synthesis there are many other species of the oligonucleotide present. Purification of the product by HPLC or PAGE was the preferred method for reducing the other products.

Quantitation of the oligonucleotides was best determined by polyacrylamide gel electrophoresis, using dilutions of the stock rehydrated oligonucleotide and visually estimating the concentration. Early studies with the first generation protocol support this, where in one case the oligonucleotide was apparently degraded and spectrophotometric readings did not show this. The values obtained by the spectrophotometric readings are
not an indication of the quality of the DNA. The breakdown of DNA results in an erroneous spectrophotometric reading due to hyperchromicity. Therefore, the quality of the DNA strands were determined by gel electrophoresis in all subsequent experiments.

The initial attempts at ligation were problematic. Many attempts to ligate the synthetic DNA were unsuccessful. Controls had been utilized indicating that the activity of the ligase was adequate to ligate Lambda Hind III digested DNA. Many reaction conditions were tested in an attempt to ligate the T7 amplification sequence components that included: 1) varying the ionic strength, 2) changing the reaction temperature, and 3) purification of the oligonucleotides. These attempts were not successful. Other theories considered were: 1) either part or all of the cohesive termini on one or both of the fragments was missing, 2) there were one or more misincorporated bases in the cohesive ends preventing annealing and subsequent ligation, 3) since synthetic pieces of DNA are typically used as promoters or probes and not in constructing larger molecules of DNA, the way in which they were synthesized may prevent ligation from occurring.

An in-depth evaluation of the synthesizing procedure showed that during synthesis of the DNA the starting nucleotide did not have a 5' phosphate on the ribose molecule. The 5' phosphate was necessary for the formation of the 3' to 5' phosphodiester bond. The phosphodiester bond involves the linking of the 3 hydroxyl with the 5' phosphate of adjacent
nucleotides. Without the 5' phosphate, the complementary cohesive termini on each of the two fragments would hybridize, but the two molecules would not be covalently linked by the T4 ligase. Because the hydrogen bonding strength between the tetrad of the cohesive termini was weak, the molecules would readily disassociate into the original two individual fragments. This explained why the control Lambda DNA fragments, were ligated and the synthesized DNA was not ligated. All subsequent ligations incorporated the addition of a 5' phosphate group to the 5' termini using T4 polynucleotide kinase or the oligonucleotides had a restriction endonuclease recognition sequence incorporated in the DNA. A restriction enzyme was used to cut the annealed oligonucleotide to generate a 5' phosphorylated end.

**First Generation T7 Amplification Sequence**

The initial sequence design was to anneal two pairs of complementary oligonucleotides that would have protruding cohesive terminus for ligation. The cohesive termini were generated when complementary oligonucleotides of different lengths were annealed. One cohesive terminus was a Sal 1 recognition site and was placed between the viral recognition sequence and the T-7 promoter recognition sequence. The second cohesive terminus was at the end of the T-7 promoter and was used to ligate the detection sequence. A drawback with this design was that the oligonucleotides had to be phosphorylated prior to ligation and when it was time to insert them into a
vector (pUC 18) there was no cohesive terminus at the viral recognition end. Work with this sequence configuration was terminated due to the low concentration of the product, generation of more than one ligated product, and the overall difficulty in producing the final product.

**Second Generation T7 amplification Sequence**

The limited amount of success in the ligation of the original fragments was theorized to be a consequence of either the high degree of oligonucleotide impurity or a problem with the cohesive termini. A modification was incorporated in the T7 amplification sequence construction to cut the annealed product prior to ligation. Both *Sal* 1 and *Pst* 1 recognition sites were included in the design. The only difference between the first and second generation T7 amplification sequence was that the second generation required a restriction digest to produce the cohesive termini.

Two pairs of oligonucleotides, each having a length of 50 bases was obtained. Additional bases were added adjacent to the recognition sequence because inefficient cutting may occur if the restriction endonuclease recognition sequence is at the very end of the sequence. The advantage of this design is that the cohesive end would be intact and the termini would already be phosphorylated, eliminating the T4 polynucleotide kinase step.

This design was successful, however, the only foreseeable problem with continuing to work with this DNA was that the actual amount of the
desired product was minimal as determined by visualization of the agarose gel. This was the same problem encountered with the first generation sequence. However, these fragments were even longer, so the purity was even less than the original 27 to 43-base oligonucleotides. In the interest of time and considering that any subsequent purification after digestion would likely yield an insufficient amount of product of interest, further work with these fragments was terminated.

It was decided that the best approach would be to purchase the synthetic DNA from a company that specializes in the synthesis of oligonucleotides. Prior work with the original synthetic DNA was a valuable learning experience, but the actual purity of those fragments can now only be perceived to hinder any imminent progress.

Companies specializing in DNA synthesis claim they can produce long fragments (greater than 100 bases) with a high efficiency, yielding microgram quantities of a purified product. This alternative, along with procedures previously developed, would yield quantities of DNA that would facilitate future cloning experiments.

**Third Generation T7 Amplification Sequence**

The sequence of the original synthetic oligomer was modified. The new T7 amplification sequence was designed with restriction sites at both ends. The original T7 amplification sequence did not contain restriction sites
at the 5' end. This modification would facilitate its eventual ligation into a vector. The T7 amplification sequence was obtained as two complementary 95-base fragments. The supplier of the new oligonucleotides had guaranteed their ability to produce a 95-base, single-stranded product. However, the initial product received did not have a single band. The oligonucleotides had to be resynthesized and ultimately the product was shown to be acceptable.

The next step was to ligate the detection sequence to the synthetic fragments. The detection sequence was obtained by PCR amplification of poliovirus cDNA. The sequence was designed to have a Pst 1 terminus to ligate to the T7 amplification synthesized sequences and a Bam H1 termini to ligate to the pUC 18 vector.

**Assembly of the T7 Amplification Components**

The final procedure used to generate the complete T7 amplification sequence is outlined in Figure 4. This procedure was successful in the generation of a T7 amplification sequence that was cloned into pUC 18 and transformed into *E. coli*. The T-7 promoter region was demonstrated to be functional.

The T7 amplification sequence was designed to have a partial single-stranded and double-stranded portion. The single-stranded portion is complementary to poliovirus RNA and the double-stranded section is for the recognition of T7 polymerase to produce RNA transcripts. A diagram of a T7
amplification sequence reaction is shown in Figure 2.

An initial question was how the partially single-stranded and double-stranded regions would be generated. There were several ways considered to construct the working T7 amplification sequence. One possible way would be as follows: 1) amplify the entire T7 amplification sequence by performing a plasmid prep, 2) isolate the T7 amplification sequence from the plasmid by digestion, 3) purify the T7 amplification sequence from the plasmid, then digest a part of the T7 amplification sequence to separate the recognition sequence from the promoter and detection sequence, 4) isolate the promoter and detection sequence by gel electrophoresis and 5) combine equal amounts of the intact T7 amplification sequence and the digested portion, then denature by heating. As the mixture cools by random annealing, it would be expected that 25% of the starting components would be of the partially single \ double stranded configuration, with a recognition sequence complementary to the target. The other 75% would be a composite of the original fragments self annealed and a single \ double stranded portion with a recognition sequence homologous to the target. The annealing reaction would then be run on a gel to isolate the T7 amplification sequence.

Another way would be to produce the T7 amplification sequence was by PCR. Figure 17 illustrates the preparation using asymmetric PCR. A pilot study to demonstrate that asymmetric PCR would work had been done. Data had shown that both of the single strands were amplified, although the
PRODUCTION OF WORKING PROBE

Figure 17. Schematic depicting one of the ways that the partially single and double stranded T7 amplification sequence could be prepared. The use of restriction digests and asymmetric PCR are shown here.
concentration of the two bands was relatively low. Since asymmetric PCR results in a linear versus a logarithmic increase in the amplified product, the results of the two are not comparable. There are two ways in which the amount of product could be increased: 1) increase the concentration of the experimental template prior to amplification or 2) amplify the product with both primers, having one at a limiting concentration. A limiting primer would result in the initial amplification of the target region, as one primer is depleted, single-stranded copies would continue to be produced.

The second alternative was undertaken, the ratio of primers was set at 50:1, the same concentration of the template was used as in the single primer amplification. The results showed a considerable increase in the amount of amplified product, hence, the limiting primer procedure worked better than only incorporating a single primer.

**Development of the Capture Sequence**

The affinity between biotin and streptavidin was one reason why this system was chosen. Few molecules exhibit the strong natural affinity observed between biotin and streptavidin ($K_d = 10^{-15} \text{M}^{-1}$). Biotin is a small water soluble vitamin that can be readily included in an oligonucleotide during synthesis by using biotinylated nucleotides. Streptavidin is a tetrameric protein (MW 60,000) isolated from the bacterium *Streptomyces avidinii* and has four biotin-binding sites. Another advantage of this nucleic
acid capture system is that biotinylation almost never interferes with biological activity, and linker arms between the biotin and the nucleotide effectively minimize steric hindrance (Farrell, 1993).

The original concept of the capture technique was to use either a polystyrene micro-titer plate or polystyrene beads coated with streptavidin. However, the microtiter plate would not permit the interaction of the biotinylated capture complex and streptavidin in solution. The beads would permit a solution interaction, but washing steps would likely result in sample loss when the supernate was aspirated. Therefore, a novel approach was taken that used superparamagnetic polystyrene beads coated with streptavidin. When the beads were added to the biotinylated capture sequence / target complex, the streptavidin bound to the biotinylated hybrid. Under the influence of a magnetic field, the whole complex was temporarily immobilized onto the sidewall of the microfuge tube. The supernatant could then be aspirated from the tube and the beads could then undergo repetitive washing. The use of a magnetic capture system would ensure the complete removal of contaminants without the use of centrifugation and minimize the amount of sample loss.

In this study, a biotinylated synthetic 25-mer was used as the capture sequence. The rational behind a sequence of this length was that hybridization assays utilize probes in the 20 to 30 base range (Maniatis, 1989). However, a concern here was that hybrids formed using short nucleotides are
easier to unwind than those formed between a longer sequence (around 200 bases) (Maniatis, 1989). If there was a need to produce a longer capture sequence either a biotinylated synthetic sequence of up to a 100 bases can be synthesized or an asymmetric PCR procedure with a biotinylated primer and poliovirus cDNA template could be used.

The first experiment for the capture sequence was to determine if the capture sequence would function as a primer in the amplification of viral nucleic acid during RT-PCR. The capture sequence would initiate the formation of cDNA using poliovirus RNA as a template during reverse transcription. From experience it was found that RNase contamination can be a problem with synthetic sequences, these experiments would show if contamination existed. Poliovirus RNA was used as the template along with the biotinylated primer and an upstream primer in the PCR procedure. Results had shown that the capture sequence along with the upstream primer was successful at amplifying the target region.

The beads were then included in the experiment to show if the biotinylated capture sequence could be captured by the magnetic beads. This would be determined following hybridization of the target and capture sequence. Controls were included to demonstrate amplification resulted from the streptavidin bead capture of the capture sequence / target complex. The controls included an identical reaction without the addition of beads. The reaction without beads should not result in any amplification since the
capture sequence / target complex would be removed during the washing steps. It was shown that the reaction without beads did not exhibit any amplification, whereas the reaction with beads was amplified. This demonstrates that the beads could capture the biotinylated hybrid.

The next issue was to determine the effect of salt concentration on the reaction. According to the bead manufacturers instructions, when working with targets greater than 1-kb, the reaction mixture should contain a final NaCl concentration of 3 molar. This was included in newly revised instructions from the bead manufacturer. Previous work had used a 1 M NaCl concentration. The poliovirus genome is greater than 7-kb, therefore, this option was pursued.

A consequence of an increased NaCl concentration is the potential for non-specific hybridization; however, subsequent washing with a lower NaCl concentration along with a primer specific PCR amplification alleviated any problems. Another problem is the volume of NaCl that needs to be added to the reaction to obtain the desired molarity. With a 2.5 M final concentration 50% of the sample would be comprised of the aqueous NaCl (when 5 M NaCl is used). Since the ability of the biotinylated capture sequence and magnetic beads to successfully hybridize and remove the target is volume-dependent, the volume of NaCl may be excessive. It was necessary to add the salt in the crystal form to the sample to minimize the increase in sample volume.

In summary, the magnetic capture approach was designed to permit
the analysis of a greater sample volume and remove enzyme inhibitors. The superparamagnetic bead approach permits for the immobilization of the biotinylated capture sequence - target complex. The beads exhibit magnetic properties when placed in a magnetic field, but have no residual magnetism when removed from the magnetic field.

All reactions are performed in solution to provide optimal reaction kinetics between the beads and the capture complex. Washing the beads is facilitated by the ability to firmly immobilize the complex while within a magnetic field. A primary concern is that, in the liberation of the viral nucleic acid for subsequent hybridization and capture, there is the potential for target RNA degradation from RNase contamination. However, whenever RNA is liberated, especially in an environmental sample, there is always the risk of RNase's being present. The incorporation of a positive control would indicate if RNase contamination was present, and therefore was included in the analysis.
REFERENCES


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


CHAPTER FOUR

Development of a Nucleic Acid Capture Probe with Reverse Transcriptase-Polymerase Chain Reaction to Detect Poliovirus in Groundwater

ABSTRACT

Clearly, there is a need to develop a practical method for the detection of viral contaminants in water supplies. Practical methods need to be developed to detect viruses of public health importance. Traditional methods, using cell culture techniques, have limitations that preclude the detection of several of these viruses. Advances in nucleic acid technology have provided the sensitivity to detect viral nucleic acid without the use of cultivation. In this study, poliovirus was used as a model to test a nucleic acid capture technique. This technique was used to recover viral RNA from concentrated groundwater samples. Poliovirus RNA was isolated using magnetic bead technology. A biotinylated oligonucleotide probe was hybridized to poliovirus-RNA in solution. Streptavidin-coated magnetic beads were then added to isolate the RNA-oligonucleotide hybrid. The procedure allows for the recovery of viral RNA suitable for amplification by reverse transcription-polymerase chain reaction (RT-PCR). This nucleic acid-capture
system was effective in both concentrating, and purifying poliovirus RNA while removing environmental RT-PCR inhibitors. A detection sensitivity of one plaque-forming unit (PFU) in 250 µl of a concentrated environmental sample was routinely attained. This was the same detection level found with seeded purified water. It was shown that the sensitivity of nucleic acid capture RT-PCR was significantly greater than direct RT-PCR, when applied to environmental samples. The amplified product was sequenced to ensure specificity. Furthermore, this technique is rapid, reliable and can be readily adapted to detect other viral pathogens.
INTRODUCTION

Outbreaks of bacterial and viral enteric diseases are an important public health problem (LeGuyader et al., 1993). The public health burden of infectious diarrhea is substantial, particularly among children, both in the United States and worldwide (MMWR, 1990). Human enteric viruses can exist for extended periods in the environment and many survive conventional water and wastewater treatment. Viral gastroenteritis outbreaks have been associated with various sources of contaminated water, including municipal and well water (MMWR, 1990). Traditional methods used to concentrate and detect viruses are often labor intensive and have limited sensitivity.

Viral cultivation is a method of choice; however, it is not practical due to the inability to cultivate several viruses of public health importance, sample toxicity, and the lack of a universal cell line (Margolin et al., 1993). Due to cell culture limitations, research was directed toward the detection of viral nucleic acid. Nucleic acid probes offered a rapid and sensitive alternative for the detection of viruses in water and other environmental samples (Bruce et al., 1989; Gerba and Margolin, 1989, Margolin et al., 1993; DeLeon et al., 1990; Shieh et al., 1991). Even with this technology and the increase in sensitivity, direct identification of viral pathogens has been
extremely rare.

Detection methods based on the ability to amplify a microorganism's nucleic acid have gained considerable attention since the development of PCR (Abraham et al., 1993; Chapman et al., 1990). Two drawbacks in testing environmental samples by PCR are the presence of enzymatic inhibitors and the limited sample volume that can be assayed (Ma et al., 1995).

The use of RT-PCR requires extensive extraction procedures to remove the inhibitory substance(s) (Atmar et al., 1993). Various sample purification protocols have been developed in response to the type of environmental sample being tested (Graff et al., 1993). Purification protocols include organic solvents (Chapman et al., 1990) and gel filtration (Abbaszadegan et al., 1993). Again, these techniques are labor-intensive and incorporate multiple steps that could affect sensitivity.

To address these issues a rapid and sensitive viral RNA isolation procedure was developed. Nucleic acid capture is a novel method to isolate and purify target nucleic acid in an environmental sample. The technique incorporates streptavidin-coated superparamagnetic beads. The beads capture the biotinylated probe-target RNA complex. The principle here is the formation of a streptavidin - biotin bond that can be immobilized against the sidewall of the microcentrifuge tube when placed in a magnetic field. Here you have the removal of the target sequence from a heterogenous suspension. This system has been used to recover viral nucleic acid in
This capture system was applied to concentrated groundwater samples.

This report describes a procedure to recover poliovirus RNA selectively, followed by RT-PCR amplification. Examined here is the efficacy of a nucleic acid capture sequence to recover poliovirus RNA from seeded groundwater concentrates. This procedure is simple, rapid, and easily adaptable to other viruses of public health importance.
MATERIALS AND METHODS

Poliovirus Preparation

The Family Picornaviridae, genus Enterovirus, poliovirus type 1 (LSc strain) was used due to its widespread occurrence in environments, such as water. This would better enable evaluation of the system with environmental samples. Poliovirus was propagated by infecting Buffalo Green Monkey Kidney (BGM) cells. Flasks were incubated at 37°C until a generalized cytopathic effect was apparent. Flasks were then freeze-thawed three times to release cell-bound viruses, and the solution clarified by centrifugation. Virus was placed in screw-cap microcentrifuge tubes and held at -70°C.

Plaque assay. Virus was titered by the plaque-forming unit (PFU) method (Dulbecco and Vogt, 1954). Twenty-five cm² flasks of confluent BGM cells were inoculated in duplicate, each with 0.1 ml of each viral dilution. Adsorption of the virus was allowed to continue for 80 min at 37°C, flasks were rocked every 15 min. Following adsorption, the monolayer was washed with phosphate-buffered saline (PBS) and overlay with Medium 199 (Sigma, ST. Louis, MO) supplemented with 2% Serum (Sigma), 2% Bacto agar.
flakes (Difco, Detroit, MI), and neutral red (Sigma). Flasks were incubated at 37°C, until plaques were formed, typically 2-3 days, then counted and averaged.

**Environmental Water Samples**

Environmental water samples were collected using the microporous filtration elution technique. Between 80 and 300 gallons of water, depending on water type, were collected in large barrels and the pH was adjusted to 3.5 with 1 N HCl. Aluminum chloride was added to a final molarity of 0.1 mM. The water was filtered through an 8” negatively charged wound filter with a nominal pore size of 1 μm (Filterite, Timonium, MD) to which viruses adsorb. Viruses were eluted using 1.5% Beef Extract V (Difco), pH 9.5 and further concentrated by flocculation to a final volume of 30 ml using a previously described technique (Katzenelson et al., 1976). Once samples were concentrated, undissolved particles were removed by centrifugation (10,000 x g for 10 min) (Beckman model J2-21M, Fullerton, CA). Samples were held at -70°C until analyzed.

**Capture Sequence and Primers**

The poliovirus primer and probe sequences were selected from a highly conserved area of the 5’ noncoding region. The capture sequence and
primers were synthesized and polyacrylamide gel purified by Bio-Synthesis, Inc. (Lewisville, TX). The capture sequence consisted of the complement to nucleotides 644 through 620 (5'-ACT TTC ACC GGA TGG CCA ATC CAA T-3'). The capture sequence was biotinylated at the 5' end by the manufacturer during synthesis. Primers used for PCR are as follows, upstream, nucleotides 444 through 460 (5'-CCT CCG GCC CCT GAA TG-3') and downstream, nucleotides 638 through 621 (5'-ACC GGA TGG CCA ATC CAA-3'). These primers generate a 195-bp product. A diagram representation of the oligonucleotide primers and probe used in the process is shown in Figure 1.

Reverse Transcription and PCR

For both reverse transcription and PCR amplification procedures, optimal conditions were determined empirically. Components contained within the GeneAmp RNA PCR kit (Perkin-Elmer by Roche Molecular Systems, Branchburg, NJ) were used in all the reactions.

Each RT reaction was done in a 20 µl final volume, with a final concentration of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 5 mM MgCl\(_2\), 1 mM each of the deoxynucleoside triphosphates, and 0.75 µM downstream primer. The RNA was liberated from the viral capsid by heating the mixture to 99°C for 5 min. Mineral oil (Sigma) was added to control evaporation. Next, 20 U of RNase inhibitor and 50 U of M-MLV reverse transcriptase were added. The
Figure 1. Oligonucleotides used for magnetic capture, reverse transcription and PCR amplification. The numbers represent the nucleotide positions in the poliovirus genome.
RT temperature profile was 42°C for 60 min, 99°C for 5 min, and 5°C for 5 min. The reaction was done in a Perkin-Elmer Thermal Cycler 480.

Following RT, 80 µl of PCR mix was added to each RT tube. The final PCR concentrations were 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM MgCl$_2$, 0.15 µM upstream primer and 2.5 U of Taq polymerase. Amplification was conducted for 35 cycles with a temperature cycling profile of 95°C for 1 min, and 55°C for 1 min, followed by a final extension at 55°C for 7 min in a Perkin-Elmer Thermal Cycler 480.

**Electrophoresis.** Ten microliters of the PCR product was mixed with gel-loading solution (Sigma) and then separated on a 2.5% agarose gel (Sigma). After electrophoresis, the gel was placed in a solution of 0.5 µg/ml ethidium bromide and then visualized under a UV light (Fotodyne, New Berlin, WI). The PCR bands were compared with a molecular weight marker, pBR322 Hae III (Sigma), to show whether the 195-bp region was amplified.

**Sequence Analysis of the Amplified Product**

The cDNA PCR 195-bp amplicon was excised from a 2.5% agarose gel following electrophoresis and ethidium bromide staining. The cDNA was purified by an electro-separation system (S&S Elutrap, Schleicher & Schuell, Keene NH). The sequence was determined using an ABI PRISM™ Dye
Terminator Cycle Sequencing Kit with AmpliTaq® DNA polymerase, FS(Perkin Elmer) and an ABI 373A automated sequencer (Perkin Elmer).

**Streptavidin coated beads**

The superparamagnetic polystyrene beads (2.8 μm ± 0.2 μm diameter, surface area 3-8 m²/g), with streptavidin covalently attached, were purchased from Dynal (Lake Success, NY). The beads were washed before use by: 1) immobilizing the beads with a Dynal magnetic particle concentrator (MPC) (Dynal), 2) aspirating the supernate while the tube was in the MPC rack, and 3) adding a resuspension buffer (5 mM Tris-HCl, pH 8.0 and 1.0 M NaCl).

**Nucleic Acid Capture with the Superparamagnetic Beads**

**Purified water.** Initially, the magnetic beads were evaluated for the recovery of a target viral nucleic acid from seeded purified water (Sigma). The reaction mix contained 250 μl of purified water with a NaCl concentration of 2.5 M, 2.5 μl of 100 mM Tris-HCl (pH 8.0), 1 μl (40 μm stock solution) of the biotinylated capture probe, and 10 μl of the appropriate viral dilution (10² through 10⁻² PFU). The reaction tube was heated to 99°C for 10 min to liberate the poliovirus RNA from the viral protein coat. The temperature was then reduced to 42°C for 20 min to permit hybridization.

The supernate was transferred to another 0.5 ml siliconized tube
containing approximately 200 ug of previously washed streptavidin magnetic beads. The sample was gently mixed to resuspend the beads and incubated at 45°C for 45 min. The beads were continuously rotated to prevent the beads from settling and to maximize the bead / target interaction rate.

The beads were then concentrated by placing the tubes in the MPC rack to immobilize the bead complex against the microfuge tube side wall. For all of the immobilization steps the beads were held in the magnetic rack for at least 1 min before aspirating the supernate. The supernate was removed while the tube was in the MPC rack. The beads were washed three times by adding 200 μl of washing buffer consisting of 1.0 M NaCl and 5 mM Tris-HCl [pH 8.0], and the beads were gently resuspended by removing the tube from the magnetic rack and mixing by inversion. After the final wash, the supernate was removed and the tube was briefly centrifuged to facilitate the removal of any remaining wash buffer.

Positive and negative controls were also incorporated and are described below under system controls. The bead / target complex was then directly used in the reverse transcription and PCR steps as described above.

**Direct Amplification of Seeded Environmental Samples**

The effect of directly incorporating an aliquot of the environmental sample in the RT-PCR assay was examined. Ten concentrated environmental groundwater samples were randomly selected. A reaction was set up with the
same component concentrations as in the RT-PCR procedure described above with the following variations: 1) 2 \( \mu l \) of the concentrated groundwater sample was added to each reverse transcription reaction, 2) each reaction was seeded with \( 10^1 \) PFU of poliovirus, 3) the M-MLV reverse transcriptase and RNase inhibitor were omitted so the reaction mix could be heat denatured at 99°C for 5 min to liberate the viral RNA, and 4) after denaturation the M-MLV reverse transcriptase and RNase inhibitor were added. The remainder of the RT and the PCR procedures were identical to those previously described.

**Sensitivity of the Capture Probe / RT-PCR System**

The same ten environmental water samples were examined using the capture procedure (Figure 2). Environmental samples were first examined to detect if poliovirus nucleic acid could be detected without seeding the sample. For these reactions 250 \( \mu l \) of an environmental sample was used. The samples were prepared as above for nucleic acid target capture and RT-PCR, except that poliovirus was not added to the samples. Also, the sample was centrifuged for 30 s at 3000 rpm in a Sorvall MC12V (Dupont, Newtown, CT) before the supernate was transferred to the magnetic beads. Samples were examined for evidence of a 195-bp band indicative of poliovirus.

The next series of reactions were with poliovirus-seeded samples.
Figure 2. Flow chart of the nucleic acid capture / amplification procedure.
Poliovirus dilutions from $10^2$ to $10^{-2}$ PFU's were prepared and used to seed the 250 µl aliquot of each concentrated environmental sample. The capture and amplification steps were as previously described. Results were visualized by agarose gel electrophoresis and ethidium bromide staining.

**System Controls**

Positive and negative control reactions were incorporated throughout the study to ensure the system was functioning properly. Positive controls (poliovirus seeded) were essential to show that RNase and other components were not inhibiting amplification (false negative). Also, negative controls (without the poliovirus target) would show that amplification was not the result of cross contamination (false positive).
RESULTS

Poliovirus RT-PCR

Initial experiments evaluated the primers and biotinylated probe to ensure RT-PCR would result in the generation of the 195-bp target; consistent amplification was demonstrated. The two-step PCR procedures, consisting in part of a 55°C anneal \ extension temperature, resulted in a single band. Nonspecific products were not detectable by agarose gel electrophoresis. These data showed that the primers and probe would consistently and specifically amplify the poliovirus RNA target region.

Sensitivity of RT-PCR for Poliovirus

To find out the sensitivity of the RT-PCR system for poliovirus, RT-PCR was done on 10-fold serial dilutions of poliovirus. Amplification was detected out to the 10^-1 dilution (Figure 3). Agarose gel electrophoresis revealed the presence of a single 195-bp band as expected. This demonstrated that the RT-PCR conditions had a sensitivity suitable for environmental sample analysis.

Sequence of Amplified Product

The PCR product was compared against previously published sequence
data (Racaniello and Baltimore, 1981). The sequenced cDNA product was found to have a 100% match when compared with the published poliovirus sequence. This confirmed the specificity of the probe and primers to isolate and amplify poliovirus. The sequence is shown in Figure 4.

**Detection of Poliovirus RNA in Purified Water**

In order to evaluate the efficacy of nucleic acid capture, poliovirus seeded purified water was initially incorporated. A minimal detection level of $10^0$ PFU of poliovirus (using a 250 µl sample size) was detected.

**Detection of Poliovirus RNA in Groundwater**

The groundwater samples were first screened for the presence of poliovirus by nucleic acid capture RT-PCR. In all of the reactions, 250 µl of an environmental sample concentrate was analyzed. There was no amplification in all of the samples. A positive control (seeded with $10^2$ PFU of poliovirus) was used with each sample to ensure that the product would not inhibit poliovirus RNA amplification. All of the positive controls exhibited amplification.

The groundwater samples were next assayed to show if poliovirus seeded at several concentrations could be recovered. This would show the efficacy and sensitivity in the recovery of poliovirus RNA from seeded groundwater samples. Sensitivity was determined by seeding 250 µl aliquots
Figure 3. Ethidium bromide stained agarose gel electrophoresis of poliovirus RNA by RT-PCR. Serial dilutions of poliovirus ranging from $10^2$ to $10^7$ PFU (lane 1 through 5), lane 6 is the negative control (no target) and M is the molecular weight marker. The arrow indicates the position of the 195-bp band.
<table>
<thead>
<tr>
<th>Sequence</th>
<th>CCGT</th>
<th>CCGT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCTCCGGCCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTGAATGCGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTAATCCCAA</td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>CCTCGGAGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GGTGGTCAACA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AACCAGTGAT</td>
<td></td>
<td>60</td>
</tr>
<tr>
<td>TGGCCTGTGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAACGCAGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTCCGTGGCCG</td>
<td></td>
<td>90</td>
</tr>
<tr>
<td>GAACCGACATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTTTGGGTGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCGTGTTTCC</td>
<td></td>
<td>120</td>
</tr>
<tr>
<td>TTTTATTTTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TTGTGGCTGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TTATGGTGAC</td>
<td></td>
<td>150</td>
</tr>
<tr>
<td>AATCACAGAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGTTATCATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAGCGAATTG</td>
<td></td>
<td>180</td>
</tr>
<tr>
<td>GATTGGCCAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCGGT</td>
<td></td>
<td>210</td>
</tr>
</tbody>
</table>

**Figure 4.** Sequence of the 195-bp PCR amplicon. Sequence was determined with an ABI PRISM™ Dye Terminator Cycle Sequencing Kit with AmpliTaq® DNA polymerase, FS (Perkin Elmer) and an ABI 373A automated sequencer (Perkin Elmer).
of the environmental sample with tenfold dilutions ($10^2$ through $10^{-2}$ PFU) of poliovirus. The nucleic acid capture procedure was employed along with RT-PCR. The results (Table 1) showed that poliovirus at $10^2$ PFU was recovered from all ten samples, $10^1$ PFU was recovered from nine of ten samples, and $10^0$ PFU recovered from eight of ten samples. At the $10^{-1}$ and $10^{-2}$ PFU concentrations, no amplification was detected in all 10 samples.

**Direct Viral RNA Detection in Seeded Groundwater**

This test evaluated the effect on target amplification when the groundwater sample was directly added to the RT-PCR assay. Nucleic acid capture RT-PCR was not used. Each RT-PCR assay was seeded with $10^2$ PFU of poliovirus. The results showed that there was no target amplification in all of the samples. This showed that the direct incorporation of the concentrated groundwater sample inhibited the enzymatic amplification of poliovirus RNA. Removal of the RT-PCR inhibitors was necessary.
Table 1. Seeded environmental sample results and controls.

<table>
<thead>
<tr>
<th>SAMPLE* #</th>
<th>Poliovirus PFU</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^2$</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
</tr>
</tbody>
</table>

$a =$ All sample reactions contain 250 µl of the environmental water sample

$b =$ negative control where poliovirus was not added to the reaction

$c =$ contained purified water in place of the environmental sample
DISCUSSION

The continued presence of human enteric viruses in the environment has led to periodic outbreaks of non-bacterial gastroenteritis. The difficulty comes when samples from these areas need to be tested for viral contamination. Viral cultivation, although a method of choice, is not practiced for routine monitoring due to factors such as: labor intensity, expense and the fact that some viruses of principle public health importance cannot be cultivated. Direct identification of viral pathogens in incriminated products has been extremely rare and difficult because of the lack of a sensitive and specific assay (Gouvea et al., 1994). Detection methods based on the presence of a microorganism's nucleic acid have gained considerable attention since the coming of PCR. The effectiveness of RT-PCR is dependent upon the ability to liberate the target organism's nucleic acid and remove components that are inhibitory to the enzymatic process.

Various environmental sample purification protocols have been developed in response to the type of environmental sample being tested (Graff et al., 1993). These protocols include, organic solvents (Regan et al., 1993), gel filtration (Abbaszadegan et al., 1993), guanidine isothiocyanate (Gouvea et al., 1994) and antigen capture (Graff et al., 1993). The development of a single technique would be advantageous for universal application.
A drawback of RT-PCR is the amount of a sample a single reaction can accommodate. Although others have scaled-up the reaction to accommodate an increased sample (Abbaszadegan et al., 1993; De Leon et al., 1990) there are limits. Experiments comparing the sensitivity of PCR and cell culture have shown that PCR was one log unit greater than cell culture (Abbaszadegan et al., 1993). Whereas an increase in sensitivity for viral detection is beneficial, it is also important to evaluate as much sample as possible, since viruses in water are not evenly distributed and viral clumping readily occurs.

This study was undertaken to develop a rapid method for the isolation of viral nucleic acid from environmental samples. The principal objective was to incorporate a procedure to remove RT-PCR inhibitors and permit the analysis of a larger sample volume. Nucleic acid capture using streptavidin-coated magnetic beads was shown to accomplish both objectives. Also, it was not labor-intensive and hazardous organic reagents are not used.

Magnetic capture systems were originally developed for use in immunoassays (Heermann et al., 1994). An advantage of the magnetic capture system was that the probe and target interact in solution, allowing for a second order kinetic reaction. A solution hybridization would eliminate the steric restraints found in systems where the probe was immobilized to a solid support. A streptavidin magnetic bead system was employed to capture the hybrids. Streptavidin bound to the magnetic beads was chosen over streptavidin bound to a fixed solid support. The magnetic beads would have
a greater interaction rate since they are in suspension with the target. The continual rotation of the mixture would further increase the interaction rate. A previous study (Suzuki et al., 1993) had demonstrated that there was a sixfold increase in the binding rate of the magnetic bead system versus one where streptavidin was bound to a microtiter plate. Magnetic beads have been successfully used for the detection of enteric viruses in clinical samples (Muir et al., 1993) and in the recovery of HIV and HBV from clinical specimens (Suzuki et al., 1993 and Heermann et al., 1994).

Initially, poliovirus was used as a model virus due to its availability and the expected widespread presence of the vaccine strain in the environment. Furthermore, in evaluating the efficacy of this assay with environmental samples, there was a greater chance of detecting poliovirus than other enteric viruses that can be grown on the BGM cell line. The detection of poliovirus would indicate the potential for other pathogenic human enteric viruses.

In this study, each environmental water sample was divided into two aliquots. One aliquot was to show the sample's inhibitory properties and the other to show the efficacy of the magnetic beads. In a 20 μl reverse transcription reaction mix, which included 2 μl of the environmental sample, target amplification was inhibited in all of the seeded samples. This demonstrated that even a small sample volume can be inhibitory to RT-PCR.

The biotinylated DNA probe used in this study was complementary to
a conserved area in the poliovirus genome. The advantage of this probe was that it also functioned as the initiation site for reverse transcription. Following RT the cDNA generated upstream from the probe would function as the PCR target area.

In this study, there were times where the beads stuck to the tube sidewall. When functioning properly, the beads should form a pellet at the base of the tube next to the magnet. All the beads were not affected by the magnet. Lightly tapping the rack and tube would free-up some beads; however, it was not completely effective. This situation could result in a loss of sensitivity. The bead manufacturer’s (Dynal) recommendation was to try another source for the microfuge tubes. Because of this, siliconized microfuge tubes (USA Plastics) were found to reduce the occurrence.

A series of poliovirus dilutions were analyzed to decide the sensitivity of RT-PCR and to ensure reaction component concentrations were optimum. The RT reactions were done in 20 μl volumes and did not employ the capture probe. Liberation of the poliovirus genome was done by heat denaturation. The poliovirus control assays demonstrated that the minimal detectable level was 10⁻¹ PFU. This was comparable to that found in another study (Abbaszadegan et al., 1993).

The efficacy of the magnetic capture system was first determined with poliovirus seeded, purified water (Sigma). The minimal level of recovery was 10⁰ PFU. This was one log less sensitive than shown in the RT-PCR
assays using poliovirus dilutions without the nucleic acid capture procedure. Loss in sensitivity was likely due to the multiple steps involved in the capture procedure. These steps include a series of washes and transfers in which target may be lost. Another area to consider was the efficiency of biotinylation during oligonucleotide synthesis. There was a chance that a non-biotinylated probe could hybridize and prevent RNA recovery.

The magnetic capture / RT-PCR system was evaluated using seeded groundwater samples. Initially, not all samples amplified with equal success. In theory, this could be the result of: 1) the presence of RNase, which would degrade the poliovirus RNA template, 2) inhibitory products in the sample or, 3) the presence of DNase, which could destroy the probe. Several experiments were done to address the above issues and included: 1) addition of a RNase inhibitor, 2) increased the number of washes and, 3) heated the sample before analysis to inactivate DNase. None of the modifications resulted in amplification. This could have been the result of enzymatic inhibitors that were binding to the beads or the microfuge tube and could not be removed in the washing steps.

An observation during these experiments was that once the samples were heat-denatured and hybridized, a precipitate was observed in the tubes. An attempt was made to remove the precipitate by centrifuging the sample for 30 s at 3k RPM. The supernate was transferred to a new microfuge tube containing the beads. The brief centrifugation step resulted in a more
consistent amplification of the poliovirus-seeded samples. For reasons unknown, inhibitory components that could not be removed by the washing steps may have been carried over. Once these inhibitory products were removed, amplification returned. Another study, which also used a magnetic capture system, incorporated a brief centrifugation step before addition of the magnetic beads (Muir et al., 1993).

A comparison between directly testing the groundwater sample (2 μl tested) and testing the groundwater sample using nucleic acid capture (250 μl tested) was done. The results had shown that as little as 2 μl of the sample prevented amplification in a reaction seeded with $10^2$ PFU of poliovirus. This confirms the inhibitory effects previously addressed by others (Abbaszadegan et al., 1993 and Ma et al., 1995). These results were the impetus for developing a more sensitive method.

In the examination of the seeded environmental samples 2 of 10 samples did not show any amplification at the $10^0$ PFU and one of those two did not exhibit amplification at the $10^1$ PFU. Despite this, most of the samples had a level of detection that was comparable to the purified water assays ($10^0$ PFU). There was no amplification at the $10^{-1}$ and $10^{-2}$ PFU in all the reactions. This was expected, whereas this level of sensitivity was not reached in the purified water assays. Overall, the decrease in sensitivity may have been the result of RNA degradation or the presence of an interfering substance that
was not removed in the washing steps.

In all of the nucleic acid recovery and amplification reactions, the amplification product was a single band of the predicted size (195-bp). Furthermore, the amplified product was isolated and sequenced to confirm the amplification of poliovirus genomic RNA. A perfect match was found between the amplified product and the published poliovirus sequence.

A concern with this type of assay was the presence of nucleases in the environmental sample. A RNase inhibitor was not included during the hybridization or capture procedures, but was added to the RT reaction. The decision not to add a RNase inhibitor was based solely on the satisfactory positive control results obtained with the seeded groundwater samples. The incorporation of a positive control (poliovirus) would show if nucleases were present.

In summary, this method has significant practical applications whereas 250 µl sample could be analyzed for poliovirus by incorporating nucleic acid capture. Furthermore, this study shows that target nucleic acid can be selectively removed from an environmental sample solution with streptavidin magnetic beads and a specific biotinylated probe. Once the sample was concentrated, the total time in which results can be obtained was less than 6 hr.

A concern with this type of assay was the presence of nucleases in the environmental sample. A RNase or DNase inhibitor was not included
during the hybridization or capture procedures. The decision was based solely on the positive results obtained with the environmental samples. The incorporation of positive controls would show if nucleases were present. In future work if nucleases are of concern, an RNase inhibitor can be added to the sample.

Genomic amplification systems cannot differentiate between infectious and noninfectious viruses directly, but can be used in conjunction with cell culture for this determination. However, these techniques should be invaluable in the surveillance of water and shellfish contamination or detection of virus following outbreaks of non-bacterial gastroenteritis. Moreover, the assay was easily adaptable to other viruses simply by generating a new biotinylated capture sequence and primer set.
REFERENCES


CONCLUSION

The microbiological safety of our food and water supply is partially dependent on the development of effective test methods to detect disease-causing agents. Through periodic monitoring, these test methods are used to evaluate food and water for microbial contaminants of public health importance. When it comes to methods available to detect viruses in the environment, there is an analytical void.

The control of infectious diseases is difficult due to diversity of disease-causing organisms present in the environment. The reliance on microbial indicators, good manufacturing processes and regulations such as Hazard Analysis and Critical Control Point (HACCP) has reduced the incidence of disease. For that reason, indicators have been used to determine the microbiological quality of a given area.

There have been two principal areas where indicators have been used in the determination of sanitary quality. One is in the water industry where coliforms and fecal coliforms have been the "gold standard". The other is in shellfish and their growing waters. Although the National Shellfish Sanitation Program (NSSP) bacterial indicator system has decreased the incidence of shellfish-associated enteric disease, its efficacy as a reliable indicator for protecting against the presence of human enteric viruses is
questionable. The inadequacy of the current bacterial indicator standard is primarily a result of the lack of parity in the survival characteristics of fecal coliforms and human enteric viruses. This addresses a larger issue, the use or reliance on a single organism “the magic bullet” to act as a predictor for anything else but other organisms of similar characteristics.

The public relies on state and federal public health officials for protection from foodborne and waterborne illnesses. When a foodborne or waterborne disease occurs, an investigation is carried out to determine the causative agent. The purpose of the investigation is to detect implicated products, identify the causative agent, and determine the factors that contributed to the contamination. In most outbreaks of gastroenteritis, the etiological agent is typically unidentified, and falls into a general category of nonbacterial gastroenteritis. In part, this is the result of inadequate methods to detect viruses in environmental samples. Without conclusive evidence of the actual cause of an illness it is extremely difficult to set up adequate safeguards.

The incidences of foodborne and waterborne illnesses are greatly underestimated due to the lack of available test methods. The number of incidents and the economic impact of foodborne and waterborne illnesses is difficult to measure. Follow-up investigations of foodborne or waterborne outbreaks are often inconclusive. Additionally, many cases are not reported due to the self-limiting nature of the illness. Sensitive and accurate analytical
methods are needed to provide accurate and conclusive information about the etiological agent implicated in an outbreak.

The focus of this research was the development and evaluation of molecular techniques used for the detection of human enteric viruses. Over the past several years several methods have been explored to detect enteric viruses in shellfish and water. Presently, there is no widely accepted methodology to detect human enteric viruses of public health significance.

Viral cultivation is a preferable means to isolate viruses, but not practical due to; long detection time, sample toxicity, lack of a universal cell line and the difficulty and inability to cultivate several viruses. Therefore, there is a need to develop other techniques to detect these viruses.

The development of molecular techniques provided a means to circumvent the need to first cultivate the virus prior to detection. Nucleic acid hybridization has been successfully used to detect viral pathogens in environmental samples. In this report, the Narragansett Bay study looked at the application of nucleic acid hybridization for the detection of poliovirus in clams. The results of that study support those reported by others, which suggest that bacterial indicators and standards, while serving to adequately protect against bacterial pathogens in shellfish, do not reliably predict the presence of human enteric viruses.

Nucleic acid hybridization procedures have a limited sensitivity. The need for extensive sample extraction only serves to further reduce the
sensitivity of the test. Other factors, such as the use of a radioactive isotope, short half-life of the probe, and the use of organic solvents, further diminished its widescale acceptance.

The advent of nucleic acid amplification using the polymerase chain reaction (PCR) technique rapidly replaced the use of nucleic acid probes alone. The ability to exponentially amplify the nucleic acid of a target microorganism was seen as a way to increase the probe sensitivity while decreasing the associated labor intensity. However, as with any new assay, it was soon determined that the degree to which PCR is effective was dependent on the type of sample assayed.

The success of PCR is dependent on the ability to recover the target from the test matrix and to effectively remove substances that inhibit the amplification process. An additional drawback of PCR is that it was originally designed to amplify DNA and not RNA. The nucleic acid of enteric viruses is RNA. The protocol for the detection of an RNA virus by PCR first requires the conversion of RNA to cDNA. The production of cDNA is a separate reaction that uses reverse transcriptase. Conventional reverse transcriptase (RT) reactions are fastidious and use enzymes that cannot tolerate temperatures above 42°C, which is a nonstringent hybridization. Also, the presence of secondary and tertiary structures in single stranded RNA, that is not disrupted at the RT incubation temperature can result in widely varying efficiencies of conversion.
While there has been the discovery of thermostable enzymes that can transcribe RNA, it must be realized that the amplification potential of RT-PCR can be severely limited or even inhibited by environmental contaminants. Therefore, the amplification potential of PCR is directly dependent on the quality of the sample.

Lastly, PCR was originally designed for use in clinical samples. Clinical samples, unlike environmental samples, usually contain a much higher concentration of target nucleic acid. This permits the evaluation of much smaller sample volumes (2-10 µl). Unfortunately, concentrated environmental samples can range from 5 to 30 ml. Evaluation of even a third of the sample by PCR would be cost ineffective and impractical.

There was much room for improvement in RNA detection by molecular techniques. Several areas explored were: 1) the use of a nucleic acid based amplification system for RNA targets that is not dependent on reverse transcriptase and is amenable to non-isotopic reporter molecules, b) an improved method to isolate and concentrate the viral target in an environmental sample, and c) a method to remove the enzymatic inhibitors inherent in many environmental samples. The second part of this research looked at the inherent problems of RT-PCR and ways to improve the analytical sensitivity.

Two molecular tools were developed and evaluated. First was the construction of a T7 polymerase-based amplification sequence to indirectly
amplify target RNA without the use of reverse transcriptase. The T7 amplification system was designed with a viral recognition sequence, a T7 polymerase recognition sequence and a detection sequence for the generation of RNA transcripts. The T7 amplification sequence was successfully constructed from nucleic acid subunits, cloned, and transformed into E. coli. The T7 amplification sequence was shown to be functional by its ability to produce radiolabeled RNA transcripts.

The T7 system was designed for easy modification to detect other viruses by the insertion of other complementary viral sequences. Longer sequences could be added to permit higher hybridization temperature to disrupt secondary and tertiary structures contained within the viral genome. Additionally, nonisotopic reporter molecules can be directly incorporated into the RNA transcript. The incorporation of labeled bases would permit amplification of not only the detection sequence, but also the reporter molecule, further enhancing the sensitivity.

The second technique was the development of a procedure to selectively isolate viral nucleic acid from an aqueous mixture. In a standard RT reaction, only 3 μl of a sample can be used. The reaction can be scaled-up, but there is also a risk of increasing the level of enzymatic inhibitors inherent in many environmental samples. The main objective here was to develop a procedure that would permit the removal of RT-PCR inhibitors and permit the analysis of a larger sample volume. Nucleic acid capture using
streptavidin coated superparamagnetic beads was shown to accomplish both objectives. Also, it is not labor-intensive, results can be obtained in one day, and hazardous organic reagents are not necessary.

The principle of the capture technique was the use of a biotinylated capture sequence to hybridize to the target viral nucleic acid. Streptavidin coated superparamagnetic beads were used to isolate the RNA-oligonucleotide hybrid. The beads are magnetic in a magnetic field, but nonmagnetic as soon as the field is removed. This procedure allows for the selective recovery of viral RNA from a sample matrix by the immobilization of the magnetic bead-target molecule complex. By immobilizing the target complex, larger sample volumes can be evaluated, the supernate containing most of the inhibitory products can be removed, the beads can be washed to remove any remaining traces of the inhibitory products, and the target can be resuspended in a minimal volume of buffer. The end result is a product suitable for amplification by RT-PCR. This system has been shown to be effective in the recovery of poliovirus from seeded groundwater samples. The success with groundwater demonstrates its potential widespread application to other environmental matrices, such as surface water, and shellfish. Combining this system with cell culture will also permit the differentiation of infectious and noninfectious viruses. It will also circumvent some of the classical problems with classical cell culture, such as long incubation times. Increased amplification will permit detection much
earlier in cells. The combined use of both systems will help establish routine monitoring of systems where determination of viral infectivity is critical, such as with treated or disinfected water.

In summary, rapid methods and automation are a dynamic area in applied microbiology. Molecular techniques are not the panacea to environmental microbiology, but currently offer the most promise in helping determine how pervasive viral contamination is in the environment. The development of a viral monitoring system to better meet the safety needs of the public is long overdue. It is envisioned that a more comprehensive system for monitoring will be multi-faceted and look not only at bacterial, but also viral pathogens to better understand the scope of the problem. Environmental virology holds many unknowns, and the key to understanding the problems are dependent on the development of rapid and sensitive methodologies. The molecular tools developed in this study were designed to be versatile. Nucleic acid sequences complementary to other human enteric viruses can easily be generated and incorporated in these techniques. Lastly, this research paves the way for further studies that can apply the capture sequence and T7 amplification sequence procedures to other areas in environmental virology. Such as the monitoring of potable water, shellfish growing areas, shellfish, and as an epidemiology tool for the investigation of outbreaks of viral origin.
LITERATURE CITED


204


Cabelli, V. J. 1988. Microbial indicator levels in shellfish, water, and sediments from the upper Narragansett Bay conditional shellfish growing area. Report to the Narragansett Bay Project, Providence, RI.


Guzewich, J. J. and D. L. Morse. 1985. Sources of Shellfish in outbreaks of...


Le Guyader, F., E. Dubois, D. Menard, and M. Pommepuy. 1994. Detection of


212

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Van Patten, P. 1990. Can the "effluent society" deal with its waste? Nor'easter Fall: 38-42.


APPENDIX A

BUFFERS AND REAGENTS

30% Acrylamide

Dissolve 29 g of acrylamide and 1 g of N, N'-methylenebisacrylamide in a total of 60 ml distilled water. Heat to 37°C to dissolve components. Adjust the volume to 100 ml with distilled water. Filter through a 0.45μm filter and store in a sterile container at 4°C.

10% Ammonium Persulfate

To 1 g of ammonium persulfate add distilled water to 10 ml. Store the solution at refrigerated temperature for up to three weeks.

Diethyl Pyrocarbonate (DEPC) Treated Water

Distilled water treated by adding 0.1% diethyl pyrocarbonate. The mixture is held overnight at room temperature and then autoclaved at 121°C for 30 min to remove the DEPC.

0.5 M EDTA

Dissolve 186.12 g of Na₂EDTA in 800-900 ml of water, adjust the pH to 8.0 with 10 N NaOH. Add distilled water to make 1 liter. Autoclave 15 min at 121°C.

Ethidium Bromide (10 mg/ml)

Add 1 g of ethidium bromide to 100 ml of distilled water. Stir until dissolved. Place the solution in a glass amber bottle and store at room temperature.
Deionized Formamide

Stir 100 ml of formamide with 5 g of AG501-X8 resin (Bio-Rad), stir for 1 hr. Filter the sample through a Whatman #1 paper.

1 N HCl

Add 89 ml of HCl (concentrated) to 800 ml of distilled water. Add additional distilled water to make 1 liter.

1 N NaOH

Transfer 40 g of NaOH to a flask, add distilled water to make 1 liter. Autoclave 15 min at 121°C.

Phenol : Chloroform : Isoamyl (PCI)

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 ml</td>
<td>phenol</td>
</tr>
<tr>
<td>48 ml</td>
<td>chloroform</td>
</tr>
<tr>
<td>2 ml</td>
<td>isoamyl alcohol</td>
</tr>
</tbody>
</table>

Mix well. Store Phenol:chloroform:Isoamyl in an amber bottle for up to 2 months at 4°C.

Phenol:Chloroform

Mix equal volumes of phenol and chloroform and place in an amber bottle at 4°C.

3.0 M Sodium Acetate

Dissolve 408.1 g of NaAc·3H₂O in 800 ml of distilled water. Adjust the pH to 5.2 with glacial acetic acid. Add additional water to make 1 liter. Dispense into aliquots and autoclave 15 min at 121°C.
**5 M Sodium Chloride**

Transfer 292.2 g of NaCl to a flask and add 800 ml of distilled water. Adjust volume to 1 liter and dispense into aliquots. Autoclave 15 min at 121°C.

**10% Sodium Dodecyl Sulfate (SDS)**

Dissolve 10 g of SDS in 50 ml of distilled water, warm to 68°C to assist in dissolution. Dilute to 100 ml and store at room temperature.

**20X SSC**

Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of distilled water. Adjust pH to 7.0 with 10 N NaOH. Bring the volume to 1 liter with distilled water. Autoclave 15 min at 121°C.

**TBE Buffer**

- Tris base 108 g
- Boric acid 55 g
- 0.5 M EDTA 40 ml

Dissolve in 800 ml of water, adjust pH to 8.0 and bring final volume to 1 liter. Autoclave 15 min at 121°C and store at room temperature.

**Trichloroacetic Acid (TCA)**

To a container with 500 g of TCA, add 227 ml of water. The resulting solution contains 100% (w/v) TCA.

**Phosphate Buffered Saline (PBS)**

Combine 8 g NaCl, 1.44 g Na₂HPO₄, and 0.24 g KH₂PO₄ in 800 ml of distilled water. Adjust the pH to 7.4. Add water to make 1 liter. Autoclave 15 min at 121°C.
APPENDIX B

MEDIA

Azide Dextrose Broth
Dissolve 34.7 g azide dextrose broth (Difco) into 1 liter distilled water.

Brilliant Green Lactose Bile Broth (BGLB)
Dissolve 40.0 g EC (Difco) into 1 liter distilled water. Transfer 10 ml to 18 x 150 mm test tubes containing inverted 10 x 75 mm fermentation tube. Autoclave for 15 min at 121°C.

Escherichia coli (EC) Broth
Dissolve 37.0 g EC (Difco) into 1 liter distilled water. Transfer 10 ml to 18 x 150 mm culture tubes containing inverted 10 x 75 mm fermentation tube. Autoclave for 15 min at 121°C.

EC-MUG
Prepare as for EC except add 50 mg 4 methylumbelliferyl-beta-D- glucuronide per liter before autoclaving.

Iron Milk Medium
Fresh whole milk 1 liter
Ferrous sulfate 7H2O 1 g
Distilled water 50 ml

Dissolve ferrous sulfate in 50 ml of distilled water. Add slowly to 1 liter of milk and mix with a magnetic stirrer. Dispense 11 ml of the medium into 18 x
150 mm culture tubes. Autoclave for 12 min at 118°C.

**Lauryl Sulfate Broth (LST)**

Dissolve 35.6 g EC (Difco) in distilled water. Transfer 10 ml to 18 x 150 mm culture tubes containing inverted 10 x 75 mm fermentation tube. Autoclave for 15 min at 121°C.

**Luria Bertani (LB) Agar**

Dissolve 40 g of LB (Difco) in distilled water and bring volume to 1 liter. Autoclave for 15 min at 121°C.

**Luria Bertani (LB) Broth**

Dissolve 25 g of LB (Difco) in distilled water and bring volume to 1 liter. Autoclave for 15 min at 121°C.