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The development of RNA probe and RT-PCR assays for the detection of enteroviruses in sludge

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THE DEVELOPMENT OF RNA PROBE AND RT-PCR ASSAYS FOR THE
DETECTION OF ENTEROVIRUSES IN SLUDGE

BY

AMY E. MOORE

B.A. University of New Hampshire, 1989

DISSERTATION

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

Doctor of Philosophy

in

Microbiology

December, 1999

UMI Number: 9953423

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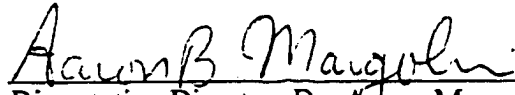
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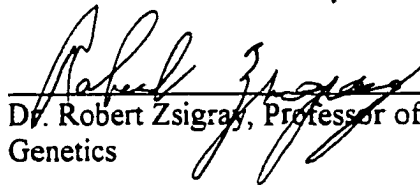
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DEDICATION

To Norm, Nicholas, Andrew, Mom, Dad, Beth, and Gram. I am very fortunate to have a family that has always encouraged and supported me. I love you all.

ACKNOWLEDGEMENTS

I would like to thank all my committee members, Dr. Aaron Margolin, Dr. Robert Zsigray, Dr. Thomas Pistole, Dr. Stephen Torosian, and Dr. James Malley for sharing their knowledge and advice during my dissertation. I would especially like to thank Aaron for standing by me on a professional, as well as personal basis.

I am thankful that I was able to share my graduate school experience with so many wonderful people. Whether near or far, my friendships with Nicola, Chris, Kim, Carlos, Shannon, and Karen will always be remembered. I would also like to thank Bob Mooney for his friendship and technical expertise.

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ABSTRACT

THE DEVELOPMENT OF RNA PROBE AND RT-PCR ASSAYS FOR THE DETECTION OF ENTEROVIRUSES IN SLUDGE

by

Amy E. Moore

University of New Hampshire, December, 1999

Many wastewater treatment plants generate more sludge than can be disposed of by conventional means. The United States Environmental Protection Agency (USEPA) has encouraged communities to dispose of sludge by land application. Sludge may contain enteric viruses that are known to survive for long periods of time in sludge-amended soil and can travel great distances, potentially contaminating surface and ground water.

Standard cell culture methods for the detection of enteric viruses are costly and results are not obtained for 30 or more days. The development of methods that provide results more quickly and with lower cost are needed.

A ³²P labeled RNA probe was developed for the detection of poliovirus in sludge. The probe detected 10 fg of poliovirus RNA transcripts and 90 pfu of poliovirus type 1 (LSc). RNA probe and plaque assays were used to evaluate beef extract elution methods for the isolation of poliovirus from sludge. Homogenization of sludge after the addition of beef extract powder at a pH of 7.0 resulted in the highest recovery of seeded poliovirus. Additionally, proteinase k digestion was found to result in a greater detection sensitivity than organic extractions.

Small reaction volumes and the presence of inhibitors in environmental samples have limited the use of the highly sensitive and rapid technique of RT-PCR. A magnetic separation procedure using oligo dT paramagnetic beads was developed to capture enterovirus RNA from 900 μ l of sample. This method resulted in a 90-fold sample concentration, removal of RT-PCR inhibitors from lime stabilized sludges, and a detection sensitivity of 5 pfu of poliovirus type 1 (LSc). Eight lime stabilized sludge concentrates were evaluated by RT-PCR with magnetic bead capture and by plaque assay. Enteroviruses were not detected by either method.

Results may be obtained from RT-PCR within hours and at a cost much lower than the plaque assay. This method could be useful as a rapid screening technique. The direct monitoring of pathogens, such as enteric viruses, instead of the reliance upon indicator organisms, may reduce the risks from land application of sludge and make this practice more acceptable to a greater number of communities.

Introduction

Over 120 viruses are members of a diverse group known as the human enteric viruses. The enteric viruses are further divided into groups based on morphological, physical, chemical, and antigenic differences (Straub et al., 1993). All enteric viruses replicate in the gastrointestinal tract and are transmitted by the fecal-oral route. Enteric viruses are excreted in high numbers by infected individuals for periods of time averaging 50 days (Rao, 1986). Enteric viral infections are very common in young children. Studies have shown that approximately 10% of children in the United States under 15 were excreting viruses (Rao and Melnick, 1986). After the initiation of the oral poliovirus vaccine, the most frequent viral isolates from wastewater have been the vaccine strains of poliovirus (Rao and Melnick, 1986). It has been estimated that a concentration of more than 7000 enteric viruses per liter is present in wastewater (Straub et al., 1993).

Enteric viruses are a leading cause of gastroenteritis and include hepatitis A and E viruses, Norwalk virus, rotaviruses, adenoviruses, reoviruses, astroviruses, and enteroviruses. The enterovirus group includes polioviruses, echoviruses, and coxsackie viruses. As few as one virus may lead to infection. Myocarditis, meningitis, encephalitis, and paralysis may also result from infection with these pathogens, although asymptomatic infections are more common. Melnick (1947) calculated the ratio of asymptomatic cases from poliovirus infection to paralytic cases to be well over 100:1. In

contrast, during outbreaks of hepatitis A virus, the symptomatic infection rate may be as high as 95% (Lednar et al., 1985).

Until the 1930's viruses were not thought to survive outside of the host and poliovirus was not recognized as an enteric pathogen. Before this time, most virologists thought the virus entered the body through the nasal passage and proceeded directly to the central nervous system (Metcalf, 1995). Re-examination of a report from 1912 by Swedish researchers that documented the isolation of poliovirus from the feces of sick and healthy people refuted this theory (Metcalf, 1995). Subsequently, poliomyelitis was considered an enteric disease (Melnick, 1946).

Spread of poliovirus by people with asymptomatic infections was then demonstrated (Rao and Melnick, 1986). Steps to limit fecal contamination in households and hospitals were undertaken to minimize exposure to poliovirus. Transmission of poliovirus through ingestion of sewage contaminated water was investigated. Poliovirus was detected in monkeys after inoculation with environmental samples, yet after extensive research, only a few outbreaks of poliomyelitis have been linked to exposure to polluted water (Rao and Melnick, 1986). However, numerous outbreaks from infection with other enteric viruses have been reported. Most noteworthy was a viral hepatitis epidemic in New Delhi, India where 30,000 people became ill due to contamination of a drinking water source with sewage (Melnick, 1957). Following this outbreak, more attention was placed on the study of enteric viruses and their transmission in the environment.

Source Water Contamination

Improperly handled wastewater can lead to the contamination of surface and ground waters that are used as sources of drinking water. There are an estimated 1 to 15 million cases of waterborne microbial illnesses in the United States each year due to viruses, bacteria, and protozoa. (Macler, 1996). An estimated 900 to 1800 of these cases result in death (Macler, 1996). This figure is only an estimate because viral gastroenteritis is not a reportable illness and many cases and outbreaks are not recognized. Additionally, individuals with asymptomatic infections can act as carriers and transmit the virus to others who may develop disease (Rao and Melnick, 1986). The socioeconomical impact from waterborne disease is great. It has been estimated that the cost to the United States is over 19 billion dollars for illnesses with no consultation from a physician, 2.75 billion for cases requiring a visit to a physician, and 760 million for cases requiring hospitalization (Young, 1996). From 1971-1994, 650 drinking waterborne outbreaks and 569,754 cases of illness were reported in the U.S. (Craun, 1996). Thirty-three percent of waterborne disease cases are associated with surface water and 58% with ground water (Craun, 1996). Viruses are the etiologic agent responsible for approximately 14% of all waterborne illness. (Craun, 1996)

Surface Water

Surface waters include reservoirs, rivers, and lakes. Untreated or inadequately treated wastewater may be discharged from an over-capacity treatment plant directly into the water. Some communities collect wastewater and stormwater in combined systems to

be transported to a wastewater treatment plant. However, during times of heavy and prolonged rain, the volume of the mixed waste exceeds the capacity of the wastewater treatment plant and is discharged to the receiving waters prior to adequate treatment (Geldreich, 1990). Pathogens, including enteric viruses, are frequently isolated from these combined sewer outfall sites (Geldreich, 1990). Fecal contamination of surface water has resulted from wild animals in the watershed area and runoff from farm animal waste. Poorly constructed or improperly located sanitary landfills also contaminate source waters with fecal material from disposable diapers and pet waste (McFeters, 1990).

Ground Water

Ground water is obtained from underground aquifers and has long been thought of safe due to the filtering capacity of the soil layer above it. Therefore, treatment of groundwater is typically minimal or nonexistent. Approximately 25% of the United States uses groundwater as the source of drinking water. Groundwater-based public water systems serve over 110 million people (Macler, 1996) and many residents of rural communities obtain drinking water from private individual wells. Occurrence data from the American Water Works Association indicate that 60-70% of groundwater sources have been contaminated with enteric pathogens (Macler, 1996). Fecal contamination originates from the surface or from sewers and septic systems. In one study 20% of wells, each with a depth from 1 to 30 meters, contained enteric viruses (Gerba et al., 1988). More recently, a study of 250 ground waters found that although many had received disinfection, 43% were contaminated with viruses (Abbaszadegan et al., 1998).

Additionally, it has been shown that viruses survive longer in groundwater than in surface water or seawater (Yates, 1985).

Treatment of Wastewater

Primary Treatment

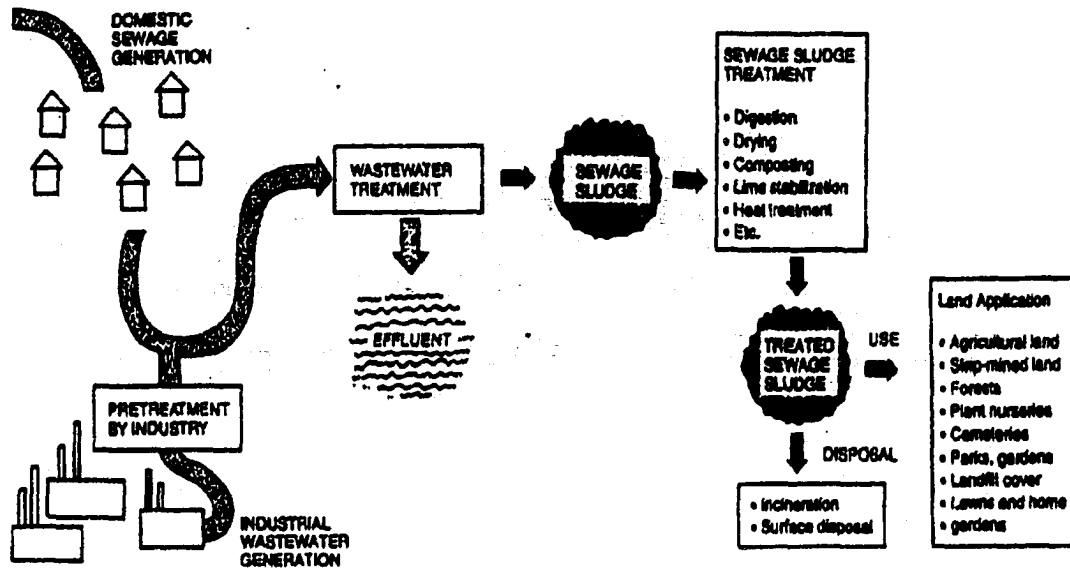
Due to the public health hazard that wastewater poses, The Clean Water Act was enacted which required the treatment of wastewater to reduce the numbers of pathogens before discharge (Figure 1). The goal of primary treatment is to reduce suspended solids. Upon entering a wastewater treatment plant, wastewater flows into a sedimentation tank, where solids are allowed to sink to the bottom. The settled material is then classified as raw sludge. The sludge is largely liquid in nature with only a three to six percent solid content. The composition of sludge is largely organic matter. Approximately 2,500 to 3,500 liters of raw sludge are generated per million liters of wastewater (USEPA, 1992).

Secondary Treatment

The sludge and effluent are separated and each receives further treatment. Secondary treatment of the effluent often involves the use of a trickling filter or activated sludge treatment. A trickling filter is a three to six foot layer of stones over which effluent is pumped. Various microorganisms grow on the stones and consume the organic matter in the effluent (Rao and Melnick, 1986). Activated sludge treatment is a more effective method. The effluent is first pumped into an aeration tank and mixed for

Figure 1

Wastewater treatment



from: USEPA, 1992

four to five hours while the bacteria contained in the effluent multiply and break down organic matter (Rao and Melnick, 1986). It is then transferred to a secondary sedimentation tank and remains for 2-3 hours while the biological solids settle. The settled material is referred to as activated sludge. The activated sludge contains many microorganisms and a portion is returned to the aeration tank to promote further consumption of organic material. The unused activated sludge can be added to raw sludge. Tertiary treatment such as coagulation, filtration, and disinfection further purify the effluent.

Treatment of Sludge

Treatment of wastewater removes pathogenic microorganisms mainly by concentrating them in the sludge. Therefore, further processing of sludge is necessary prior to disposal. A variety of methods is available and two or more processes are often used together. Aerobic and anaerobic digestion are common means to reduce the pathogen content of sludge. Aerobic digestion reduces organic matter to carbon dioxide, water, and nitrate nitrogen after 10-40 days of aeration with temperatures ranging from 10 to 30°C (USEPA, 1992). Anaerobic digestion takes place in a sealed reactor at temperatures of 30 to 60°C for 10 to 60 days (USEPA, 1992). Ammonia is produced during anaerobic digestion and has been shown to be virucidal (Ward and Ashley, 1977a). Methane is generated during anaerobic digestion and can be used to generate power for the treatment plant. Another method is composting, where previously dewatered sludge is combined with wood chips or leaves and allowed to decompose. The greatest reduction of pathogens occurs if a high temperature is maintained in the compost

pile. Viruses are susceptible to high temperature and some sludges are treated by thermal processes. Drying sludge is also used as a means of reducing the pathogen content of sludge. The water content of the sludge must be reduced to less than 30% for the process to be effective (Ward and Ashley, 1977b). Treatment of sludge with lime (calcium hydroxide) also reduces pathogen content. A sufficient quantity of lime is added to achieve a pH of 12 or greater. This process has been shown to drastically reduce the levels of bacteria and viruses (Grabow et al., 1978; Sattar et al., 1976).

Sludge Disposal

Disposal Practices

After sludge receives treatment, disposal is necessary. In the past, sludge was frequently disposed of in the ocean. However this practice was linked with beach pollution and damage to marine organisms and was banned after 1992. Approximately 20 percent of sludge is incinerated (USEPA, 1992). Incineration significantly reduces the volume of sludge that must be disposed, however the concentration of pollutants generated must be carefully monitored. Landfilled sludge is placed in a dedicated area, alone or with solid waste, and buried. Forty-one percent of the sewage sludge disposed of by publicly owned treatment works (POTWs) is landfilled with solid waste (USEPA, 1992). A small number of POTWs dispose of sludge in monofills, which are landfills containing only sludge. Surface disposal of sludge onto dedicated land sites is often used. These sites often receive large quantities of sludge for many years.

Reuse Practices

Land application of raw sewage sludge onto farmland was commonplace before the industrial revolution. After chemical fertilizers were developed, land application of sludge became unnecessary (Sattar, 1983). Due to rapid growth of our population, many POTWs are over capacity and generate much more sludge than can be disposed of by the previously mentioned methods. The USEPA has encouraged all communities to use the sludge in a beneficial manner (USEPA, 1992). Many communities apply sludge to land as a means of disposal, while also taking advantage of its nutrient content. Sludge contains organic matter and nutrients such as nitrogen and phosphorus that make it useful as a fertilizer.

Many communities use sludge on golf courses, median strips, and for covering landfills. Sludge has been successfully used in land reclamation of strip-mined areas (USEPA, 1992). Sludge has also been used to re-vegetate forests destroyed by fire and to accelerate tree growth for shorter wood production periods. A University of Washington study demonstrated a two-fold growth rate increase for trees grown in sludge amended soil (USEPA, 1992). The majority of sludge applied to land is used to improve the yield of agricultural crops for grazing, animal feed, and food for human consumption. A USEPA survey showed that 33% of the sewage sludge generated in the United States is applied to land (USEPA, 1992). This method of sludge disposal is also cost effective. The cost of land disposal was found to be 72% lower than incineration (Sattar, 1983).

Survival of Viruses in Sludge-amended Soil

After sludge is applied to land, the soil matrix serves as a filter for many pathogens. Protozoa and bacteria are large enough to be trapped at or near the soil surface (Gilbert et al., 1976 and Edmonds, 1976). Sunlight and desiccation eliminates these microorganisms. Viruses, however, are much smaller and are not retained by the soil on the basis of size. Some viruses may become immobilized at the soil surface when embedded within sludge solids (Bitton, 1984). However, the most significant soil retention of viruses is caused by the adsorption of viruses onto the soil surface. Survival of viruses retained in the soil depends on many environmental conditions including the temperature and moisture content, soil microorganisms, characteristics of the soil, organic matter content, pH, and ionic strength.

Temperature and moisture

Temperature and moisture are the two most important factors that affect survival of viruses in soil. Yeager and O'Brien (1979) found poliovirus type 1 and coxsackie B3 virus were reduced to undetectable levels in soil after 12 days at 37°C. However at 4°C, the viruses were still detectable after 180 days. Straub et al (1992) also demonstrated the importance of temperature and moisture for the survival of viruses in a desert soil. Sagik et al (1979) demonstrated that a one-log decrease of poliovirus 1 occurred after 3 months at 4°C, 1 month at 20°C, and less than 1 week at 30°C. Additionally, if the moisture of the soil was lowered from 15-25% to 10%, the time required for a 1 log decrease of poliovirus 1 was reduced from 1 month to 1 week. Another study by Bitton et al (1984)

further demonstrates the importance of moisture for survival of viruses. They found that echovirus and poliovirus survived 35 days in warm wet weather during the summer in Florida, but only 8 days in the fall when the weather was warm and dry. Yeager and O'Brien (1979) determined that the critical moisture content for a sandy loam soil was 2.9%. At soil moisture contents above 2.9% virus survival was dependent on temperature. Below 2.9% viral inactivation was due to desiccation. Furthermore, at or below this moisture, viral nucleic acid was ejected after conformational changes occurred in the protein coat. Viral nucleic acid was rapidly degraded by indigenous soil microbe nucleases.

Soil Microorganisms

Microorganisms in the soil and their enzymes reduce virus survival (Rao and Melnick, 1986). Poliovirus survived longer in sterile soils versus non-sterile soils, regardless of soil type (Rao and Melnick, 1986). Soil microbes degrade viral structural proteins for use as a metabolic substrate (Rao and Melnick, 1986). It has been suggested that the clay particles stabilize the nucleic acid of an adsorbed virus (Gerba et al., 1981 and Hurst et al., 1980).

Soil characteristics

Viruses are charged colloidal particles that are able to adsorb to other particles. Soils contain many other colloidal particles such as sand and clay. Viruses associated with particulate matter have increased survival rates (Sattar, 1983). Hurst et al (1980) demonstrated that virus adsorption to soil was one of the most important factors affecting

virus survival. Adsorption is highly dependent on the composition of the soil. Soils contain different amounts of sand, silt, and clay. An average soil contains 20% clay and 40% of both silt and sand (Harpstead et al., 1988).

Clay. Each clay particle is composed of irregularly stacked layers of ions with fractured edges due to weathering. The result is a large surface area due to external and internal surfaces. Clays have two basic structures. Montmorillonite, illite, and vermiculite are 2:1 clays containing an alumina layer (aluminum plus oxygen) which is sandwiched between two silica layers (silicon plus oxygen). Kaolinite is an example of a 1:1 clay. These clays contain a single silica layer bonded to a single alumina layer. The 2:1 and 1:1 clays are referred to as layer lattice silicate clays due to their geometric structure.

Variations within the geometric pattern of ions form the charge on the surface of the particle. Most clays have a negative charge due to oxygen ions with an unsatisfied charge along the edge of the particle. Additionally, ions can be substituted during the formation of the clay particle in a process known as isomorphous substitution. For example, approximately 25% of the aluminum ions in montmorillonite clay may be replaced with magnesium or iron that produces negative charges at the surface of the particle (Harpstead et al., 1988). The ion exchange capacity of a soil indicates the number of adsorbed ion charges that can be desorbed from the soil (Sposito, 1989). These sites are available to adsorb other ions in the soil solution. A particle with a net negative charge adsorbs cations, hence the ion exchange capacity of clays is typically referred to as the cation exchange capacity. Clays with a 2:1 structure have a much higher cation exchange capacity than 1:1 clay.

In one study, reovirus was found to adsorb to montmorillonite and kaolinite clays almost immediately (Lipson and Stotzky, 1983). The degree of adsorption correlated to the cation exchange capacity of each clay demonstrating that adsorption was primarily due to negatively charged sites on the clays. The authors suggest that because the pH at the clay surface is much lower than the soil suspension, hydrogen ions from the clay surface shifted to the virus. Thus a cation exchange reaction between the negatively charged clay and the now net positively charged virus could occur.

Sand. Sand is a poor virus adsorbent due to the small surface area of each relatively spherical particle. Yeager and O'Brien (1979) found that a sandy soil adsorbed less poliovirus than a sandy loam soil that contained 19% clay. Additionally, they found that poliovirus was inactivated more rapidly in the sandy soil. Other researchers have demonstrated that viruses survived longer in sludge amended clay loam soils than in sludge-amended sandy loam soils (Gerba et al., 1981, Hurst et al., 1980, Powelson et al., 1991). Viruses that are not adsorbed to soil particles remain in the soil solution and are vulnerable to the proteolytic activity of microorganisms.

Organic Matter

In general, soils with a high organic content do not adsorb viruses as well as clay soils (Rao and Melnick, 1986). Soluble organic compounds also compete with viruses for adsorption sites on soil particles (Carlson, 1968). Sobsey et al. (1980) studied poliovirus and reovirus adsorption to a soil primarily composed mainly of organic matter. This soil was found to adsorb both viruses poorly. Stagg et al. (1977) demonstrated that the adsorption of bacteriophage MS-2 to bentonite clay was reduced from 97% to 35% after

the addition of organic carbon. Furthermore, organic matter was found to complex with MS-2 and prevent its adsorption to soil. Organic compounds in sludge were also found to reduce adsorption of poliovirus to soil. Dizer et al (1984) noted reduced adsorption of poliovirus 1, coxsackie viruses A9 and B1, and echovirus 7 to soil columns previously washed with secondary effluent. No reduction occurred when soil columns were washed with groundwater or tertiary effluent. The adsorption of enteroviruses, rotavirus, and several bacteriophages to soil was reduced when suspended in secondarily treated sewage versus water or soil extract (Goyal, 1979). Moore et al (1981) also found soil organic matter to be a poor adsorbent for poliovirus. The low pI of organic matter results in a negative charge at most natural soil pH's. Therefore the similarly charged virus and organic molecule would repel each other.

pH

Very little viral adsorption occurs above pH 8. Enteroviruses and soil particles are negatively charged at an alkaline pH and thus repel each other. Sobsey et al (1980) demonstrated that adsorption of poliovirus to sandy soils and soils with a high organic matter content was much greater at a low pH. Soils with high concentrations of clay were found to effectively adsorb virus over a wide range of pH values. Goyal and Gerba (1979) found soil pH to be the most important factor affecting viral adsorption to soil. A soil pH below 5 favored adsorption of a variety of viruses.

Ionic strength

Cations, especially divalent and trivalent cations, decrease the diffuse double-layer composed of the inner layer of negatively charged soil particles and the outer layer of ions in the soil solution (Sposito, 1989). Divalent and trivalent cations with a small diameter such as calcium, are held more closely to the soil particle surface, while monovalent cations with large diameters such as sodium, remain farther away (Sposito, 1989). This cation bridging helps neutralize excess negative charge on the surface of the virus and clay particle allowing the virus and clay particles to approach each other and be bound by physical forces such as electrostatic and Van der Waals forces. (Bitton, 1984; Lipson and Stotzky, 1983). Van der Waals force is an attractive interaction produced when two nonpolar molecules collide through Brownian motion and induce dipole moments in each molecule (Sposito, 1989). Van der Waals forces may be significant in the absence of proton and cation exchange, particularly when viruses are at or near their isoelectric point (Lipson and Stotzky, 1983). Sobsey et al. (1980) showed that the addition of $MgCl_2$ greatly increased the adsorption of poliovirus to sand. Lipson and Stotzky (1983) demonstrated increased adsorption of reovirus to clays after the addition of cations. Divalent cations were found to be more effective than monovalent cations.

Virus Transport from Land Application Sites

Viruses have been shown to travel significant distances once they enter the soil matrix. Migration of the viruses may result in the contamination of ground water. The first isolation of poliovirus in contaminated ground water was in 1972 from a 30.5 meter

deep well (Mack et al.,1972). It was believed that the water became contaminated from seepage of a wastewater drainfield located 91 meters from the well. Viruses contaminating groundwater at a site that received secondary effluent, were thought to have traveled 30 meters vertically and 183 meters laterally (Schaub and Sorber, 1977). Another wastewater infiltration site showed evidence of viral movement to a 18.3 meter deep well (Keswick and Gerba, 1980). Vaughn et al. (1978) isolated poliovirus, echoviruses, and coxsackieviruses from effluent used to recharge three aquifers. Viruses were subsequently isolated from two of the aquifers with depths of 18 and 34 feet. No virus was found in the third aquifer that was located at a depth of 80 feet. Wellings et al (1975) isolated coxsackie B4 virus from a 10 meter deep well located 7 meters away from a Florida cypress dome effluent discharge site. Further research (Wellings et al., 1978) at a sludge disposal site revealed the presence of poliovirus from 8.5 and 17.7 meter deep wells located 18 meters from the edge of the site.

More recently, an epidemiological investigation of a small community outbreak of hepatitis A virus was undertaken (De Serres,1999). Several people who lived adjacent to the index patient and consumed well water became ill. HAV was found in these wells which were located up to 60 m from the index patient's septic tank. Straub et al. (1995) collected soil cores from a farm that had received sewage sludge for 5 years and found enterovirus nucleic acid at a depth of 20 meters with significant lateral movement. Other researchers have not found evidence of virus migration (Damgaard-Larsen et al.,1977; Bitton et al.,1984; and Pancorbo et al.,1988) and believe that most viruses in sludge are retained in the uppermost soil layer. Many factors influence the transport of viruses once

they are deposited in the soil, including soil type and characteristics of percolating fluid (Figure 2).

Soil Subsurface Structure

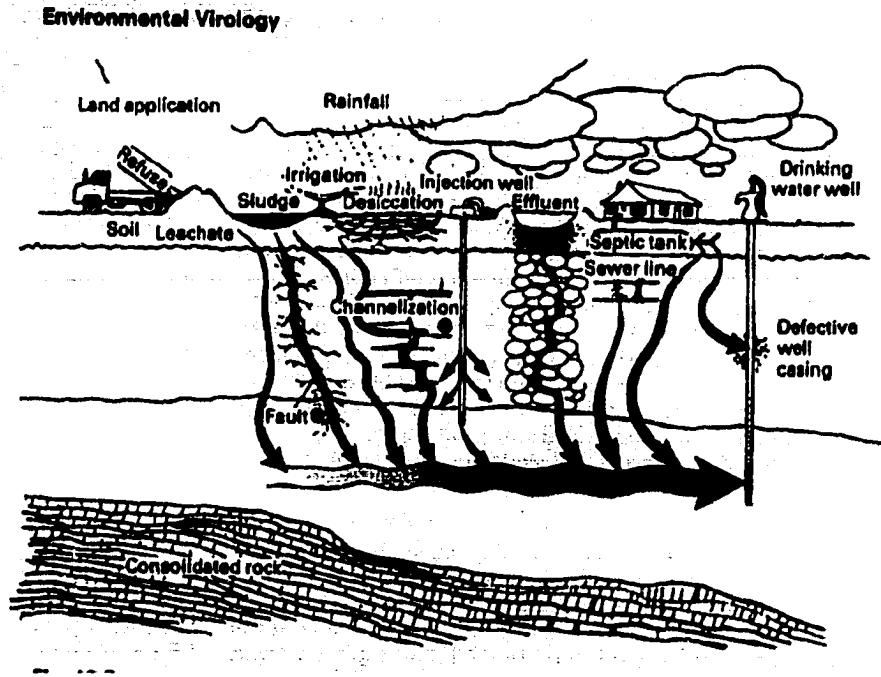
Fractures, channels and cracks may occur below the surface in some soils. Montmorillonite clay has a high affinity for water that causes the clay layers to slip past each other (Harpstead et al., 1988). When these soils dry, cracks of 2 inches or more may appear (Harpstead et al., 1988). Rapid dispersal of bacteria through fracture lines has been demonstrated (Keswick and Gerba, 1980). Many researchers employ soil columns to study the movement of viruses through soil. These columns attempt to recreate the natural soil matrix in the laboratory, however this type of soil substructure cannot be represented in a soil column. Therefore, data acquired with soil columns may not be an accurate predictor of viral movement in natural conditions.

Moisture

Soil that is saturated with water does not retain viruses effectively. Lance and Gerba (1984) demonstrated movement of viruses to a depth of 40 cm in a column of loamy sand soil under unsaturated conditions. However, viruses were found at the depth of 160 cm during saturated conditions. High infiltration rates (75-100 cm/hr) of a coarse sandy soil during groundwater recharge were found to lead to poor retention of poliovirus (Vaughn et al, 1981). At these high rates, a significant amount of poliovirus traveled 7.5 meters to the groundwater within 2.5 hours. At lower rates (6 cm/hr and 1 cm/hr) very little poliovirus reached the groundwater by twenty-four hours. Wang et al (1981) also

Figure 2

Factors influencing the survival and migration of enteric viruses after land application of sludge



from: Rao, V.C. and J.L. Melnick. 1986. Environmental Virology

found virus adsorption in soil columns to be negatively correlated with the flow rate of sewage effluent. During saturated soil conditions there is a greater thickness of water surrounding soil particles which reduces the probability of contact between soil and virus and decreases adsorption.

Desorption

Organic matter. Organic matter contained in the soil and sludge may compete with viruses for adsorption sites in the soil. A soil with high organic matter has a lower capacity to adsorb virus and may allow virus to travel further into the soil. Powelson et al. (1991) found that soil humic material and sludge organic matter increased the transport of MS-2 bacteriophage. Soluble humus such as fulvic acid forms complexes with other organic compounds that can travel with the percolating water through the soil (Sposito, 1989). Bacteriophage MS-2 has been shown to complex with fulvic acid. (Bixby and O'Brien, 1979). It was shown that 80% of the poliovirus in a water sample with high concentrations of humic and fulvic acids passed through soil columns (Rao and Melnick, 1986). After the water was treated with activated carbon to adsorb the organic acids, the poliovirus was retained.

Ionic strength. The concentration of ions in the infiltrating liquid may affect desorption of viruses from soil particles. Duboise et al demonstrated the desorption of T7 bacteriophage from a soil column after it was rinsed with deionized water. Lance et al (1976) also reported desorption of viruses from a soil column after flooding with deionized water. However, addition of calcium chloride to the deionized water prevented most of the virus desorption. Rainwater has a low ionic strength and may similarly

desorb viruses from soil particles. Enteric viruses were isolated in the groundwater below an effluent land disposal site after a heavy rainfall of 71 cm (Rao and Melnick, 1986). Wellings et al (1975) suggests that heavy rainfall may have contributed to desorption of virus from a cypress dome. Landry et al (1979) demonstrated mobilization of poliovirus 3 Leon and poliovirus 1 and 3 field isolates after application of rainwater to a column containing sandy soil.

Aerosols

Sewage treatment and application of sludge to land generates aerosols. Viruses can be encapsulated within small airborne droplets of (Rao and Melnick, 1986). Inhalation of the aerosols or contact with contaminated surfaces may result in infection. The greatest amount of aerosolization occurs during application of sludge with a low solids content via high volume spray guns (Straub et al., 1993). Particles from dried sludges may be small and light enough to be blown from the site of land application (Straub et al., 1993). One study found the incidence of typhoid fever, salmonellosis, shigellosis, and hepatitis was 2 to 4 times higher in small agricultural communities that practiced sewage irrigation than control groups (Katzenelson et al., 1976). Another epidemiological study demonstrated that frequent occupational exposure to raw sewage was a significant risk factor for hepatitis A virus (HAV) infection (Heptonstall et al., 1998). Of the 50 sewage treatment plant workers with the most exposure, 60% had antibody against HAV indicating previous exposure. Carducci et al (1995) monitored the air adjacent to an activated sludge plant and found enteroviruses in 25% of the samples. Furthermore, coliphages were detected 20 meters away from the plant. Another

study showed a 40 meter spread of endogenous enterovirus 7 from a sprinkler during spray irrigation with wastewater (Teltsch and Katzenelson, 1978). However Sorber et al. (1984) found a lower concentration of microbes in aerosols generated from application of liquid sludge than from wastewater spray application sites.

Plants

Many of the viruses present in sludge are retained in the first few centimeters of soil. However, this portion is the most likely to come into direct contact with food crops. Many researchers have shown that viruses can survive for long periods of time on food crops (Sattar, 1983). Tierney et al. (1977) demonstrated poliovirus survival for 36 days on lettuce and radishes after crops were spray irrigated with sludge. Additionally, poliovirus was isolated after the plants were grown in soil flooded with sludge. Internal contamination of plants may result if viruses penetrate the root system. Ward and Mahler (1982) exposed plants to a nutrient solution containing 10^{10} pfu/ml of f2 bacteriophage during hydroponic growth. Plants whose roots had been freshly cut, contained 10^6 pfu/g of plant tissue. Plants whose roots were not cut only contained 10^2 pfu/g of tissue. Damaged roots were quickly repaired by the plant. Therefore, the authors concluded that the transmission of virus through plant uptake was minimal and not a significant public health risk.

Animals

It is known that grazing animals consume significant amounts of soil during feeding. These animals may serve as a reservoir for pathogens from sludge-amended

soil. They may even support the replication of the pathogens, particularly parasites such as those within the *Taeniidae* family commonly known as tapeworms (Sattar, 1983 and Barbier, 1990) and the protozoan *Cryptosporidium* (Tzipori, 1983; Current, 1987). Burrowing animals and birds could also come into contact with land applied sludge and spread viruses to other areas (Straub et al.,1993). Soil macroinvertebrates such as millipedes, slugs, and worms are also exposed to sludge-amended soil. Metcalf et al.(1978) demonstrated that these macroinvertebrates could become virus carriers when fed virus contaminated food in the laboratory. Therefore these species have the potential to be carriers in the natural environment and spread viruses to other areas as they travel and to their predators upon ingestion.

EPA Sludge Regulations

Due to the potential harm to the environment and public health from the pollutants in sludge, the USEPA issued new rules for the use and disposal of sludge under the authority of the Clean Water Act as amended in 1977 and the 1976 Resource Conservation and Recovery Act. (USEPA, 1992). The regulation encompasses sludge that is applied to land, distributed and marketed, placed in monofills, surface disposal sites, and incinerated. Limits on pollutant concentration, pathogen reduction, and management practices of the sludge are described.

Pollutant Levels

The use or disposal of any sludge that may cause an underground-drinking source to exceed maximum containment levels of ten heavy metals and six organic compounds is prohibited. The maximum containment levels were established by employing algorithms that model the movement of the pollutant into the soil, water, and air, and ultimately to animals, plants, and humans.

Pathogen Reduction Requirements

Pathogen reduction may be achieved by treatment alone or in combination with management practices that limit access to the site of sludge application. The direct measurement of pathogens including viable helminth ova, *Salmonella* sp., and enteric viruses may be required or indicator organisms may be substituted. Fecal coliform bacteria are used as an indicator for fecal pathogens. Fecal coliforms are abundant in raw sewage and their presence demonstrates fecal contamination. Fecal coliforms are easily and inexpensively measured (USEPA, 1992). However, enteric viruses are more resistant to many sludge treatments (Straub et al., 1993) and disinfection than fecal coliform bacteria (Rao and Melnick, 1986). Viruses are protected from inactivation by chlorine disinfection when occluded within particulates (Hejkal et al., 1979). Numerous studies have demonstrated the presence of enteric viruses in environmental samples that did not contain fecal coliform bacteria (Rao and Melnick, 1986). Therefore, a sludge that meets fecal coliform standards, may contain unacceptable levels of enteric viruses. The USEPA has defined two levels of pathogen reduction.

Class A. Class A pathogen reduction is reached solely by treatment processes. The USEPA's goal for the Class A requirements is the reduction of pathogens to below detectable levels, which are defined as fewer than 3 *Salmonella* colony forming units, 1 plaque forming unit, and 1 helminth ova per 4 grams of total sludge solids. Class A requirements may be met by narrowly defined processes, including a high pH-high temperature treatment, composting, heat drying, heat treatment, thermophilic aerobic digestion, beta ray irradiation, and pasteurization. These processes require the demonstration of fewer than 1,000 fecal coliform most probable number (mpn) or fewer than 3 *Salmonella* colony forming units (cfu) per gram of total sludge solids. Sludge generated from processes that do not meet these conditions may require monitoring of fecal coliforms, enteric viruses and helminth ova. The USEPA believes that the Class A pathogen requirements reduce pathogen levels to an amount less than the infectious dose and that no access to the site is necessary.

Class B. The USEPA's objective for Class B pathogen requirements is the adequate reduction, but not elimination, of pathogens. Processes that generate Class B sludge may only reduce enteric virus levels by a factor of ten (USEPA, 1992). Pathogens are assumed to be sufficiently reduced when fecal coliform levels are at or below 2 million cfu or mpn/g of total sludge solids. Processes that generate Class B sludge include aerobic digestion, air drying, anaerobic digestion, and lime stabilization. The sludge generated by eleven of the thirteen wastewater treatment plants applying sludge to land in New Hampshire are lime stabilized (Margolin, 1999).

The likelihood of pathogens remaining in Class B sludge is high, therefore access is limited to the application site to permit pathogen reduction by environmental factors.

Food crops that have harvested parts above ground and touching the sludge soil mixture cannot be grown for 14 months after application of sludge. There is no time restriction for crops with harvested parts that do not touch the ground. Food crops with harvested parts below the ground cannot be grown for 38 months after the application of sludge. Feed crops may not be harvested and animals may not graze on the site until 30 days after application of the sludge. Additionally, public access to the land is prevented for 12 months.

Vector Attraction Reduction Requirements

Untreated sludge can serve as a food source for vectors, such as insects and rodents. Organic compounds in the sludge emit odors that attract the vectors. Vectors may spread pathogens to other sites. Composting, digesting, raising the pH, reducing moisture content, or injecting the sludge beneath the surface reduce vector attraction. Reduction of the volatile solids by 38% or digestion at mesophilic temperatures with a 15% or less volatile solids reduction after 40 additional days of digestion meets the vector attraction reduction requirement. Another route is to reduce the specific oxygen uptake rate (SOUR) of aerobically digested liquid sludge to 1 mg or less of oxygen per hour per gram of sludge solids. Addition of sufficient alkali to raise the pH of sludge to 12 or above, without further addition of alkali for 2 hours followed by 22 hours at a pH of 11.5 or greater also satisfies the vector attraction reduction requirement. The pH requirements are sufficient to reduce the bacterial content of sludge and to prevent regrowth before the sludge is used or disposed. Drying the sludge to produce a 75% or greater solids content

also achieves the requirement by greatly reducing bacterial growth. Finally, vector attraction reduction may be attained by injecting sludge below the soil surface.

Site Requirements

Monofills. Sludge that meets Class A or Class B pathogen reduction requirements may be used. These sites must receive a daily soil cover that reduces runoff and methane gas production.

Surface Disposal. Sludge must meet Class A or Class B pathogen reduction requirements and one method of vector reduction. Crops cannot be grown on the site, public access is restricted, and methane gas generation must be limited.

Distribution and Marketing. Any sludge or product derived from sludge that is given away or sold must meet Class A pathogen reduction requirements and one of the vector attraction reduction requirements. The package must contain a list of pollutants, nitrogen concentrations, and appropriate application rates.

Land Application. Sludge that is liquid, composted, dried, or dewatered can be applied to land. The application of sludge cannot harm any endangered species or habitat. Sludge cannot be applied to frozen, snow covered, or flooded land. Additionally it cannot be applied within 10 meters of surface water or at rates above the nitrogen requirements of the vegetative growth. Pathogen and vector attraction reduction requirements, as previously stated, must be met.

Recovery of Viruses from Sludge

Elution

Many studies have shown that the majority of indigenous virus in sludge are solids associated (Lund, 1970, Lund and Ronne, 1973, and Wellings et al., 1976). Furthermore, when viruses are added to sludge they rapidly adsorb to sludge solids (Hurst et al., 1978 and Moore et al., 1975). Therefore the removal or elution of viruses from the solids is a necessary step. Many eluants such as fetal calf serum in saline (Sattar and Westwood, 1979), high pH glycine buffer (Hurst et al., 1978), skim milk (Goddard et al., 1981), freon (Brashear and Ward, 1982) and urea-lysine (Farrah et al., 1981) have been used. However, elution with beef extract has been employed most frequently (Berg and Sullivan, 1988, Berman et al., 1981, and Glass et al., 1978). Beef extract is a non-defined proteinaceous solution that displaces virus from sludge particles.

Prior to elution, a step known as conditioning is often performed to ensure virus adsorption to sludge solids. The pH of the sludge is reduced to 3.5 with 1 N HCl, $AlCl_3$ is added, and the sludge is mixed (Berman et al., 1981). This step promotes the electrostatic attraction of viruses to sludge solids by the creation of cationic salt bridges and reduction of the net negative charge of the virus. Hurst and Goyke (1986) recommended the reduction of pH prior to the addition of $AlCl_3$. The sludge solids are collected by centrifugation and eluted.

Many eluants were used at pH levels of 9 or greater and early studies also used alkaline beef extract (Berg et al., 1988). To determine the optimum pH for the elution of enteroviruses from sludge, Berg et al. (1988) compared viral recovery with beef extract at

pH 7.2, 9.2, and 10.2. Recovery efficiencies were equal to or in many instances better at pH 7.2 than the alkaline pH's. Use of beef extract at a neutral pH also eliminated the rapid inactivation of enteroviruses known to occur at high pH levels (Belshe, 1991). Beef extract has been used at concentrations ranging from 3% to 10% (Safferman et al., 1988 and Brashear and Ward, 1982) and volumes equaling and up to five times the sludge volume (Berman et al., 1981; Hurst and Goyke, 1986). Elution is enhanced with thorough mixing either by stirring, homogenization, or sonication (Berman et al., 1981; Brasher and Ward, 1982; Albert and Schwartzbrod, 1991). Centrifugation separates the sludge solids from the supernate, which is commonly referred to as the eluate. The majority of viruses are found in the eluate fraction.

Concentration

A large volume of eluate is produced after elution with beef extract. The proteins in the beef extract solution behave as colloids. Colloids do not dissolve in solutions but remain as a solid phase in suspension (Sposito, 1989). Beef extraction was previously known to be stable at neutral or alkaline pH's but became unstable when the pH was lowered, causing coagulation or flocculation of proteins. Viruses also act as colloids and are co-precipitated in a procedure introduced by Katzenelson et al. (1976) known as organic flocculation. During organic flocculation the eluate is mixed and the pH maintained at 3.5 for 30 minutes. The viruses are contained within the floc, collected by centrifugation, and resuspended in a small volume of Na_2HPO_4 . Approximately 75% of poliovirus seeded in a beef extract solution was recovered with this method. A method for the recovery of viruses from sludge is described in current regulations from the

United States Environmental Protection Agency (1992). This method utilizes sludge conditioning, elution with beef extract, and organic flocculation.

Detection of Enteric Viruses

Cell Culture

The only detection method available during early research with enteroviruses was animal inoculation. Primates and suckling mice were found to be susceptible to infection with enteroviruses. A major breakthrough in the study of poliovirus occurred in 1949 when Enders, Weller, and Robbins reported growth of poliovirus in human embryonic tissues.

Many types of cell cultures have since been used for the detection of enteric viruses. Primary cell cultures are composed of cells from the tissues or organs of sacrificed animals. These cells can be grown only for a finite period of time. Until the 1970's, virus research in environmental and clinical laboratories relied on primary cell cultures of rhesus monkey and African green monkey kidney cells, resulting in the death of hundreds to thousands of primates weekly (Dahling et al., 1974). The loss of life and cost were great.

Continuous cell lines are derived from cell cultures that have been transformed and can then be passed hundreds of times. A continuous cell line that was comparable to the primary cell cultures for the detection and growth of enteric viruses was sought for over 20 years. In 1974 Dahling et al. studied a continuous cell line derived from the African green monkey and designated it Buffalo green monkey (BGM) due to its

discovery in Buffalo, New York (Barron et al., 1970). Enteroviruses and reoviruses from sewage and natural waters were found to grow as well or better in the BGM cell line than in primary cell cultures. Later studies demonstrated that the BGM cell line was unsatisfactory for the isolation of coxsackie A viruses, reoviruses, and echoviruses (Schmidt et al., 1978 and Chonmaitree et al., 1988). Nevertheless, cell culture with the BGM cell line has become the standard method for the detection of enteric viruses from the environment (ASTM, 1992 and USEPA, 1992).

Cell culture assays begin with inoculation of the sample onto confluent monolayers of cells grown on the surface of cell culture flasks. Prior treatment of environmental samples with antibiotics or filtration is usually necessary to minimize contamination from bacteria and fungi. The inoculum is kept on the cell monolayer to allow binding of viral particles to receptors on the cell surface. Addition of a nutrient media is then necessary for the maintenance of the cells. The plaque assay is a common method in which cell cultures inoculated with a sample are covered with a solidifying agar medium. The solid medium localizes lysed cells that form clear areas or plaques, and are visualized with a vital stain. The number of plaques is related to the number of viruses in the sample (Cooper, 1961 and Berg et al., 1963). The concentration of virus is expressed as plaque forming units per milliliter. Infected cell cultures that receive liquid overlays do not form plaques, but exhibit cytopathic effects such as cell rounding, shrinking, nuclear pyknosis, refractility and cell lysis that may be viewed microscopically (Belshe, 1991). Quantal assays with liquid overlays include the 50% tissue culture infectious dose (TCID₅₀) and the most probable number of cytopathic units (MPNCU). The TCID₅₀ measures the amount of virus required to cause CPE in 50% of the cell

cultures. The MPNCU is based on a mathematical equation relating the number of cultures that exhibit CPE with the total number of cultures.

Cell culture assays are very sensitive and have the potential to detect as few as one infectious virus. However many problems exist. There is no universal cell line that supports the growth of all enteric viruses that may be found in environmental samples (Schmidt, 1978). Two studies have shown that the use of multiple cell lines is necessary for the sensitive detection of enteric viruses (Schmidt, 1978 and Chonmaitree, 1988). Many enteric viruses isolated from the environment, such as hepatitis A virus and Norwalk virus, replicate slowly or not at all in cell culture. Other isolates may replicate but not produce cytopathic effects. Measures taken to reduce bacterial contamination may not be effective. Contamination often prevents plaque development. Environmental samples, particularly sludge concentrates, can be toxic to cell cultures (Hurst and Goyke, 1983). Substances in sludge such as heavy metals and organic acids as well as beef extract (Richards and Weinheimer, 1985) may be toxic or reduce virus yields. Cytotoxicity can be mistaken for the cytopathic effects due to viruses. Some studies have shown that viruses could not be isolated from approximately one third of the plaques from wastewater concentrates (Sobsey, 1982). These “false-positive” plaques are likely due to toxicity, therefore plaques should be confirmed by secondary passage in cell culture (USEPA, 1992). Cell destruction due to sample toxicity may also occur with liquid overlay assays, therefore these cultures also need to be confirmed (USEPA, 1995). Procedures to reduce toxicity also reduces virus titer (Hurst and Goyke, 1983). Inoculation and monitoring of multiple samples is cumbersome and labor intensive. Current USEPA regulations for the detection of viruses from sludge (USEPA, 1992)

require monitoring of a BGM cell plaque assay for 16 days, followed by one or two confirmation passages of 7 days. Therefore, the cell culture assay may take up to 30 days or more to complete. Additionally, the material costs for the cell culture assay are high.

Nucleic Acid Probes

Nucleic acid probes are labeled segments of DNA or RNA that bind specifically to target nucleic acid. The target nucleic acid can be applied to nylon membranes with the use of a dot blot apparatus. Probes may be labeled through the incorporation of radionucleotides or digoxigenin and conjugation of biotin or horseradish peroxidase. DNA probes are often labeled by nick translation or random priming (Sambrook et al., 1989). RNA probes are synthesized *in vitro* by T7 and SP6 phage RNA polymerases. These polymerases initiate RNA synthesis only at the appropriate promoter site in a plasmid vector (Melton et al., 1984). Many vectors contain two promoters arranged in opposite orientations. These promoters typically flank a multiple cloning region where the DNA segment of interest is inserted. Prior to transcription, the plasmid is linearized at a site downstream of the promoter and inserted DNA. Defined, runoff single-stranded RNA transcripts are formed. Vectors containing two promoters can produce two complementary single-stranded RNA molecules. RNA transcripts may serve as a probe when labeled ribonucleosides are included in the transcription reaction.

Before the probe is allowed to anneal or hybridize to the target nucleic acid, a pre-hybridization step is often performed. Pre-hybridization solutions often contain agents such as Denhardt's reagent, non-fat dried milk, sheared salmon sperm DNA, and detergents such as sodium dodecyl sulfate that help to prevent non-specific binding of

probe to the membrane (Sambrook, 1992). After pre-hybridization, a high ionic strength hybridization solution and the probe are added. A high salt concentration maximizes the annealing rate of probe to target (Sambrook, 1992). To ensure specific binding, hybridization with RNA probes is usually conducted at 5 to 15°C below the T_m of the hybrid (Titus, 1991). The hybridization fluid may also contain dextran sulfate and formamide. Formamide decreases the T_m of the hybrid and allows a lower incubation temperature during hybridization (Sambrook, 1992). The rate of hybridization is increased tenfold with the inclusion of dextran sulfate in the hybridization solution. The effective concentration of the probe is increased because it is excluded from the portion of the solution taken up by the dextran sulfate. After hybridization, the membrane is washed to remove unbound and non-specifically bound probe. Areas where a radiolabeled probe annealed can be viewed after exposing x-ray film to the membrane in a process known as autoradiography. X-ray film is sensitive to the energy emitted by radioactive molecules and becomes darker in its presence.

Radiolabeled probes have been shown to be more sensitive than non-radiolabeled probes (Verbeek and Tijssen, 1988; Moore and Margolin, 1993). Synthesis of RNA probes *in vitro* efficiently incorporates radiolabeled ribonucleosides and very high specific activities can be reached (Melton et al., 1984). RNA-RNA duplexes have been found to be more stable than RNA-DNA or DNA-DNA (Melton et al., 1984). The melting temperature (T_m) of a DNA:DNA hybrid is 10°C lower than that of the comparable RNA:RNA hybrid (Sambrook, 1992). Many studies have shown that RNA probes are more sensitive than DNA probes (Melton et al., 1984; Kopecka et al., 1988; Jiang et al., 1987).

Nucleic acid probes have been used to detect a variety of enteric viruses in clinical samples (Hyypia, et al., 1984; Lin et al., 1985; Stalhandske, et al., 1985; Takiff et al., 1985; Kulski and Norval, 1985; Ticehurst et al., 1987). These methods have been adapted for the detection of enteric viruses in water (Jiang et al., 1986; Jiang et al., 1987; Richardson et al., 1988; Shieh et al., 1991; Margolin et al., 1991), shellfish (Guyader et al., 1993; McCabe, 1996) and wastewater (Dubrou et al., 1991). Multiple samples may be processed simultaneously and results obtained within 3-4 days.

Reverse Transcription and the Polymerase Chain Reaction

The polymerase chain reaction is an *in vitro* DNA amplification procedure first described in 1988 by Saiki et al. Two oligonucleotide primers are selected that flank the DNA segment to be amplified. Temperature cycles resulting in denaturation of DNA, annealing of primers, and extension of annealed primers are repeated. *Taq* polymerase, a thermostable DNA polymerase purified from the thermophilic bacterium *Thermus aquaticus*, catalyzes the extension of the annealed primers. The result is the exponential increase of target DNA. RNA segments may also be amplified by the polymerase chain reaction, however RNA must first be converted to cDNA by reverse transcription. This highly sensitive method results in the amplification of target DNA by a factor of 10^6 or greater (Sambrook et al., 1989). The DNA segment can then be detected by agarose gel electrophoresis and ethidium bromide staining or Southern hybridization. Results may be obtained in as little as five hours.

The polymerase chain reaction has been used for the detection of many enteric viruses (Chapman et al., 1990; Abraham et al., 1993; Birkenmeyer and Mushahwar,

1994). Procedures for the detection of enteric viruses in environmental samples by the polymerase chain reaction have also been described (Atmar et al., 1993; Jaykus et al., 1993; Le Guyader et al., 1994; Kopecka et al., 1993; Abbaszadegan et al., 1993; Schwab et al., 1993; Green and Lewis, 1995; Straub et al., 1994). However, RT-PCR is limited by small reaction volumes. Only 10 to 20 μ l of a sample concentrate can be used per reaction. This volume may not be an adequate representation of the sample. Additionally, substances that inhibit enzymatic reactions, such as metals and organic compounds, may be present in the sample concentrate.

A variety of methods to remove these substances prior to PCR have been described. Column chromatography with resins that remove metals and exclude high molecular weight substances have been widely utilized (Abbaszadegan et al., 1993; Hale et al., 1996; Straub et al., 1994; Reynolds et al., 1998). Other researchers have extracted samples with guanidine thiocyanate which denatures proteins including nucleases (Gilgen et al., 1995; Beaulieux et al., 1997; Shieh et al., 1995). Immunomagnetic separation has also been used successfully (Monceyron and Grinde, 1994; Schwab et al., 1996; Graff et al., 1993). This method uses paramagnetic beads linked to a virus specific antibody that can be hybridized to viral capsid epitopes. The magnetic beads can then be washed, thereby removing inhibitors. Alternatively, streptavidin linked magnetic beads have been used to capture biotin labeled oligomers hybridized to viral nucleic acid (Gilgen et al., 1995, Beaulieux et al., 1997; Muir et al., 1993; Reagan and Margolin, 1997).

Magnetic beads covalently linked to an oligodT tract have been used for the isolation of mRNA from a variety of sources. They are also capable of hybridization with enteroviruses and hepatitis A virus which contain a polyA tail (Belshe, 1991). Oligo dT

magnetic beads have the ability to simultaneously capture these viruses without the need for virus specific oligomers. After washing the beads, they may be resuspended in as little as 10 μ l of liquid. This concentration increases the amount of the original sample that can be included per polymerase chain reaction. RT-PCR is not inhibited by the presence of the oligo dT paramagnetic beads. The oligo dT paramagnetic beads may be reused after treatment with 0.1M NaOH. The capture of viral RNA by oligo dT paramagnetic beads can be completed in as little as 5 minutes.

METHODOLOGY

Development of a ³²P labeled ssRNA Probe for the Detection of Poliovirus in Sludge

Preparation of plasticware, glassware, and water

Plasticware that was purchased sterile, such as centrifuge tubes and pipette tips was used as much as possible. Other plasticware, including non-sterile pipette tips and microcentrifuge tubes were immersed in a 0.1% solution of diethylpyrocarbonate (DEPC) (Sigma, St. Louis, MO) and incubated at 37°C overnight. The material was then autoclaved until no odor remained. The DEPC solution was removed and the plasticware was dried in an oven at 80°C. All glassware was baked at 100°C for 24 hours. DEPC was added to MilliQ water (Millipore, Bedford, MA) to a final concentration of 0.1% and autoclaved to eliminate RNases.

Poliovirus propagation

Buffalo Green Monkey (BGM) kidney cells (Biowhittaker, Walkersville, MD) were grown in equal parts Minimum Essential Medium (MEM)(Sigma) and L-15 medium (Sigma) supplemented with 5-8% fetal bovine serum or iron supplemented calf serum (Sigma). The cells were grown to confluency in 25 cm², 75 cm², or 225 cm² cell culture flasks (Corning, Corning, N.Y.). Cells were rinsed with 1X phosphate buffered saline at a pH of 7.2 (Sigma). Poliovirus type 1 (Lsc) was added to confluent cell monolayers at a multiplicity of 10. The cells were incubated at room temperature or 37°C for 80 minutes

with rocking every 15 minutes. MEM/L-15 medium supplemented with 2% serum was added to the flasks and the cells were incubated at 37°C for 24 to 72 hours. When 75% of the cells exhibited cytopathic effects, flasks were frozen at -80°C. Poliovirus was liberated from the cells by three successive freeze thaw cycles. The lysate was placed in sterile polypropylene Oakridge tubes (Nalgene, Rochester, NY) and centrifuged (Beckman) at 7,500 x g for 10 minutes to pellet cellular debris. The supernate was collected and stored in aliquots at -80°C until further use.

Poliovirus quantification

BGM cells were grown to confluency in 25 cm² flasks (Corning, Corning, NY). Ten-fold dilutions of poliovirus type 1 (Lsc) were prepared in 1X PBS, pH 7.2. Duplicate flasks of BGM cells were rinsed with 1X PBS and infected with 0.2 ml of each poliovirus dilution. Two negative control flasks were inoculated with 0.2 ml of 1X PBS. The inoculum was incubated for eighty minutes at 25°C with rocking every 15 minutes. Unadsorbed virus was rinsed off with 1X PBS and 10 mls of an agar overlay consisting of 1% flake agar (Difco), Medium 199 (Sigma), 2% fetal calf serum, 1% MgCl₂, and neutral red dye (Sigma) was added. Once the overlay solidified, the flasks were inverted and incubated at 37°C. Plaques were counted after approximately three days.

Preparation of poliovirus cDNA

Poliovirus cDNA between base pair 115 and 7440 was previously cloned into the Pst-1 site of pBR322 producing the plasmid pVR 104. This plasmid and *Escherichia coli* HB101 cells transformed with pVR 104 were supplied by Charles Gerba (Department of

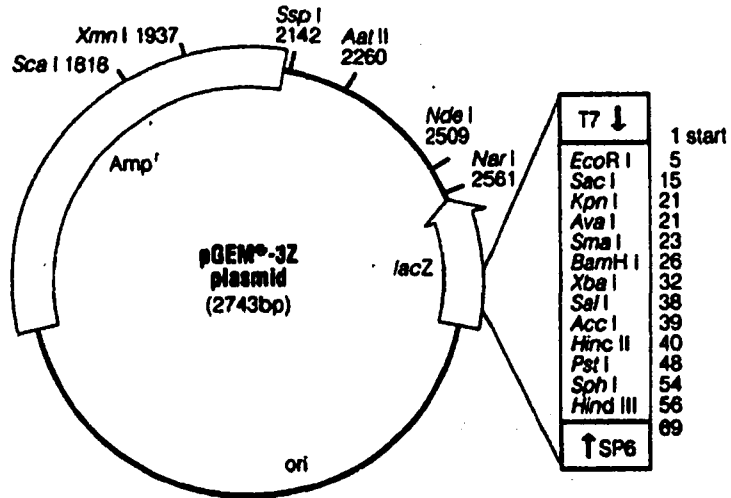
Soil and Water Science, University of Arizona, Tucson, Arizona). The *E. coli* HB101 cells containing pVR104 were grown overnight in Luria-Bertani (LB) medium (Difco, Detroit, MI) supplemented with 50 µg/ml tetracycline. Cells were centrifuged at 5,000 x g for 15 minutes at 4°C. Plasmid DNA was released from the cells by the alkaline lysis method (Sambrook et al., 1992). Plasmid DNA was purified from cesium chloride gradients or by RNase (Sigma) treatment followed by phenol/chloroform extraction (Amersco, Solon, OH) and precipitation with 2.5 volumes of 100% ethanol and 0.3M sodium acetate at -20°C. Poliovirus cDNA was isolated from the pBR322 vector by Pst-1 digestion (Boehringer Mannheim, Indianapolis, IN). Pst-1 digestion yielded four fragments of 4300 bp, 1689 bp, 1174 bp, and 434 bp. The 1174 bp fragment was sliced from agarose gels (Sigma) and purified by electroelution (Schleicher and Schuell, Keene, NH) or by a freeze squeeze procedure (Sambrook et al., 1992). Briefly, the freeze squeeze procedure consisted of addition of 0.3M sodium acetate and 1 mM EDTA to a polypropylene tube containing the gel slices. The gel slices were then broken up with a pipet tip and frozen at -80°C. The mixture was thawed and the liquid portion placed in another tube. The DNA was extracted with phenol/chloroform, and precipitated with 95% ethanol. DNA was recovered by centrifugation at 12,000 x g for twenty minutes at 4°C. The DNA pellet was then resuspended in TE buffer or DEPC treated water.

Construction of recombinant plasmid pGEM-3Z/PV 1174

The vector pGEM-3Z (Promega, Madison, WI) (Figure 3) was digested with Pst-1 restriction endonuclease (Boehringer Mannheim). The linearized plasmid DNA was treated

Figure 3

Map of vector pGEM-3Z



from: Promega, 1991

with calf intestinal alkaline phosphatase (Boehringer Mannheim) to remove 5' phosphate groups and prevent recirculization. Plasmid DNA was phenol/chloroform extracted and ethanol precipitated. Linearized pGEM-3Z and the PV 1174 bp cDNA fragment were ligated with T4 DNA ligase (Boehringer Mannheim) in a 1:3 vector to insert ratio. Ligation reactions, linearized pGEM-3Z, 1174 bp poliovirus cDNA, and lambda DNA digested with Hind III and EcoRI (Sigma) were loaded into a 1% agarose gel (Sigma) and electrophoresed for 1 hour at 70V in 1X Tris Borate EDTA (TBE) running buffer. The gel was submerged in a 0.1% ethidium bromide solution (Sigma) for 10-30 minutes. The gel was placed on a transilluminator and the DNA was viewed.

Transformation of *E. coli* JM 101

E. coli JM 101 cells were grown in LB broth with vigorous shaking to early log phase. Competent cells were prepared by the CaCl₂ method (Sambrook, 1992). A 30-50 ng amount of pGEM-3Z/PV 1174 DNA was added to 200 µl of competent cells. The mixture was heated to 42°C for 90 seconds and chilled on ice for 1-2 minutes. Eight hundred µl of LB broth was added and the cells were incubated at 37°C for 1 hour to allow for expression of ampicillin resistance. Cells were plated onto Violet Red Bile (VRB) medium (Difco) supplemented with 50 µg/ml ampicillin and incubated at 37°C for approximately 16 hours.

Selection of transformants

Colonies were selected on the basis of blue/white color screening. Colonies containing the recombinant plasmid do not ferment lactose and appeared white or colorless due to loss of β-galactosidase activity resulting from the insertion of PV cDNA within the

lacZ gene of pGEM-3Z. Blue colonies retained β -galactosidase activity because there was no insertion of poliovirus cDNA within the lacZ gene of pGEM-3Z. White colonies were selected and grown in 5 ml of LB broth overnight. Plasmid DNA was isolated using the following mini-prep procedure (Titus, 1991). One ml of cells was centrifuged for 30 seconds at 12,000 x g. The pellet was resuspended in 100 μ l of TE (pH 7.6) and 200 μ l of a 0.2N NaOH/1% SDS solution was added. The mixture was gently inverted several times and placed on ice for 5 minutes. One hundred and fifty μ l of 5M potassium acetate was added and the lysate was briefly vortexed and placed on ice for 5 minutes. Cell debris and chromosomal DNA were pelleted by centrifugation at 12,000 x g for 1 minute. The supernate was carefully removed and mixed with 100% ethanol. The plasmid DNA was precipitated by centrifugation at 12,000 x g at 4°C and resuspended in TE buffer. Plasmid DNA was digested with Pst-1 restriction endonuclease (Boehringer Mannheim) to excise the poliovirus cDNA insert. The DNA fragments were separated by agarose gel electrophoresis and viewed after ethidium bromide staining. Plasmid pGEM 3Z/PV 1174 was isolated using the large scale plasmid preparation procedures previously mentioned for subsequent experimentation.

Determination of insert orientation

The PV 1174 cDNA was inserted in one of two possible orientations because the plasmid and insert were digested with a single restriction endonuclease. It was necessary to know the insert orientation to determine which RNA polymerase would produce negative sense RNA transcripts and which would produce positive sense transcripts. The poliovirus cDNA contained a Kpn I restriction site at the 3' end of the segment (Rancaniello and

Baltimore, 1981) and the pGEM-3Z multiple cloning site also contained a Kpn I restriction site. Using this sequence data, it was determined that digestion of pGEM-3Z/PV 1174 with Kpn I would result in two DNA band patterns dependent on the insert orientation (Figure 4). Orientation 1 would produce DNA fragments of 800 and 3100 bp, while orientation 2 would produce DNA fragments of 450 and 3500 bp.

The plasmid pGEM-3Z/PV 1174 was digested with the restriction endonuclease Kpn I (Boehringer Mannheim) and the fragments were separated by 1% agarose gel electrophoresis and viewed after ethidium bromide staining. The size of the DNA fragments was determined after comparison with a Hind III and EcoRI digest of lamda DNA (Sigma).

Production of positive sense poliovirus RNA transcripts

The plasmid pGEM-3Z/PV1174 was linearized with Hind III restriction endonuclease (Boehringer Mannheim). The restriction site is located in the multiple cloning region and downstream of the T7 promoter (Figure 5). The template was added at a final concentration of 20 µg/ml to the following reaction mixture: 1X T7 RNA polymerase buffer (Boehringer Mannheim), 2 mM UTP, GTP, CTP, ATP (BM), 1 µl RNasin (Promega), 100 µg/ml bovine serum albumin (Sigma), 5 mM DTT (Boehringer Mannheim), 125 units T7 RNA polymerase (Boehringer Mannheim), and DEPC treated water to a final volume of 25 µl. The mixture was incubated at 37°C for 90 minutes. The RNA transcription reaction, linearized pGEM-3Z/PV 1174, and a molecular weight marker of Hind III and EcoRI digested lamda DNA were separated by agarose gel electrophoresis and viewed after ethidium bromide staining.

Figure 4

Restriction mapping of pGEM-3Z/PV 1174

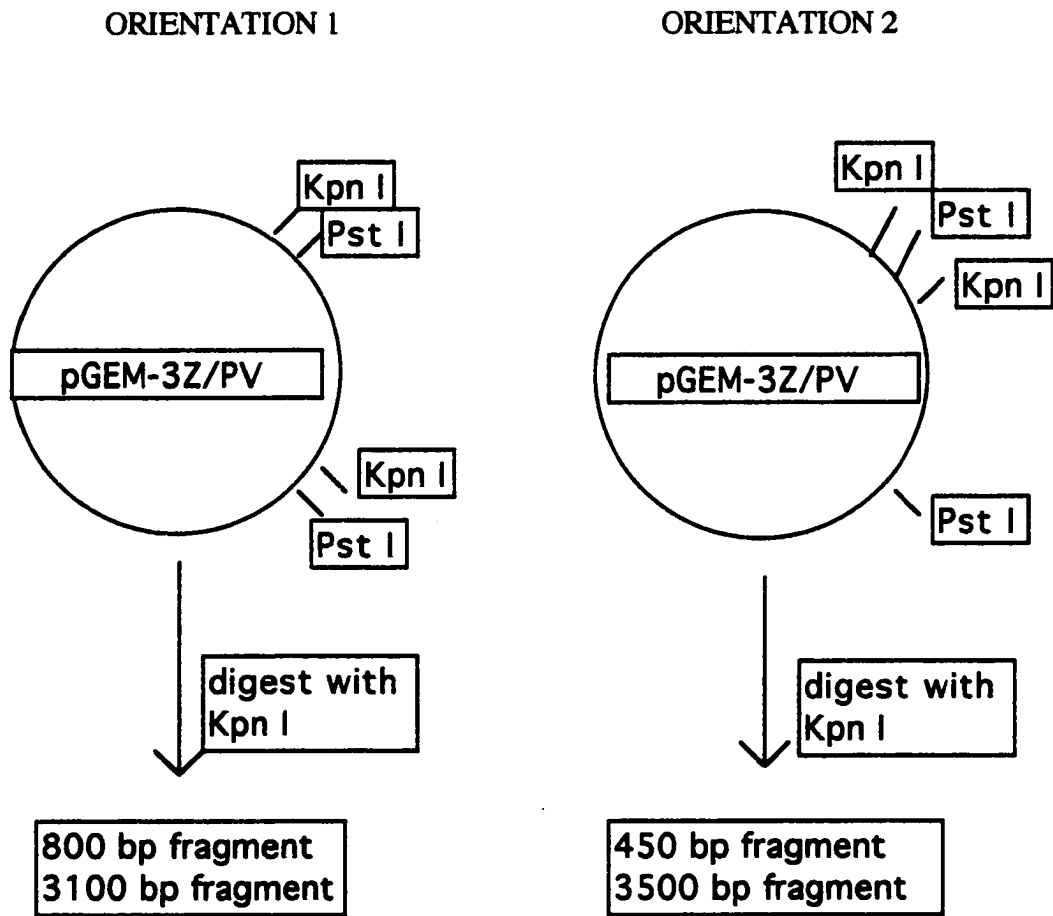
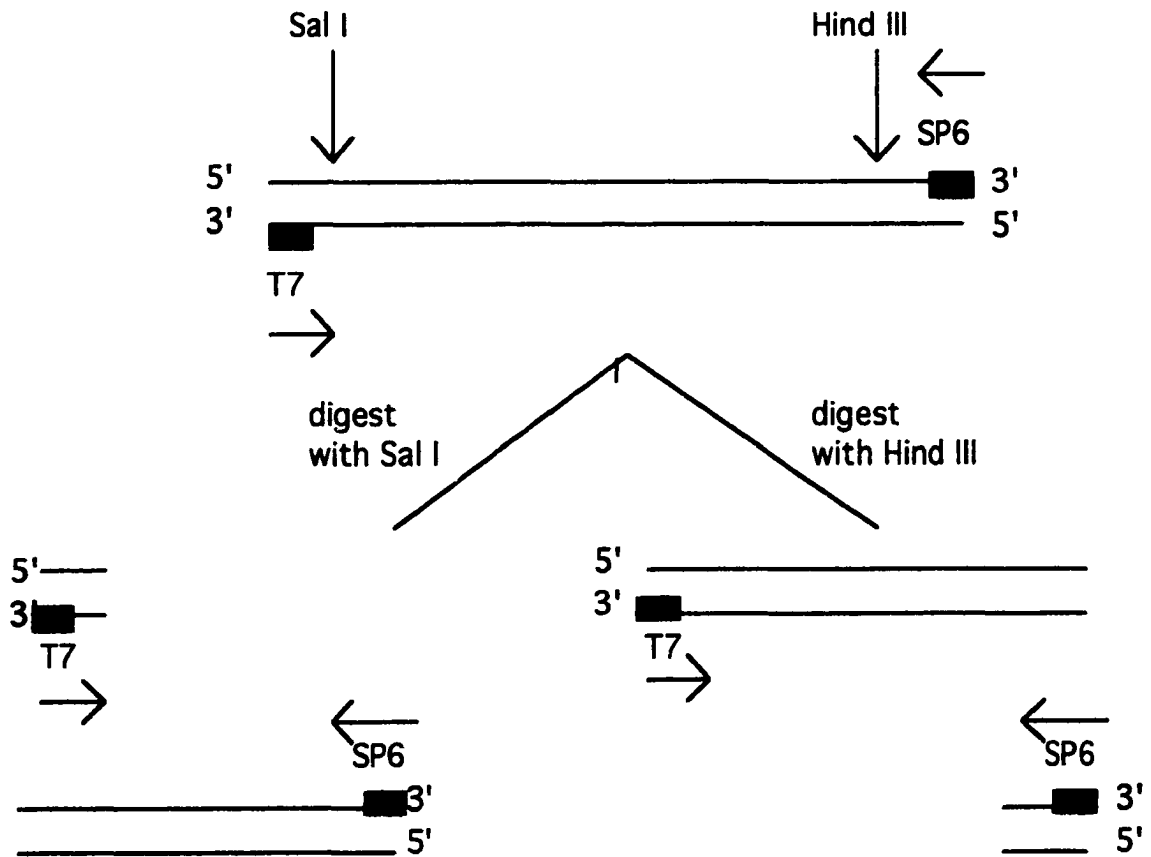


Figure 5

Synthesis of positive and negative sense RNA transcripts



Optimization of negative sense poliovirus RNA transcript production

The plasmid pGEM-3Z/PV1174 was linearized to completion with Sal I restriction endonuclease (BM). The restriction site is located in the multiple cloning region and is downstream of the SP6 promoter (Figure 5). The template was added at a final concentration of 20 µg/ml to the following reaction mixture: 1X SP6 RNA polymerase reaction buffer (Boehringer Mannheim), 400-1200 µM ATP, CTP, GTP, UTP, 1 µl RNasin, 100 µg/ml bovine serum albumin (Boehringer Mannheim), 1 mM DTT (Boehringer Mannheim), 7.5 units of SP6 RNA polymerase (Boehringer Mannheim), and DEPC treated water to a final volume of 25 µl. Tubes containing the mixture were incubated at 37°C for 30-120 minutes. RNA transcription reactions, linearized pGEM-3Z/PV 1174, and a molecular weight marker of Hind III and EcoRI digested lamda DNA were separated by agarose gel electrophoresis and viewed after ethidium bromide staining. Optimization of RNA transcript production was repeated twice to ensure reproducibility.

Removal of DNA template

One to two units of DNase I (RNase free) (Sigma) was added per µg of plasmid DNA and incubated for 10-15 minutes at 37°C. The mixture was extracted with phenol/chloroform and the RNA was precipitated with 2.5 volumes of 100% ethanol and 0.3M sodium acetate at -20°C. The RNA was collected by centrifugation at 12,000 x g for 20 minutes at 4°C. RNA pellets were resuspended in DEPC treated water and stored at -80°C.

Determination of RNA transcript yield

RNA yields were determined by spectrophotometric readings (Beckman DU/Gilford Detector) at 260 nm. An optical density of 1 corresponded to 40 µg/ml of RNA (Sambrook et al, 1989). Alternatively, RNA yields were estimated after viewing RNA bands from a 1% agarose gel stained with ethidium bromide.

Production of negative sense ³²P labeled RNA probe

SP6 RNA polymerase reaction buffer (Boehringer Mannheim), 500 µM ATP, GTP, CTP (Boehringer Mannheim), 1 mM DTT (Boehringer Mannheim), 20 µg/ml of Sal I linearized pGEM-3Z/PV1174, 15 units SP6 RNA polymerase (Boehringer Mannheim), and 150 µCi ³²P-UTP (3000 Ci/mmol) (NEN-Dupont) were combined in a volume of 25 µl and incubated at 37°C for 120 minutes with an extra addition of polymerase at 60 minutes. The reaction mixture was treated with DNase I as previously described.

Determination of RNA yield and specific activity

A 1 µl aliquot of the RNA transcription reaction was removed and added to 5 µl of 1 mg/ml yeast tRNA (Sigma) and 94 µl of DEPC-treated water. Two 5 µl aliquots of this dilution were spotted onto two small glass fiber filters (Whatman, Clifton, NJ). RNA was precipitated from one filter by placing the filter in 5 ml of cold 10% trichloroacetic acid/1% sodium pyrophosphate (Sigma) and swirling for 5 minutes. The filter was washed four times by swirling for 5 minutes in 5 ml of 5% trichloroacetic acid per wash. The filter was then washed in 100% ethanol for 2 minutes. The remaining unwashed filter and the washed filter were allowed to air dry. The filters were placed in scintillation vials with 5 ml of

scintillation fluid (Sigma) and counts per minute (cpm) were determined on a Beckman scintillation counter.

Calculation of the specific activity of radioisotope:

$$\frac{\text{cpm unwashed filter}}{\text{total pmol labeled NTP}} \times \frac{\text{total reaction volume}}{\text{volume sampled}} = \frac{\text{cpm/pmol labeled NTP}}{\text{NTP}}$$

Calculation of the RNA yield:

$$\frac{\text{precipitable cpm}}{\text{specific activity}} \times \frac{\text{total reaction volume}}{\text{volume sampled}} \times \frac{* 4 \text{ pmol NTP}}{\text{pmol labeled NTP}} \times \frac{**1}{3225} = \mu\text{g}$$

* An average of four nucleotides will be incorporated into the RNA for every labeled NTP that is scored by this assay.

** 1 μg RNA \cong 3225 pmol

Calculation of Specific Activity of RNA probe:

specific activity X dilution factor = cpm/ μg RNA
of radioisotope

Purification of RNA probes

Unincorporated NTP's were removed with a Nensorb 20 Nucleic Acid Purification Cartridge (NEN-Dupont). The cartridge was equilibrated with methanol and 50 mM triethylamine. The sample was loaded and washed with 50 mM triethylamine. The RNA probe was eluted with 50% n-propanol. The probe was used immediately or stored at -20°C for no longer than one week.

Preparation of dot blot apparatus

The dot blot apparatus (Biorad, Hercules, CA) was autoclaved for 20 minute and placed in a 10% solution of sodium dodecyl sulfate (SDS) for 30 minutes. Each piece of the apparatus was rinsed with MilliQ water until all SDS was removed. Each piece was placed in an 80° C oven until dry.

Application of sample to hybridization membranes

Samples were digested with proteinase K (Sigma) at a final concentration of 400 µg/ml for 60 minutes at 56°C. The sample was placed on ice until it reached room temperature. The appropriate size piece of Gene Screen Plus™ nylon hybridization membrane (NEN-Dupont) was cut, wetted with DEPC treated water, and placed between the upper and lower portions of the dot blot apparatus. The dot blot apparatus was assembled according to manufacturer's instructions. Multiple layers of perfilm were placed on areas of the dot blot apparatus not used. A maximum of 1 ml was applied to each well. After sample application, membranes were baked at 80°C for 2 hours in a Fisher Isotemp® 500 series oven. Membranes were completely covered with plastic wrap and stored at room temperature until use.

Pre-hybridization

Membranes were placed in 50 ml disposable polypropylene centrifuge tubes (Corning). Five ml of a pre-hybridization fluid consisting of 6X SSPE, 0.5% SDS, 10% dextran sulfate, and 50% deionized formamide was added to the tubes and allowed to coat the membranes. Membranes were secured to the sides of the tubes and freed of any air

bubbles. Tubes were placed into a rotisserie-type hybridization oven (Vanguard International, Inc.) and rotated for at least two hours at 50°C.

Hybridization and optimization of RNA probe concentration

Tubes were removed from the oven and RNA probe was added to the hybridization fluid. The tubes were returned to the oven and rotated for 12 hours at 50°C. To determine the optimum probe concentration, dilutions of positive sense poliovirus RNA transcripts were spotted onto separate hybridization membranes. Each membrane was probed with 1×10^6 , 5×10^6 , or 1×10^7 cpm/ml of hybridization fluid with a negative sense poliovirus RNA probe with a specific activity of 1.7×10^9 cpm/ μ g. Comparison of RNA probe concentration was repeated twice to ensure reproducibility.

Washing hybridization membranes

Tubes were removed from the hybridization oven. Hybridization fluid was poured out and quickly replaced with 10 ml of 2X SSPE. The tubes were placed on a cell production roller apparatus (Bellco Glass, Inc.) and rotated for 5 minutes at room temperature. This step was repeated followed by two washes with 5 ml of 2X SSPE/0.5% SDS for 30 minutes at 65°C in the hybridization oven. The final washing consisted of two 10 ml washes of 0.1X SSPE for 30 minutes each, at room temperature. The membranes and wash fluid were checked with a geiger counter to determine if the background was adequately reduced. Membranes were allowed to dry on filter paper at room temperature.

Autoradiography

Membranes were wrapped in a single layer of plastic wrap and placed on a X-ray cassette with an intensifying screen (NEN-Dupont). The membranes were exposed to Kodak XAR-5 x-ray film (Eastman Kodak Co.) for 24-48 hours, at -80°C. The film was processed with Kodak D-11 developer (Eastman Kodak Co.) for 5 minutes followed by a 30 second wash with lukewarm water, and fixed for 5 minutes in Kodak fixer (Eastman Kodak Co.). The autoradiograph was then submerged in lukewarm water for 15 minutes and hung to dry.

Evaluation of poliovirus negative sense RNA probe sensitivity

Positive sense RNA transcripts. Positive sense RNA transcripts were quantified spectrophotometrically, diluted with DEPC treated water, and applied to duplicate hybridization membranes with the dot blot apparatus. The membranes were hybridized with the negative sense RNA probe for 12 hours and exposed to Kodak XAR-5 x-ray film (Eastman Kodak Co.) for 36 hours. Sensitivity determination was repeated twice to ensure reproducibility.

Poliovirus type 1(Lsc). Poliovirus type 1 (Lsc) with a titer of 1.8×10^8 pfu/ml, was treated with proteinase K as previously described. Ten-fold dilutions of liberated poliovirus RNA were prepared in DEPC water and 500 μ l was applied to duplicate hybridization membranes with the dot blot apparatus. The membranes were hybridized with the negative sense RNA probe for 12 hours and exposed to Kodak XAR-5 x-ray film (Eastman Kodak Co.) for 36 hours. Sensitivity determination was repeated three times to ensure reproducibility.

Evaluation of negative sense RNA probe specificity

Propagation and quantification of MS-2. *Escherichia coli* C3000 was grown overnight with shaking in heart infusion broth (HIB)(Difco) supplemented with 5×10^{-3} M $MgCl_2$ and $CaCl_2$. The culture was diluted 1:50 in HIB and incubated for 3 hours with shaking. MS-2 bacteriophage was added to the culture at a multiplicity of infection of 2 and shaken slowly at 37°C until clearing was apparent. The lysates were chloroform treated and centrifuged at 7,500 x g for 10 minutes at 4°C. The supernate was retained and the MS-2 titer was determined by a standard double agar overlay plaque assay.

Propagation and quantification of HAV. Fetal rhesus kidney (FRhK-4) cells (ATCC, Rockville, MD) were grown in equal parts of MEM and L-15 medium (Sigma), supplemented with 20% fetal bovine serum (Sigma). Confluent monolayers of FRhK-4 cells grown in 75cm² flasks were rinsed with 1X PBS and inoculated with HAV strain HM-175 at a multiplicity of 10. Flasks were incubated at 37°C for 60 minutes with rocking every 15 minutes. Cells were freeze/thawed 3 times after 75% CPE was reached. Cells were pelleted at 7500 x g and the supernate was stored in aliquots at -80°C. HAV was quantified using the same plaque assay procedure for poliovirus, however plaques were viewed after 7 to 10 days.

Propagation of other Enteroviruses. Confluent monolayers of BGM cells were grown in 75 cm² flasks and inoculated with coxsackie A7 (ATCC VR-1012), coxsackie B3 (strain Nancy)(ATCC VR-30), poliovirus 2 (strain MEF-1)(ATCC VR-61), poliovirus 3 (strain Leon)(ATCC VR-1004), and echovirus 7 (strain Wallace)(ATCC VR-1047) as previously described for poliovirus. Cells were incubated at 37°C until 75% CPE was

reached. Cells were freeze/thawed three times and centrifuged to pellet cellular debris. The supernates were stored in aliquots at -80°C.

Isolation of viral nucleic acid. All viruses were phenol/chloroform extracted to remove any proteinaceous material followed by ether extraction to remove any residual chloroform.

Evaluation of cross-reactivity. Ten-fold dilutions of viral nucleic acid were prepared in DEPC water and applied to a hybridization membrane with the dot blot apparatus. The membrane was probed with the ³²P labeled negative sense RNA probe, hybridized for 12 hours, and autoradiographed for 36 hours. Evaluation of cross-reactivity of the negative sense probe was repeated twice to ensure reproducibility.

Elution of seeded poliovirus from sludge

Sludge characteristics. The sludge was obtained from a municipal wastewater treatment plant in Ogunquit, Maine. The sludge contained approximately 2% solids and had been aerobically digested.

Adsorption of seeded poliovirus to sludge. One ml of poliovirus type 1 (LSc) with a titer of 3.7×10^7 pfu/ml was added to 180 ml of room temperature sludge. The seeded sludge was kept at room temperature for 1 hour to allow adsorption of poliovirus to sludge solids. Twenty ml samples were collected while the sample was gently mixed on a magnetic stirrer.

Eluant. Beef extract V (Becton Dickinson) was used to elute poliovirus type 1 (Lsc) from the sludge. The use of beef extract in liquid or powder form and at two pH's was compared. A sterile 3% beef extract solution supplemented with 90 mM glycine was used

at pH 7.0 or 9.5. Alternatively, beef extract powder was added directly to the sludge to a final concentration of 3% with subsequent adjustment of the pH to 7.0 or 9.5.

Elution of poliovirus with beef extract solution. Sludge solids were collected after centrifugation at 1700 x g for 30 minutes. The supernate was discarded and the sludge solids were mixed on a magnetic stirrer with 20 ml of 3% beef extract supplemented with 90 mM glycine. Once the solids were resuspended the pH was adjusted to 7.0 or 9.5 and mixed for 10 minutes at room temperature. The sludge was centrifuged at 5000 x g for 10 minutes. The sludge solids were discarded and the pH of the eluate was adjusted to 7.0 if necessary.

Elution of virus with beef extract powder. Beef extract powder was added to sludge to a final concentration of 3% and mixed until dissolved. The pH was adjusted to 7.0 or 9.5 and the sludge was mixed on a magnetic stirrer for 10 minutes and centrifuged at 5000 x g for 10 minutes. The sludge solids were discarded and the pH of the eluate was adjusted to 7.0 if necessary.

Concentration of eluate. The eluates were concentrated by organic flocculation (Katzenelson, 1976). AlCl_3 was added to each eluate to a final concentration of 0.005M and the pH was lowered to 3.5. The solution was mixed on a magnetic stirrer for 10 minutes and centrifuged at 7500 x g for 10 minutes. The floc was resuspended in 0.15M NaH_2P_4 at a pH of 9.5, mixed for 5 minutes, and centrifuged at 7500 rpm for 10 minutes. The pH of the supernate was lowered to 7.5 and stored at -80°C .

Detection of recovered poliovirus. The sludge concentrates were diluted with 1X PBS and recovered poliovirus was detected by plaque assay with BGM cells, using a 1 ml inoculum of sludge concentrate per 25 cm^2 flask. Additionally, 100 μl of each sample was

proteinase k treated, spotted onto a hybridization membrane and probed with the ^{32}P labeled negative sense poliovirus RNA probe. The entire procedure was repeated twice.

Liberation of viral nucleic acid from sludge concentrates

Three methods were compared for their ability to efficiently liberate poliovirus nucleic acid from sludge concentrates. Nine ml portions of a sludge concentrate were seeded with 1 ml of poliovirus type 1 (LSc) with a titer of 1.8×10^8 pfu/ml or 1 ml of serially diluted poliovirus. One third of each seeded sludge concentrate aliquot was proteinase k treated as previously described. Another third of each portion was proteinase k treated then extracted with phenol/chloroform until no protein layer was visible, followed by extraction with chloroform and ether. The remaining third of each seeded sludge concentrate aliquot was only phenol/chloroform, chloroform, and ether extracted. One hundred μl aliquots of each sample was spotted onto duplicate hybridization membranes as previously described. The membranes were hybridized with the negative sense poliovirus RNA probe and autoradiographed as previously described. Comparison of viral liberation methods was repeated twice to ensure reproducibility.

Development of a Reverse Transcription-Polymerase Chain Reaction Assay for the Detection of Enteroviruses in Sludge

Preparation of plasticware, water, and work area

All plasticware was purchased pre-sterilized and RNase/DNase free. As an added precaution microfuge tubes (U.S.A. Scientific) were exposed to ultraviolet (UV) light in a biological safety hood for 20 minutes prior to use. All pipet tips were purchased pre-sterilized and with aerosol resistant plugs (CLP, San Diego, CA). Molecular biology grade water (Sigma) that was certified RNase/DNase free by the manufacturer was used. Twenty to thirty ml portions of this water were poured into sterile 50 ml disposable centrifuge tubes (Fisher, Pittsburgh, PA) and exposed to UV light in a biological safety hood for 20 minutes prior to use. All reverse transcription and polymerase chain reaction work was performed in a dedicated area. The area, including benchtop and pipettors, was sprayed with 70% ethanol before work was begun.

Reverse transcription

The sample was placed in a 0.2 ml UV treated thin walled microtube and put in a Perkin Elmer Thermal Cycler model 2400 (Perkin Elmer, Norwalk CT). Alternatively, the sample was placed in a 0.65 ml UV treated microtube, overlaid with 50 μ l of RNase/DNase free mineral oil (Sigma), and placed in a Perkin Elmer Thermal Cycler. This thermal cycler was the first model produced by Perkin Elmer and it was not given a model number. Viral RNA was liberated by digestion with 400 μ g/ml proteinase K at 56°C for 60 minutes. After digestion, the sample was heated to 99°C for 5 minutes to inactivate the proteinase K and placed on ice.

The reverse transcription (RT) procedure was modified from Morteza et al.(1996). The reverse transcription reaction mixture consisted of up to 10 µl of sample RNA, 50 µM downstream primer (5'-ACC GGA TGG CCA ATC CAA-3'), 3 µl of 10X PCR buffer (Perkin Elmer), 7 µl of dNTP's (Perkin Elmer), 6 µl of MgCl₂, 1.5 µl of RNase inhibitor (Perkin Elmer), 1.5 µl of MuLV reverse transcriptase (Perkin Elmer) and UV treated water to achieve a final volume of 30 µl. The mixture was placed in either thermal cycler and heated to 42°C for 45 minutes followed by 99°C for 5 minutes and then immediately placed on ice.

Polymerase chain reaction

The polymerase chain reaction (PCR) reagents were added directly to the completed reverse transcription reaction. To each tube 50 µM of upstream primer (5'-CCT CCG GCC CCT GAA TG-3'), 7 µl of 10X PCR buffer, 2 µl of MgCl₂, and UV treated water were added for a total reaction volume of 100 µl. The tubes were heated to 85°C for 2-3 minutes and 0.5 µl of AmpliTaq DNA polymerase (Perkin Elmer) was added. An initial denaturation at 95°C for 5 minutes was performed and then PCR continued for 35 cycles of denaturation, annealing and extension. Reactions in the Perkin Elmer Thermal Cycler 2400 were cycled at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 7 minutes. Reactions in the Perkin Elmer Thermal Cycler were cycled at 95°C for 1 minute and 55°C for 1 minute, followed by a final extension at 72°C for 7 minutes. Completed reactions were stored at 4°C until they were loaded with a 100 bp DNA ladder (Gibco) onto a 1.5% agarose gel

containing ethidium bromide and electrophoresed for 1-2 hours at 70-100 volts. The gel was placed on a UV transilluminator and 196 bp RT-PCR products were viewed.

Determination of sensitivity of RT-PCR assay

Poliovirus type 1 (LSc) was previously quantified by plaque assay and the titer was determined to be 1×10^8 plaque forming units/ml. Poliovirus was proteinase K digested as previously described and viral RNA was serially diluted in UV treated water. These dilutions, a negative control consisting of UV treated water, and a positive control of 10^3 plaque forming units of proteinase k digested poliovirus type 1 (LSc) were analyzed by RT-PCR. Determination of RT-PCR sensitivity was repeated 5 times to ensure reproducibility.

Demonstration of RT-PCR inhibitors in sludge

Sludge concentrates are known to contain substances that inhibit RT-PCR. To demonstrate the presence of RT-PCR inhibitors, 100 μ l of a sludge concentrate was seeded with 10^4 pfu of poliovirus type 1 (LSc) to achieve a concentration of 10^3 pfu/ μ l. One hundred μ l of UV treated water was also seeded with 10^4 pfu of poliovirus to serve as a positive control. These two samples and a negative control consisting of UV treated water were proteinase k digested and analyzed by RT-PCR. Demonstration of RT-PCR inhibitors in sludge was repeated twice to ensure reproducibility.

Column purification

Columns composed of Sephadex G-100 (Sigma) and Chelex 100 (Bio-Rad) resins were made for purification of sludge concentrates prior to RT-PCR. Slurries of both resins were made with UV treated water. Sterile glass wool (Fisher Scientific, Springfield, NJ) was placed at the bottom of a sterile 1 cc plastic syringe. Depending on the thickness of the resin slurry, 0.5 to 1.0 ml of Chelex 100 was added to the syringe. The syringe was placed in a sterile 15 ml disposable centrifuge tube without a cap and centrifuged at 1000 x g for 5 minutes at room temperature. This step was repeated, if necessary, until a packed Chelex resin volume was achieved. The Sephadex slurry was added on top of the packed Chelex resin and the centrifugation was repeated. The total packed volume of the column was approximately 0.8 ml. The column was transferred to a clean 15 ml centrifuge tube. A 200 μ l portion of the sample was placed onto the column and centrifuged at 1500 x g for 5 minutes at room temperature. The column was discarded and the filtrate was immediately analyzed by RT-PCR.

Determination of sensitivity of RT-PCR with column purification

The sensitivity of RT-PCR with and without column purification was compared. Serial dilutions of poliovirus type 1 (LSc) with a titer of 1×10^8 pfu/ml were made in sterile 1X PBS. A 200 μ l portion of dilutions containing 10^3 , 10^2 , 10, 1, and 0.1 pfu/ml were purified with Chelex 100/Sephadex G-100 columns. The remaining portion of each dilution was untreated. Column purified poliovirus dilutions, non-purified poliovirus dilutions, a negative control consisting of UV treated water, and a positive control of 10^3 pfu of poliovirus were proteinase K digested and analyzed by RT-PCR. Determination of RT-

PCR sensitivity with column purification was repeated twice to ensure reproducibility.

Oligo dT paramagnetic bead capture: 100 μ l sample volume

Before use, oligo dT paramagnetic beads (Dynal) were gently resuspended in the manufacturer's storage buffer. Ten to 50 μ l of the paramagnetic beads was placed in a 1.5 ml microfuge tube. The tube was placed in a magnetic bead capture rack (Promega) for 30 seconds. The storage buffer was carefully withdrawn with a pipet tip and the tube was removed from the rack. The magnetic beads were washed with two hundred μ l of 2X binding buffer containing 20 mM Tris-HCl (pH 7.5), 1.0M LiCl, and 2 mM EDTA. The magnetic beads were resuspended in the buffer by gently shaking the tube. The tube was placed in the magnetic bead capture rack for 30 seconds and the buffer was removed. This washing procedure was then repeated. After the last wash, the magnetic beads were resuspended in 100 μ l of 2X binding buffer.

At the same time, 100 μ l of sample was proteinase K digested and heated to 65°C for 2 minutes. The heated sample was added immediately to the washed magnetic beads and incubated for 10 minutes at room temperature to allow for hybridization of the viral RNA 5' poly A tail and oligo dT tract on the magnetic beads.

The mixture was then placed in the magnetic capture rack for 30 seconds and the binding buffer was carefully withdrawn with a pipet tip. The tube was removed and 200 μ l of washing buffer composed of 10 mM Tris-HCl (pH 7.5), 0.15 M LiCl, and 1 mM EDTA was added. The beads were gently resuspended in the washing buffer and returned to the magnetic bead capture rack. The washing procedure was repeated. After the last wash, buffer was completely removed and 10 μ l of UV treated water was added.

The magnetic beads and water mixture was placed on ice and immediately analyzed by RT-PCR.

Determination of optimum paramagnetic bead volume. Three volumes of oligo dT paramagnetic beads were compared to determine the amount that was optimum for the capture of poliovirus RNA from 100 μ l of sample. Oligo dT magnetic beads were washed as previously described. Ten, twenty five, or fifty μ l of washed magnetic beads was placed into 1.5 ml microfuge tubes. Poliovirus type 1 (LSc) with a titer of 7.5×10^7 pfu/ml was diluted with 1X PBS. Dilutions containing 7.5×10^4 , 7.5×10^3 , 7.5×10^2 pfu/ml were proteinase k digested and heated as previously described. One hundred μ l of each dilution was added to tubes each containing one of the three volumes of washed oligo dT paramagnetic beads and incubated for 10 minutes at room temperature. Washing was performed as previously described. The captured RNA, a negative control consisting of UV treated water, and a positive control of 10^3 plaque forming units of proteinase k digested poliovirus LSc-1 were analyzed by RT-PCR. Optimization of paramagnetic bead volume was repeated twice to ensure reproducibility.

Determination of optimum incubation conditions. Three different incubation conditions were compared to determine the optimum incubation for the capture of poliovirus RNA from 100 μ l of sample. Oligo dT magnetic beads were washed as previously described. Twenty five μ l of washed magnetic beads was placed into 6 1.5 ml microfuge tubes. Poliovirus type 1 (LSc) with a titer of 7.5×10^7 pfu/ml was diluted with 1X PBS. Dilutions containing 7.5×10^3 and 7.5×10^4 pfu/ml were proteinase k digested and heated. One hundred μ l of each dilution was added to three tubes containing washed oligo dT paramagnetic beads. One tube from each dilution was incubated for 10 minutes at room

temperature, 10 minutes on ice, or 5 minutes at room temperature followed by 5 minutes on ice. Washing was performed as previously described. The captured RNA, a negative control consisting of UV treated water, and a positive control of 10^3 pfu of proteinase k digested poliovirus LSc-1 were analyzed by RT-PCR. Optimization of incubation conditions during magnetic bead capture was repeated twice to ensure reproducibility.

Oligo dT paramagnetic bead capture: 900 μ l sample volume

A modification of the previously described 100 μ l oligo dT paramagnetic bead capture method was used. Before use, oligo dT paramagnetic beads (Dynal) were gently resuspended in the manufacturer's storage buffer. Fifty to 150 μ l of the paramagnetic beads was placed in a 1.5 ml microfuge tube. The tube was placed in a magnetic bead capture rack (Promega) for 30 seconds. The storage buffer was carefully withdrawn with a pipet tip and the tube was removed from the rack. The magnetic beads were washed with 1 ml of 10X binding buffer containing 1M Tris-HCl (pH 7.5), 5.0 M LiCl, and 10 mM EDTA. The magnetic beads were resuspended in the buffer by gently shaking the tube. The tube was placed in the magnetic bead capture rack for 30 seconds and the buffer was removed. This washing procedure was then repeated. After the last wash, the magnetic beads were resuspended in 100 μ l of 10X binding buffer.

At the same time, 900 μ l of sample was proteinase K digested and heated to 65°C for 5 minutes. The heated sample was added immediately to the washed magnetic beads and incubated for 15 minutes at room temperature followed by 5 minutes on ice. The mixture was then placed in the magnetic capture rack for 30 seconds and the binding buffer was carefully withdrawn with a pipet tip. The tube was removed and 1 ml of

washing buffer composed of 10 mM Tris-HCl (pH 7.5), 0.15 M LiCl, and 1 mM EDTA was added. The beads were gently resuspended in the washing buffer and returned to the magnetic bead capture rack. The washing procedure was repeated. After the last wash, buffer was completely removed and 10 μ l of UV treated water was added. The magnetic beads and water mixture were placed on ice and immediately analyzed by RT-PCR.

Determination of optimum paramagnetic bead volume. Three volumes of oligo dT paramagnetic beads were compared to determine the optimum volume for the capture of poliovirus RNA from 900 μ l of sample. Oligo dT magnetic beads were washed as previously described. Fifty, 100, and 150 μ l of washed magnetic beads were placed into 3 1.5 ml microfuge tubes. Poliovirus type 1 (LSc) with a titer of 5×10^7 pfu/ml was diluted with 1X PBS. Dilutions containing 500 and 50 pfu/ml of poliovirus were proteinase k digested and heated. Nine hundred μ l of each dilution was added to a tube containing washed oligo dT paramagnetic beads and incubated for 15 minutes at room temperature and 5 minutes on ice. Washing was performed as previously described. The captured RNA, a negative control consisting of UV treated water, and a positive control of 10^3 pfu of proteinase k digested poliovirus type 1 (LSc) were analyzed by RT-PCR. Optimization of paramagnetic bead volume was repeated twice to ensure reproducibility.

Comparison of rocked or stationary conditions during incubation. Rocked or stationary incubations were compared to determine the optimum condition for the capture of poliovirus RNA from 900 μ l of sample. Poliovirus type 1 (LSc) with a titer of 5×10^7 pfu/ml was serially diluted with 1X PBS. Dilutions containing 5×10^3 and 5×10^2 pfu of poliovirus were proteinase k digested and heated. Nine hundred μ l of each dilution was added to two tubes containing 100 μ l of washed oligo dT paramagnetic beads. One tube

from each dilution was incubated at room temperature on a platform rocker (Speci Mix) for 15 minutes, while the other tubes remained stationary. All tubes were then placed on ice for 5 minutes. Washing was performed as previously described. The captured RNA, a negative control consisting of UV treated water, and a positive control of 10^3 pfu of proteinase k digested poliovirus type 1 (LSc) were analyzed by RT-PCR. Comparison of rocked or stationary conditions during magnetic capture was repeated twice to ensure reproducibility.

Determination of RT-PCR sensitivity in a sludge concentrate with the 900 μ l oligo dT paramagnetic bead capture method

A poliovirus seeded sludge concentrate was used to determine the sensitivity of RT-PCR with the 900 μ l sample volume magnetic bead capture methods. Sludge concentrates contain RT-PCR inhibitors that may affect the sensitivity of the methods. Prior to seeding, it was necessary to remove any indigenous nucleic acid. This must be accomplished without elimination of the RT-PCR inhibitors.

Removal of indigenous nucleic acid from sludge concentrate. The pH of the sludge concentrate was raised to 12 or above with 5N NaOH. After 60 minutes, the pH was reduced to 7.0 with 5N HCl. Concentrated NaOH and HCl were used to reduce dilution of the sample from pH adjustment. The pH treated sludge concentrate was proteinase K treated as previously described. Two 900 μ l aliquots were placed in 1.5 ml microfuge tubes and subjected to magnetic bead capture. To determine if indigenous viral nucleic acid was eliminated by the high pH treatment, one tube of magnetic beads was resuspended in 10 μ l of UV treated water. Oligo dT paramagnetic beads in the other were resuspended in 9 μ l of UV treated water and seeded with 1 μ l of 10^5 pfu/ml of

proteinase k digested poliovirus type 1(LSc) to demonstrate the absence of RT-PCR inhibitors. Additionally, nine μl of sludge concentrate that had not undergone magnetic bead capture was seeded with 1 μl of 10^5 pfu/ml of proteinase k digested poliovirus type 1 (LSc) to determine whether the high pH treatment eliminated RT-PCR inhibitors. These samples, a negative control consisting of UV treated water, and a positive control of 10^3 pfu of proteinase k digested poliovirus type 1 (LSc) were analyzed by RT-PCR.

Determination of sensitivity. To determine the least amount of poliovirus in a sludge concentrate that could be detected using RT-PCR with the 900 μl oligo dT paramagnetic bead capture method, a sludge concentrate was seeded with a known quantity of poliovirus. A lime stabilized sludge concentrate that had been pH treated and shown to be free of any indigenous nucleic acid by the previously described method was used. Poliovirus type 1 (LSc) with a titer of 5×10^7 pfu/ml was diluted in the pH treated sludge concentrate. The first dilution was a 1:100 dilution comprised of 990 μl of pH treated sludge concentrate and 10 μl of poliovirus. The remaining dilutions were 1:10 with 100 μl of diluted poliovirus added to 900 μl of pH treated sludge concentrate. Nine hundred μl of sludge concentrate dilutions containing 500, 50, 5, and 0.5 plaque forming units/ml of poliovirus were proteinase k digested, heated and added to 100 μl of washed magnetic beads. The magnetic bead mixture was incubated for 15 minutes at room temperature on a platform rocker followed by 5 minutes on ice. Magnetic beads were washed and resuspended in 10 μl of UV treated water as previously described. The captured RNA, a negative control consisting of UV treated water, and a positive control of 10^3 pfu of proteinase k digested poliovirus type 1 (LSc) were analyzed by RT-PCR. Determination of sensitivity was repeated three times to ensure reproducibility.

Comparison of 100 µl and 900 µl sample volume oligo dT paramagnetic bead capture methods in a sludge concentrate seeded with poliovirus

To compare the sensitivity of RT-PCR with 100 µl and 900 µl sample volume oligo dT paramagnetic bead capture methods in a sludge concentrate, a mock sample consisting of a sludge concentrate seeded with a known quantity of poliovirus was used. Twenty-five and 100 µl aliquots of oligo dT magnetic beads were washed as previously described. A lime stabilized sludge concentrate that had been pH treated and shown to be free of any indigenous nucleic acid by the previously described method was used. Poliovirus type 1 (LSc) was diluted in the pH treated sludge concentrate to ensure that the RT-PCR inhibitors were not reduced by dilution. The first dilution was a 1:100 dilution comprised of 1.98 ml of pH treated sludge concentrate and 20 µl of poliovirus. The remaining dilutions were 1:10 with 200 µl of diluted poliovirus added to 1.8 ml of pH treated sludge concentrate. Sludge concentrate dilutions containing 500 and 50 pfu /ml of poliovirus were proteinase K digested and heated. One hundred µl aliquots of each dilution was placed in the tubes containing 25 µl of magnetic beads, while 900 µl of each dilution was placed in the tubes containing 100 µl of magnetic beads. Reactions with a 100 µl sample volume were incubated for 5 min at room temperature followed by 5 minutes on ice. Reactions with a 900 µl sample volume were incubated for 15 minutes at room temperature on a platform rocker followed by 5 minutes on ice. All reactions were washed as previously described. The captured RNA, a negative control consisting of UV treated water, and a positive control of 10^3 pfu of proteinase k digested poliovirus type 1(LSc) were analyzed by RT-PCR. Magnetic capture methods were compared twice to ensure reproducibility.

Comparison of column purification and oligo dT paramagnetic bead capture methods

A lime stabilized sludge concentrate that had been pH treated and shown to be free of any indigenous nucleic acid by the previously described method was used. Poliovirus type 1 (LSc) was diluted in the pH treated sludge concentrate. The first dilution was a 1:100 dilution comprised of 1.98 ml of pH treated sludge concentrate and 20 μ l of poliovirus. The remaining dilutions were 1:10 with 200 μ l of diluted poliovirus added to 1.8 ml of pH treated sludge concentrate. One hundred μ l of the dilution containing 100 plaque forming units/ml of poliovirus type 1 (LSc) was proteinase K digested, heated, and added to 25 μ l of washed magnetic beads as previously described. After capture was completed, the magnetic beads were resuspended in 10 μ l of UV treated water. Nine hundred μ l of the same dilution was also proteinase K digested, heated, and added to 100 μ l of washed magnetic beads as previously described. After capture was completed, the magnetic beads were resuspended in 10 μ l of UV treated water. Two hundred μ l of the dilution was purified with a Chelex 100/Sephadex G-100 column and then proteinase K digested. The RNA samples, a negative control consisting of UV treated water, and a positive control of RNA from approximately 10^3 plaque forming units of poliovirus type 1 (LSc) were analyzed by RT-PCR. Comparison of columns and magnetic bead capture methods was repeated twice to ensure reproducibility.

Evaluation of sludge concentrates by RT-PCR with the 900 μ l sample volume oligo dT paramagnetic bead capture method and by plaque assay

Eight lime stabilized sludges and three untreated (raw) sludges were eluted with 3% beef extract and concentrated by organic flocculation (EPA, 1992). Raw sludge concentrates were diluted 1:2, 1:5, and 1:10 with 1X PBS while lime stabilized sludge concentrates were used undiluted. Two 900 μ l aliquots of each concentrate or dilution were proteinase K digested, heated, and added to 100 μ l of washed magnetic beads as previously described. After capture was completed, one set of captured magnetic beads from each sample was resuspended in 10 μ l of UV treated water. The other set was resuspended in 9 μ l of UV treated water and seeded with 1 μ l of 10^5 plaque forming units/ml of poliovirus type 1 (LSc) that had been proteinase k digested. The RNA samples, a negative control consisting of UV treated water, and a positive control of RNA from approximately 10^3 plaque forming units of poliovirus type 1 (LSc) were analyzed by RT-PCR. Evaluation of sludge concentrates by RT-PCR with magnetic bead capture was repeated twice to ensure reproducibility.

Additionally, the sludge concentrates were analyzed by plaque assay with BGM cells as previously described. The sludge concentrates were diluted with 1X PBS and viruses was detected by plaque assay with BGM cells, using a 1 ml inoculum of sludge concentrate per 25 cm² flask. Four flasks were used per dilution.

RESULTS

Detection of poliovirus in sludge with a ³²P labeled RNA probe

Orientation of pGEM 3Z/PV 1174

The plasmid pGEM 3Z/PV 1174 was digested with restriction endonuclease Kpn I and the fragments were separated by agarose gel electrophoresis and viewed after ethidium bromide staining (Figure 6). Bands of 3100 and 800 bp were produced and demonstrated that the insert was in orientation 1 (Figure 4). Based on the arrangement of the promoters within pGEM-3Z, it was determined that SP6 RNA polymerase would result in the production of negative sense RNA transcripts, while T7 RNA polymerase would produce positive sense RNA transcripts.

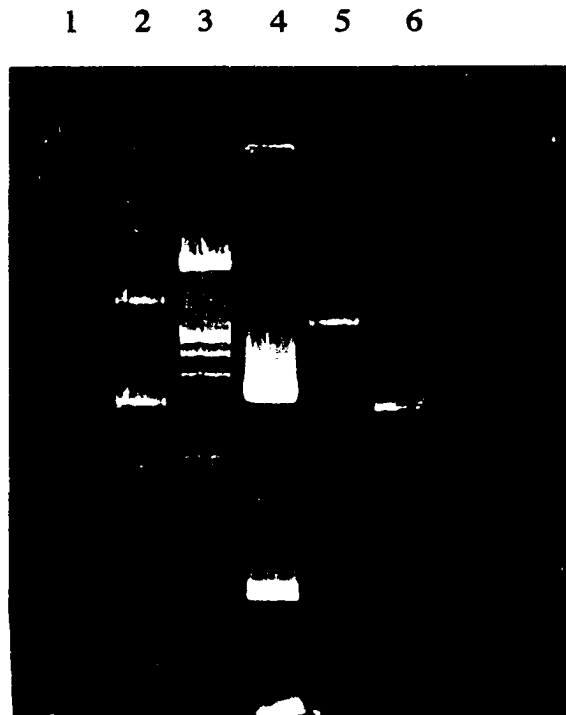
Optimization of negative sense poliovirus RNA transcript production

The RNA yields from reactions containing five different concentrations of NTP's were compared. RNA yields were determined by spectrophotometric readings. It was determined that a 500 µM NTP concentration resulted in the greatest RNA transcript production (Figure 7).

The RNA yields from reactions incubated at 37°C for 15, 30, 60, 90, 120 minutes, and 120 minutes with an extra addition of SP6 RNA polymerase midway through the incubation were compared. RNA yields were compared after agarose gel electrophoresis

Figure 6

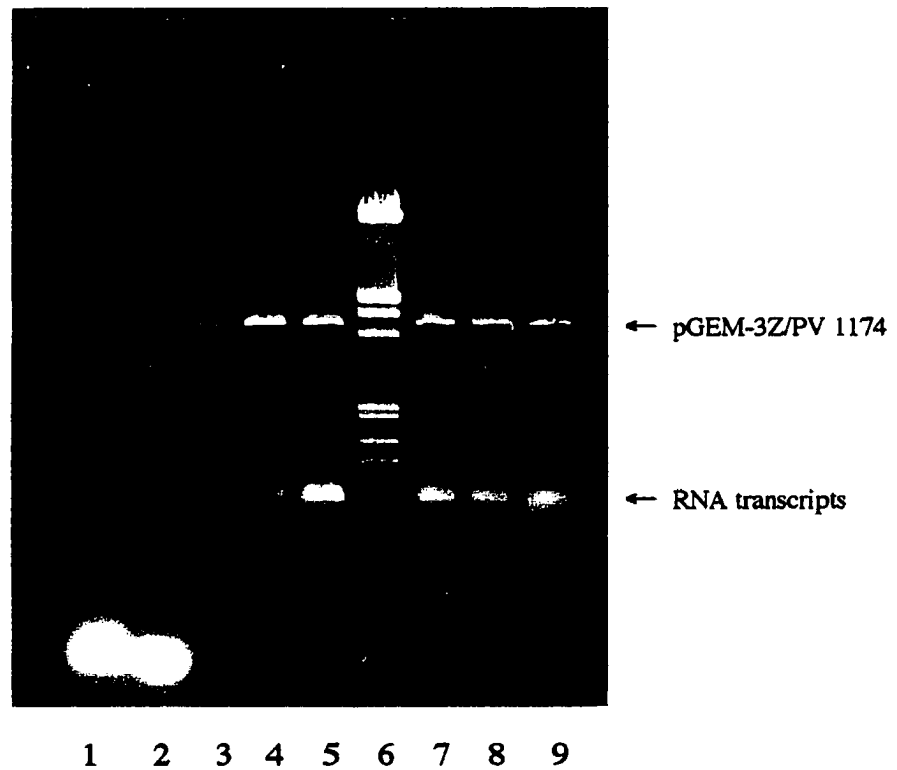
Determination of poliovirus cDNA insert orientation



Agarose gel stained with ethidium bromide showing restriction digests of the vector pGEM-3Z/PV 1174. Lanes: 1, empty; 2, pGEM-3Z/PV 1174; 3, lambda Hind III and EcoRI digest; 4, pGEM-3Z/PV 1174 digested with Kpn I; 5, pGEM-3Z/PV 1174 digested with Pst I.

Figure 7

Optimization of negative sense poliovirus RNA transcript production
NTP concentration



Agarose gel analysis demonstrating poliovirus *in vitro* RNA synthesis with SP6 RNA polymerase with varied NTP concentrations. Lanes: 1, yeast tRNA; 2, yeast tRNA; 3, linearized pGEM-3Z/PV 1174; 4, RNA transcription with 400 μM NTP's; 5, RNA transcription with 500 μM NTP's; 6, Hind III/EcoRI digest of lambda DNA; 7, RNA transcription with 600 μM NTP's; 8, RNA transcription with 800 μM NTP's; 9, RNA transcription with 1200 μM NTP's.

and ethidium bromide staining (Figure 8). It was determined that a 120 minute incubation with extra enzyme produced the greatest amount of RNA

Production of positive sense poliovirus RNA transcripts

RNA transcript production from reactions incubated at 37°C for 90 minutes were evaluated after agarose gel electrophoresis and ethidium bromide staining (Figure 9).

RNA yields were determined to be consistently high using this procedure.

Optimization of RNA probe concentration

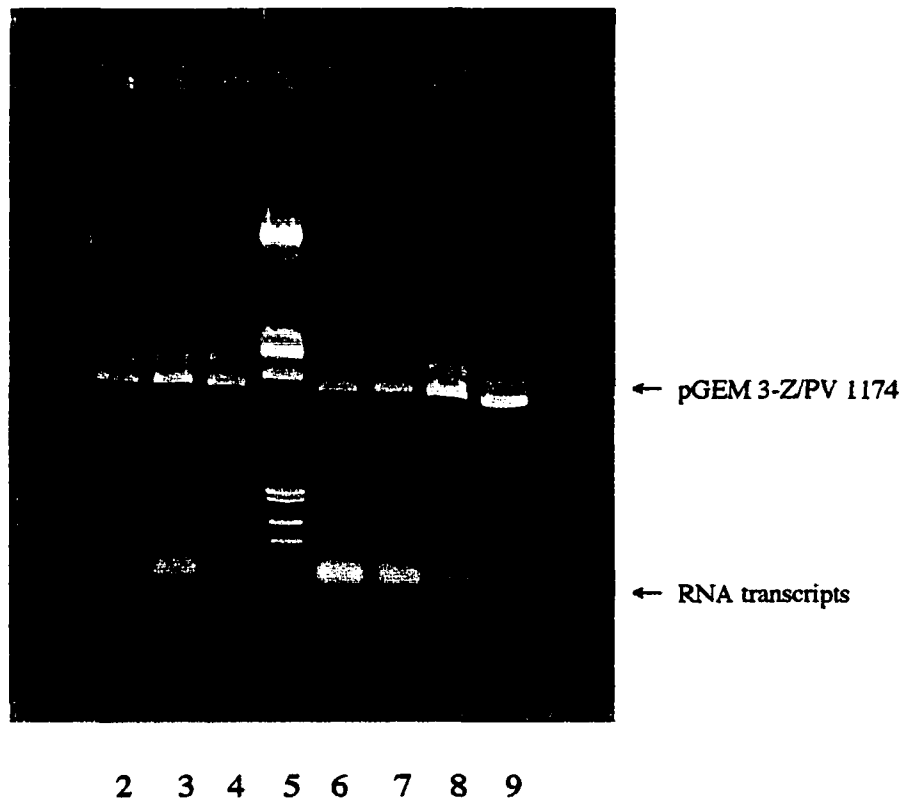
Membranes spotted with positive sense poliovirus RNA transcripts were hybridized with a negative sense poliovirus RNA probe with a specific activity of 1.7×10^9 cpm/ μ g at concentrations of 1×10^6 , 5×10^6 , and 1×10^7 cpm/ml of hybridization fluid. It was determined that probe concentrations of 5×10^6 and 1×10^7 cpm/ml resulted in the greatest sensitivity, however, the 1×10^7 cpm/ml probe concentration produced significant background as seen after autoradiography (Figure 10). Therefore, the 5×10^6 cpm/ml probe concentration was used in subsequent experimentation.

Sensitivity of negative sense RNA probe

Dilutions of positive sense RNA transcripts and proteinase K digested poliovirus type 1 (LSc) were applied to hybridization membranes and probed with the negative sense RNA probe. The detection limit of the probe was 10 fg of positive sense RNA transcripts and 90 plaque forming units of poliovirus LSc-1 (Table 1).

Figure 8

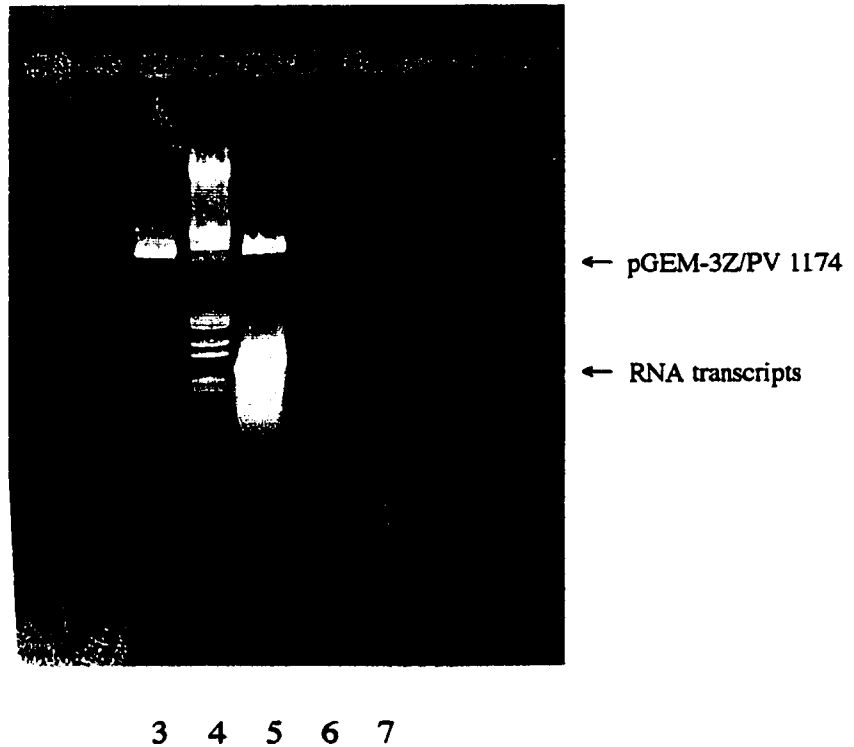
Optimization of negative sense poliovirus RNA transcript production
Incubation conditions



Agarose gel analysis demonstrating poliovirus *in vitro* RNA synthesis with SP6 RNA polymerase with varied incubation conditions. Lanes: 2, RNA transcription for 30 minutes; 3, RNA transcription for 60 minutes; 4, RNA transcription for 15 minutes; 5, Hind III/EcoRI digest of lambda DNA; 6, RNA transcription for 120 minutes with extra enzyme; 7, RNA transcription for 90 minutes; 8, RNA transcription for 120 minutes; 9, linearized pGEM-3Z/PV 174.

Figure 9

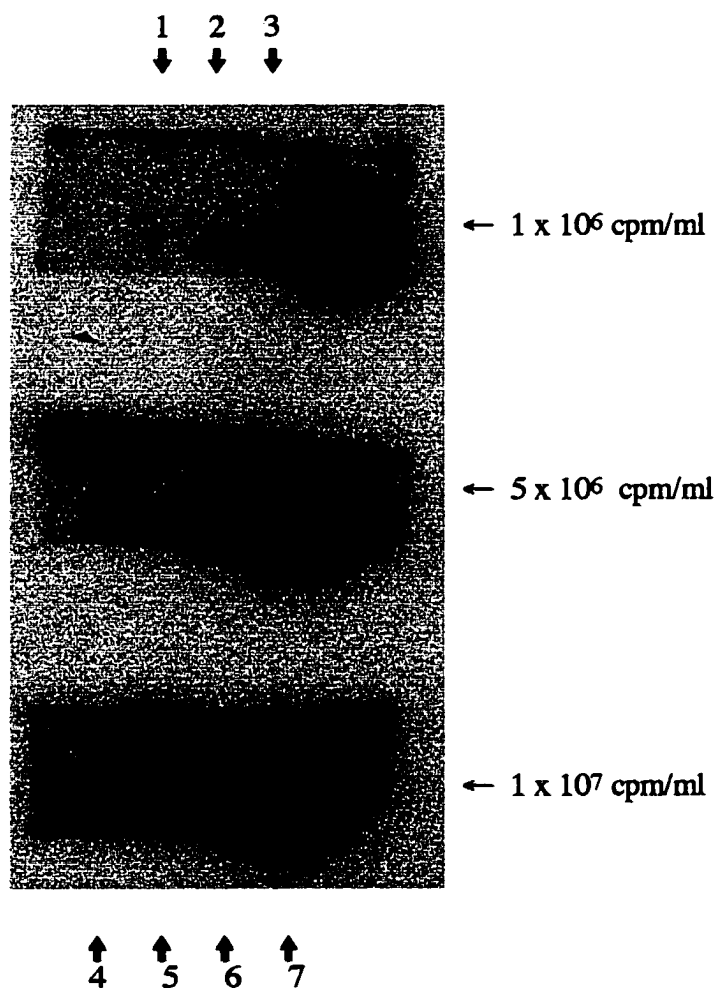
Production of positive sense RNA transcripts



Agarose gel analysis demonstrating poliovirus *in vitro* RNA synthesis with T7 RNA polymerase. Lanes: 3, pGEM-3Z/PV 1174 digested with Hind III; 4, lambda DNA digested with Hind III and EcoRI; 5, pGEM-3Z/PV 1174 digested with Hind III and positive sense RNA transcripts generated with T7 RNA polymerase; 6, yeast tRNA; 7, yeast tRNA.

Figure 10

Optimization of RNA probe concentration



Autoradiograph of three identical hybridization membranes containing positive sense RNA transcript dilutions, applied with the dot blot apparatus, and individually probed with three concentrations of negative sense RNA probe. Top row of wells: 1, negative control (DEPC treated water); 2, 0.5 fg RNA; 3, 5.0 fg. Bottom row of wells: 4, 50 fg; 5, 500 fg; 6, 5 pg; 7, 50 pg.

Table 1

Detection of poliovirus type 1 (LSc) and RNA transcripts with a ^{32}P labeled RNA probe

	poliovirus pfu					
Lowest RNA detected	9×10^5	9×10^4	9×10^3	9×10^2	9×10^1	9×10^0
10 fg	+	+	+	+	+	-

+ viral RNA detected

- viral RNA not detected

Specificity of poliovirus RNA probe

Dilutions of bacteriophage MS-2, hepatitis A virus, coxsackie B3 virus, poliovirus 1, poliovirus 2, poliovirus 3, and echovirus 7 RNA were applied to a hybridization membrane and probed with the negative sense RNA probe. The negative sense poliovirus RNA probe strongly hybridized with poliovirus 1, 2, and 3 RNA (Table 2). There was a weak signal produced after hybridization with echovirus 7 RNA.

Comparison of beef extract elution methods

Poliovirus type 1 (LSc) seeded sludge was eluted with a 3% solution of beef extract V supplemented with 90 mM glycine at pH 7.0 or 9.5. Alternatively, beef extract V powder was added directly to the sludge and the pH was adjusted to 7.0 or 9.5. During elution, the sludge was mixed on a magnetic stirrer for 10 minutes or homogenized for 2 minutes. Poliovirus recovery was determined by plaque assay and hybridization with the negative sense poliovirus RNA probe (Table 3). Overall, beef extract powder at either pH or mixing technique yielded a higher poliovirus recovery as measured by plaque assay and RNA probe than elution with beef extract solution. Use of beef extract powder at pH 7.0 with homogenization resulted in slightly higher poliovirus recovery as measured by plaque assay than other elution methods.

Comparison of viral nucleic acid liberation methods from sludge concentrates

Proteinase k digestion, phenol/chloroform/ether extraction, and proteinase k digestion followed by phenol/chloroform/ether extraction were compared for the liberation of poliovirus RNA from sludge concentrates. RNA from the three methods

were spotted onto a hybridization membrane and probed with the negative sense RNA probe (Table 4). Phenol/chloroform/ether extraction, with or without proteinase k digestion, resulted in the detection of 1.8×10^4 pfu of poliovirus. However proteinase k digestion alone resulted in the detection of 1.8×10^2 pfu of poliovirus.

Table 2

Specificity of poliovirus ³²P labeled RNA probe

Virus	Dilution		
	10 ⁻²	10 ⁻³	10 ⁻⁴
Poliovirus-1	+	+	+
Poliovirus-2	+	+	+
Poliovirus-3	+	+	-
Echovirus 7	+	-	-
Coxsackie A7	-	-	-
Coxsackie B3	-	-	-
Hepatitis A virus	-	-	-

+ viral RNA detected

- viral RNA not detected

Table 3

Comparison of eluant and mixing technique for the recovery of poliovirus type 1 (LSc) from sludge

			plaque assay	RNA probe		
eluant	mixing	pH		dilution		
			pfu/ml	^a 10 ⁰	10 ⁻¹	10 ⁻²
^b 3% be (l)	mag. stirrer	7.0	1.3 x 10 ⁵	+	+	+
	mag. stirrer	9.5	1.0 x 10 ⁵	+	+	-
	homogenizer	7.0	1.0 x 10 ⁵	+	+	-
	homogenizer	9.5	1.1 x 10 ⁵	+	+	-
^c 3% be (s)	mag. stirrer	7.0	1.3 x 10 ⁵	+	+	+
	mag. stirrer	9.5	1.7 x 10 ⁵	+	+	+
	homogenizer	7.0	2.6 x 10 ⁵	+	+	+
	homogenizer	9.5	2.4 x 10 ⁵	+	+	+

^a 100 µl of each dilution was spotted onto hybridization membrane

^b 3% beef extract solution supplemented with 90 mM glycine

^c beef extract powder added to a final concentration of 3%

+ viral RNA detected

- viral RNA not detected

Table 4

Comparison of viral liberation method for the detection of
Poliovirus with ^{32}P labeled RNA probe

method	Poliovirus pfu/100 μl					
	1.8×10^7	1.8×10^6	1.8×10^5	1.8×10^4	1.8×10^3	1.8×10^2
^a prot. k	^c +	+	+	+	+	+
^b p/c/e	+	+	+	+	-	-
Prot. K + p/c/e	+	+	+	+	-	-

^a proteinase k digestion

^b phenol/chloroform and ether extraction

+ viral RNA detected

- viral RNA not detected

Detection of Enteroviruses in Sludge with RT-PCR

Sensitivity of RT-PCR assay

Poliovirus type 1(LSc) was proteinase K digested and diluted in UV treated water. RT-PCR was performed with negative and positive controls. After agarose gel electrophoresis and ethidium bromide staining, 196 bp RT-PCR products were viewed (Table 5). One plaque forming unit of poliovirus per 10 µl was the lowest amount detected.

Demonstration of RT-PCR inhibitors in sludge

A sludge concentrate was seeded with poliovirus type 1(LSc) to obtain a concentration of 10^3 pfu/10 µl of concentrate. The seeded concentrate, a positive control containing 10^3 pfu poliovirus/10 µl of UV treated water, and a negative control were proteinase k digested and assayed by RT-PCR. After agarose gel electrophoresis and ethidium bromide staining, 196 bp RT-PCR products were viewed. Amplification occurred in the positive control but not in the concentrate, demonstrating the presence of RT-PCR inhibitors in the sludge concentrate.

Sensitivity of RT-PCR with column purification

Aliquots of poliovirus type 1 (LSc) dilutions were purified with Chelex 100/Sephadex G-100 columns. Non-column purified dilutions, column purified dilutions, and controls were proteinase K digested and assayed by RT-PCR. After agarose gel electrophoresis and ethidium bromide staining, 196 bp RT-PCR products were viewed

Table 5

Sensitivity of RT-PCR assay for the detection of poliovirus

Poliovirus (pfu/10 μ l)			
100	10	1.0	0.1
+	+	+	-

+ 196 bp RT-PCR product detected by agarose gel analysis

- 196 bp RT-PCR product not detected by agarose gel analysis

(Table 6). The dilution that contained 5 pfu/10 μ l of poliovirus and did not receive column purification was the last dilution that yielded a product. However, with column purification, the last dilution of poliovirus that yielded a product contained 5×10^2 pfu/10 μ l.

Optimization of oligo dT paramagnetic bead capture: 100 μ l sample volume

Three volumes of magnetic beads were compared to determine the optimum amount required for the capture poliovirus RNA. Dilutions of proteinase k digested poliovirus type 1 (LSc) were added to tubes containing 10, 25, or 50 μ l of magnetic beads. Magnetic bead capture and RT-PCR were performed. . After agarose gel electrophoresis and ethidium bromide staining, 196 bp RT-PCR products were viewed (Table 7). Use of 25 or 50 μ l of magnetic beads resulted in a greater sensitivity than the use of 10 μ l, and resulted in the detection of 75 pfu of poliovirus per 100 μ l. There was no difference in the detection sensitivity with the use of 25 or 50 μ l volumes of magnetic beads. Therefore, to conserve the reagent, 25 μ l of oligo dT paramagnetic beads was used in subsequent experiments.

Three temperatures were compared to determine the optimum hybridization conditions for the capture of poliovirus RNA. Dilutions of poliovirus containing 7.5×10^3 and 7.5×10^2 pfu/100 μ l were added to 25 μ l of magnetic beads and incubated for 10 minutes at room temperature, 10 minutes on ice, or 5 minutes at room temperature followed by 5 minutes on ice. Magnetic bead capture and RT-PCR were performed. . After agarose gel electrophoresis and ethidium bromide staining, 196 bp RT-PCR

Table 6

Sensitivity of RT-PCR with Chelex 100/Sephadex G-100 columns
for the detection of poliovirus

method	Poliovirus (pfu/10 μ l)				
	10 ³	1 x 10 ²	10	1.0	0.1
no column	+	+	+	+	-
column	+	+	-	-	-

+ 196 bp RT-PCR product detected by agarose gel analysis

- 196 bp RT-PCR product not detected by agarose gel analysis

Table 7

Optimization of oligo dT paramagnetic bead volume
100 μ l sample volume

volume of mag. beads	Poliovirus (pfu/100 μ l)		
	7.5×10^3	7.5×10^2	7.5×10^1
10 μ l	+	+	-
25 μ l	+	+	+
50 μ l	+	+	+

- + 196 bp PCR product detected by agarose gel analysis
- 196 bp PCR product not detected by agarose gel analysis

products were viewed (Figure 11 and Table 8). Both dilutions were detected using all three hybridization temperatures, although incubation for 5 minutes at room temperature followed by 5 minutes on ice resulted in DNA bands of much greater intensity.

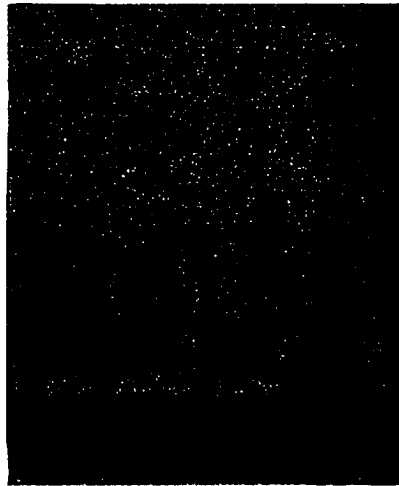
Optimization of oligo dT paramagnetic bead capture: 900 µl sample volume

Three volumes of magnetic beads were compared to determine the optimum amount required for the capture poliovirus RNA. Dilutions of proteinase k digested poliovirus type 1 (LSc) containing 500 and 50 pfu/ml were added to tubes with 50, 100, or 150 µl of magnetic beads. Magnetic bead capture and RT-PCR were performed. After agarose gel electrophoresis and ethidium bromide staining, 196 bp RT-PCR products were viewed (Table 9). All three volumes yielded products from the dilution containing 500 pfu/ml of poliovirus, however only 100 and 150 µl magnetic bead volumes yielded products from the dilution containing 50 pfu/ml. There was no difference in the detection sensitivity of assays using 100 or 150 µl of magnetic beads; therefore 100 µl was used in subsequent experiments.

Hybridizations with or without rocking were compared to determine the optimum conditions required for the capture of poliovirus RNA. Dilutions of proteinase k digested and heated poliovirus type 1 (LSc) containing 5×10^3 and 5×10^2 pfu/ml were added to tubes with 100 µl of magnetic beads. During room temperature hybridization, one set of tubes was rocked on a platform rocker while the other remained stationary. The remaining portions of the procedure were followed and RT-PCR was performed. After agarose gel electrophoresis and ethidium bromide staining, 196 bp RT-PCR

Figure 11

Optimization of hybridization temperature during oligo dT magnetic bead capture
100 μ l sample volume



1 2 3 4 5 6 7 8 9 10

Agarose gel analysis of RT-PCR products from samples containing dilutions of poliovirus type 1 (Lsc) in 1X PBS after 100 μ l sample volume oligo dT paramagnetic bead capture. Samples were incubated during magnetic bead capture for 10 minutes at room temperature (r.t.), 10 minutes on ice, or 5 minutes at r.t. followed by 5 minutes on ice. Lanes: 1, negative control (U.V. treated water); 2, 10 min. r.t. with 7.5×10^3 pfu poliovirus; 3, 10 min. ice with 7.5×10^3 pfu poliovirus; 4, 5 min. r.t./5 min ice with 7.5×10^3 pfu poliovirus; 5, 10 min r.t. with 7.5×10^2 pfu poliovirus; 6, 10 min. ice with 7.5×10^2 pfu poliovirus; 7, 5 min. r.t./5 min. ice with 7.5×10^2 pfu poliovirus; 8, 100 bp DNA ladder; 9, negative control (U.V. treated water).

Table 8

Optimization of hybridization temperature during oligo dT magnetic bead capture
100 μ l sample volume

Temperature	Poliovirus (pfu/100 μ l)	
	7.5×10^3	7.5×10^2
10 min room temp (RT)	^a -	-
10 min ice	+	+
5 min RT/5 min ice	+++	++

^a representative of DNA band intensity as seen on a 1.5% agarose gel after ethidium bromide staining
 +++ high intensity
 ++ mid intensity
 + low intensity
 - negative

Table 9

Optimization of oligo dT paramagnetic bead volume
900 μ l sample volume

volume of mag. beads	Poliovirus (pfu/ml)	
	500	50
50	+	-
100	+	+
150	+	+

+ 196 bp PCR product detected by agarose gel analysis

- 196 bp PCR product not detected by agarose gel analysis

products were viewed (Table 10). Rocked and stationary conditions resulted in the detection of 5×10^3 pfu of poliovirus, however 5×10^2 pfu of poliovirus were only detected when tubes were rocked during hybridization.

Sensitivity of RT-PCR in a sludge concentrate using the 900 μ l sample volume oligo dT paramagnetic bead capture method

Removal of indigenous nucleic acid from a sludge concentrate. Indigenous nucleic acid from a limed sludge concentrate was removed by raising the pH to 12. Magnetic bead capture was performed with two 900 μ l aliquots of the pH treated sludge concentrate. After capture, one of the two aliquots was seeded with poliovirus type 1 (LSc). A 9 μ l aliquot of sludge concentrate that did not undergo magnetic capture was also seeded with poliovirus. RT-PCR was performed on all reactions and 196 bp RT-PCR products were viewed after agarose gel electrophoresis and ethidium bromide staining. Non-seeded sludge that underwent magnetic capture did not yield a product, demonstrating that any indigenous nucleic acid was removed. Seeded sludge that underwent magnetic capture yielded a product, demonstrating that the method removed RT-PCR inhibitors that would have prevented detection of any indigenous nucleic acid. No product was formed with seeded sludge concentrate that did not undergo magnetic capture, demonstrating that the high pH treatment did not eliminate the RT-PCR inhibitors in the sludge.

Sensitivity of RT-PCR in a sludge concentrate with the 900 μ l oligo dT paramagnetic bead capture method. Indigenous virus was removed from a sludge concentrate as previously described. The sludge concentrate was seeded with 500, 50, 5, or 0.5 pfu of poliovirus type 1 (LSc) per ml. Magnetic capture and RT-PCR were

Table 10

Comparison of rocked and stationary hybridization for oligo dT magnetic bead capture
900 μ l sample volume

hybridization condition	poliovirus (pfu/ml)	
	5×10^3	5×10^2
rocked	+	+
stationary	+	-

- + 196 bp PCR product detected by agarose gel analysis
- 196 bp PCR product not detected by agarose gel analysis

performed on all reactions. RT-PCR products were viewed after agarose gel electrophoresis and ethidium bromide staining (Table 11). The lowest poliovirus concentration detected with this method was 5 pfu/ml of sludge concentrate.

Comparison of 100 µl and 900 µl sample volume oligo dT paramagnetic bead capture methods using a poliovirus seeded sludge concentrate

Comparison of oligo dT magnetic bead methods. Indigenous virus was removed from a sludge concentrate as previously described. Sludge was seeded with 50 or 500 pfu of poliovirus per ml. Magnetic capture with 100 µl and 900 µl sample volumes and RT-PCR were performed on all reactions. RT-PCR products were viewed after agarose gel electrophoresis and ethidium bromide staining (Table 12). Fifty pfu of poliovirus per ml of sludge concentrate was easily detected with the 900 µl sample volume magnetic bead capture method. The same amount of poliovirus was not detected with the 100 µl sample volume magnetic bead capture method.

Comparison of 100 µl and 900 µl sample volume oligo dT paramagnetic bead capture methods and Chelex 100/Sephadex G-100 columns using a poliovirus seeded sludge concentrate

Indigenous virus was removed from a sludge concentrate as previously described. The sludge concentrate was seeded with 100 pfu of poliovirus type 1 (LSc) per ml. Column purification and magnetic capture using 100 µl and 900 µl sample volumes were performed. All reactions were assayed by RT-PCR and 196 bp RT-PCR products were viewed after agarose gel electrophoresis and ethidium bromide staining (Figure 12). Poliovirus was detected only with the 900 µl sample volume magnetic bead capture method.

Table 11

Sensitivity of RT-PCR with 900 μ l sample volume oligo dT magnetic bead capture for the detection of poliovirus in a sludge concentrate

poliovirus (pfu/ml)			
500	50	5	0.5
+	+	+	-

+ 196 bp PCR product detected by agarose gel analysis

- 196 bp PCR product not detected by agarose gel analysis

Table 12

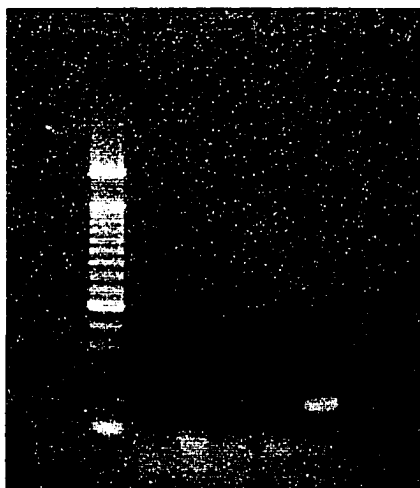
Comparison of 100 μ l and 900 μ l oligo dT paramagnetic bead capture methods

	poliovirus (pfu/ml)	
	500	50
100 μ l sample volume	+	-
900 μ l sample volume	+	+

- + 196 bp PCR product detected by agarose gel analysis
- 196 bp PCR product not detected by agarose gel analysis

Figure 12

Comparison of 100 μ l and 900 μ l oligo dT paramagnetic bead capture methods and Chelex 100/Sephadex G-100 columns



3 4 5 6 7 8

Agarose gel analysis of RT-PCR products from a lime stabilized sludge concentrate seeded with 100 pfu of poliovirus type 1 (LSc). The seeded sludge sample was treated by Chelex 100/Sephadex G-100 columns or oligo dT paramagnetic bead capture methods using 100 μ l or 900 μ l sample volumes. Lanes: 3, 100 bp DNA ladder; 4, column; 5, 100 μ l sample volume magnetic bead capture; 6, 900 μ l sample volume magnetic bead capture; 7, negative control (UV treated water); 8, positive control (10^3 pfu of poliovirus type 1).

Evaluation of sludge concentrates by RT-PCR with the 900 µl sample volume oligo dT paramagnetic bead capture method and by plaque assay

Eight lime stabilized sludge concentrates and three raw sludge concentrates were evaluated by plaque assay and RT-PCR with oligo dT magnetic bead capture for the presence of enteroviruses. Enteroviruses were not detected in the lime stabilized sludge concentrates by plaque assay (Table 13) or RT-PCR with magnetic bead capture (Table 14). When seeded, the concentrates yielded a RT-PCR product, demonstrating the removal of inhibitors in the lime stabilized sludges by the oligo dT magnetic bead capture method. No toxicity from the lime stabilized sludge concentrates was observed during plaque assay.

Enteroviruses were detected in the raw sludge concentrates by plaque assay (Table 13). Plaques were not formed with the undiluted concentrates due to sample toxicity that resulted in the destruction of the cell monolayer. Sample toxicity was reduced after 1:10 dilution of concentrates. Low numbers of plaques were counted in each of the four flasks of BGM cells inoculated with 1 ml of sample. Enteroviruses were not detected in the raw sludge concentrates by RT-PCR with magnetic capture (Table 15). When seeded, one of the concentrates did not yield a RT-PCR product until diluted 1:2, while the remaining two required a 1:10 dilution. This demonstrates the incomplete removal of inhibitors in these raw sludges by the oligo dT magnetic bead capture method.

Table 13

Evaluation of lime stabilized and raw sludge concentrates
Plaque assay

Sample	Enterovirus dilution (pfu/ml)	
	Undiluted	10-1
Limed sludge 1	0	0
Limed sludge 2	0	0
Limed sludge 3	0	0
Limed sludge 4	0	0
Limed sludge 5	0	0
Limed sludge 6	0	0
Limed sludge 7	0	0
Limed sludge 8	0	0
Raw sludge 1	^a 0	4.3
Raw sludge 2	^a 0	1.5
Raw sludge 3	^a 0	5.0

^a BGM cell monolayer was destroyed due to sample toxicity

Table 14

Evaluation of lime stabilized concentrates
 RT-PCR with 900 µl sample volume oligo dT paramagnetic bead capture

Sample	Non-seeded	Seeded with poliovirus
Limed sludge 1	-	+
Limed sludge 2	-	+
Limed sludge 3	-	+
Limed sludge 4	-	+
Limed sludge 5	-	+
Limed sludge 6	-	+
Limed sludge 7	-	+
Limed sludge 8	-	+

+ 196 bp PCR product detected by agarose gel analysis
 - 196 bp PCR product by agarose gel analysis

Table 15

Evaluation of raw sludge concentrates
 RT-PCR with 900 µl sample volume oligo dT magnetic bead capture

sample	Dilution of non-seeded concentrate				Dilution of seeded concentrate			
	Undil.	1:2	1:5	1:10	Undil.	1:2	1:5	1:10
Raw 1	-	-	-	-	-	-	-	+
Raw 2	-	-	-	-	-	+	+	+
Raw 3	-	-	-	-	-	-	-	+

- + 196 bp PCR product detected by agarose gel analysis
- 196 bp PCR product not detected by agarose gel analysis

DISCUSSION

Introduction to the Detection of Viruses in Sludge

Due to rapid population growth, many wastewater treatment plants generate much more sludge than can be disposed of by traditional practices, such as incineration and landfilling. In response, the USEPA has encouraged communities to use sludge in a beneficial manner. Sludge contains organic matter and nutrients that make it useful as a fertilizer. Sludge has been used to fortify the soil in golf courses, median strips, forests, and agricultural crops (USEPA, 1992).

However, land application of sludge may result in the contamination of surface and groundwater with pathogens. Although sludge must be treated prior to land application, these treatments do not completely eliminate pathogens. Protozoa and bacteria are large enough to be trapped at or near the soil surface where they are destroyed by sunlight and desiccation (Gilbert et al., 1976 and Edmonds, 1976). Viruses, however, are much smaller and are not retained by the soil on the basis of size. Viruses may travel with the infiltrating fluids through deep channels in the soil. Spread of viruses through aerosolization, ingestion of contaminated crops, and by animals located in the area may occur.

Due to the potential risk to the public health from the pollutants in sludge, the USEPA issued regulations that control the application of sludge to land. Sludge must meet pathogen reduction standards with or without site restrictions. The direct

measurement of pathogen levels, such as enteric viruses may be required. However, most of the sludge that is disposed of through land application only requires monitoring of fecal coliform levels. It is known that the presence of fecal coliform bacteria does not correlate with the presence of pathogens, such as enteric viruses (Rao and Melnick, 1986). However, methods to detect and quantify fecal coliforms are inexpensive and easy to perform.

The standard method for the detection of enteric viruses in sludge is the plaque assay with Buffalo green monkey (BGM) kidney cells. Sludge samples are inoculated onto monolayers of BGM cells and covered with a solid medium that localizes lysed cells that form clear areas or plaques. The plaques are counted and the concentration of viruses is determined. Cell culture assays, such as the plaque assay, are very sensitive and have the potential to detect as few as one infectious virus. However, there are many shortcomings as well. As yet, there is no cell line that supports the growth of all enteric viruses that may be found in sludge. Some viruses, such as hepatitis A virus and Norwalk virus, replicate slowly or not at all in cell culture. Other viral isolates may replicate but not produce cytopathic effects such as plaques. Sludge concentrates must be treated prior to inoculation of cell cultures to reduce bacterial contamination. These treatments may reduce the viral titer and are sometimes ineffectual. Additionally, sludge concentrates can be so toxic to cell cultures that the cells are destroyed within minutes of sample inoculation. Cytotoxicity may result in plaque-like areas of clearing that can be mistaken for true plaques. The USEPA regulations require monitoring of a BGM cell plaque assay for 16 days, followed by one or two confirmation passages of 7 days. Therefore, the BGM cell plaque assay may take up to 30 days or more to complete.

Inoculation and monitoring of multiple samples is cumbersome and labor intensive. The material cost is very high due to the expense of cell culture plasticware and serum.

Molecular techniques, such as ssRNA probes and RT-PCR, detect the nucleic acid of the virus and avoid many of the problems associated with cell culture assays.

Radiolabeled RNA probes are efficiently synthesized *in vitro* to a high specific activity by T3, T7, or SP6 bacteriophage RNA polymerases with the addition of one or more ³²P labeled rNTP's to the transcription reaction. Additionally, multiple samples can be processed simultaneously and results obtained within 3-4 days. These factors help to lower the cost of the RNA probe assay.

The polymerase chain reaction is an *in vitro* DNA amplification procedure that results in the exponential increase of target DNA. RNA must be converted to cDNA by reverse transcription prior to PCR. The amplified DNA can then be viewed after agarose gel electrophoresis and ethidium bromide staining. Depending on the thermal cycler model and assay, twenty-four or more samples can be processed at the same time. Results may be obtained within hours and material costs are low.

Although PCR has a high degree of sensitivity, its use in environmental samples is limited by the presence of substances in the samples that inhibit PCR and small reaction volumes. Magnetic separation procedures using paramagnetic beads linked to virus specific antibody or streptavidin have also been used for hybridization of viral capsid epitopes or biotin labeled oligomers bound to viral nucleic acid. Once hybridization is complete the magnetic beads are washed and the inhibitors are removed. Paramagnetic beads covalently linked to an oligo dT tract have primarily been used for the isolation of mRNA. However they can also hybridize with the polyA tail of the

enteroviruses and hepatitis A virus. Oligo dT magnetic beads have the ability to simultaneously capture these viruses without the need for virus specific oligomers during hybridization. After washing the beads, they may be resuspended in as little as 10 μ l of liquid or added directly to the RT-PCR reaction. This concentration increases the amount of the original sample that can be included per polymerase chain reaction

Development of ssRNA Probe for the Detection of Poliovirus in Sludge

Optimization of negative sense RNA probe

Optimization of negative sense RNA transcript yields was initially conducted with non-labeled rNTP's. RNA yields were greatly increased when the polymerase reaction was extended to 120 minutes with further addition of RNA polymerase after 60 minutes. A concentration of the probe equaling 5×10^6 cpm/ml of hybridization fluid, was found produce a good signal without excess background following autoradiography of hybridization membranes. The probe was able to detect 10 fg of positive sense RNA transcripts and 90 plaque forming units of poliovirus type 1 (LSc).

Specificity of negative sense RNA probe

Following hybridization with MS-2 bacteriophage, several enteroviruses, and hepatitis A virus, the probe was found to strongly hybridize with only poliovirus types 1, 2, and 3. The probe hybridizes to the region of poliovirus RNA that is translated into viral protein 1 (VP 1). VP 1 is the major neutralizing epitope of the enteroviruses and thus the area where they share the least homology (Belshe, 1991). The probe was also

found to weakly hybridize with echovirus 7. This may be due to the approximately 20% RNA homology between groups of enteroviruses (Belshe, 1991).

Elution of seeded poliovirus from sludge with beef extract

Many methods for the elution of viruses from environmental samples used beef extract at alkaline pH's (Berg and Sullivan, 1988). It was determined by plaque assay that elution of poliovirus at pH 7.0 was no less effective and may have resulted in a greater recovery of poliovirus from sludge than elution at pH 9.5. The difference between the poliovirus recovery at pH 7.0 and 9.5 may be due to the instability of enteroviruses at highly alkaline pH's.

Elution of poliovirus from sludge with beef extract powder resulted in nearly two times the recovery of poliovirus than elution with a beef extract solution. Although elution with a beef extract solution is a more common practice, the simple addition of beef extract powder reduces the time required for preparing and performing the procedure.

Mixing during elution by homogenization or magnetic stirring was also compared. Little difference between the mixing techniques was noted during elution with 3% beef extract solution. However, approximately 60% more poliovirus was recovered during elution with beef extract powder with homogenization. This may be due to better incorporation of the beef extract within the sludge resulting in a greater amount of poliovirus eluted from the sludge. Most likely it is not from the release of embedded viruses, as this would have been evident during elution with beef extract solution.

Poliovirus recoveries ranging from 12% to 31% were obtained. The elution method that resulted in the most recovered poliovirus was the use of beef extract powder at a pH of 7.0 with homogenization. The amount of poliovirus recovered by this procedure is comparable or greater than recoveries by other methods (Farrah et al., 1981; Pancorbo et al., 1981). A sterile beef extract solution does not need to be prepared and mixing time is reduced with this method. Therefore, this method substantially reduces the time required for elution.

Viral liberation methods

Proteinase k digestion was found to result in a greater detection sensitivity of poliovirus in a sludge concentrate than phenol/chloroform/ether extraction or proteinase k digestion followed by organic solvent extraction. Sludge concentrates generated from beef extract elution and organic flocculation, remain highly proteinaceous. Sludge concentrates required several extractions with phenol/chloroform to eliminate the protein layer. Poliovirus RNA may have been retained in the extensive protein layer during extractions. Multiple extractions may have resulted in loss of poliovirus RNA. Additionally, proteinase k digestion is preferred because the method is simple, less time consuming, and does not require the use or disposal of organic solvents.

Detection of poliovirus from sludge with plaque assay and RNA probe

Poliovirus recovered by the previously described elution procedures were detected by plaque assay and RNA probe. Many of the problems often encountered with cell culture assays such as toxicity, false positives, and lack of cytopathic effect

development did not occur. The sludge was seeded with a high concentration of poliovirus type 1 (LSc) and the concentrate required dilution prior to inoculation of the cells to achieve a countable number of plaques. Dilution also reduces toxic substances consequently eliminating the possibility of cell destruction and false-positives. Additionally, poliovirus type 1 (LSc) is a fast growing lab strain that produces large plaques. Use of this virus simplified experimentation, yet it did not accurately model the behavior of viruses that do not grow well in cell culture.

The RNA probe had previously been demonstrated to detect 90 pfu of poliovirus, but was unable to detect 100 pfu of poliovirus from sludge. However, it was able to detect 130 pfu of poliovirus from sludge. This slight discrepancy in sensitivity may be the result of viral RNA destruction due to RNase activity from contaminating microorganisms within the concentrate. Additionally, only 100 μ l of each dilution was able to be spotted onto hybridization membranes. Greater volumes, such as 500 or 1000 μ l, did not pass through the membrane or did so with great difficulty. A non-specific signal around the circumference of the well often resulted when a sample required an extended period under vacuum before it passed through the membrane. Smaller sample volumes may not be representative of the sample due to the tendency of viruses to form aggregates with each other and other particles in solution.

Conclusions

Overall, results from the RNA probe assay can be obtained within 3 days as compared to 30 or more days for cell culture assay. The RNA probe assay can be scaled up to easily accommodate multiple sludge samples. Evaluation of multiple samples with

the cell culture assay significantly increases the cost and the amount of labor required to perform the assay. The RNA probe assay is less sensitive than the cell culture assay, but may be useful as a quick screening method.

Development of RT-PCR Assays for the Detection of Enteroviruses in Sludge

RT-PCR without prior treatment

It has been demonstrated that reverse transcription is only approximately 5% efficient (Zhang et al., 1991). Therefore 20 RNA templates may be necessary to produce one cDNA. It has been estimated that 10-100 copies of template cDNA are required to produce a sufficient amount of DNA after 30 cycles of PCR to be visualized in an agarose gel (Hale et al., 1996). Therefore, approximately 200 viral genomes may be required as a starting template for successful RT-PCR. Due to aggregation and defective interfering particles, there are about 100 poliovirus genome copies per plaque (Richardson, 1989) for cell culture-adapted viruses.

Poliovirus type 1(LSc) was diluted in 1X PBS and viral RNA was liberated by digestion with proteinase k. Using the previously described reaction conditions, dilutions of poliovirus containing 1 pfu/10 μ l were consistently detected by RT-PCR. Given the probable minimum amounts of RNA necessary for successful RT-PCR, this result approaches or is equal to the sensitivity limit that may be obtained by reverse transcription with a single round of PCR. The sensitivity obtained with RT-PCR is equivalent to the sensitivity obtained with the cell culture plaque assay. However, the sensitivity of RT-PCR was greatly diminished when poliovirus was present in sludge

concentrates. Sludge concentrates contain inhibitors of RT-PCR and include metals and organic acids that may bind divalent magnesium required by the polymerases (Tsai and Olson, 1992). Additionally, the beef extract used as an eluant in the standard methods for the isolation of viruses from sludge is also inhibitory.

RT-PCR with column purification

In an effort to reduce the inhibitors in sludge concentrates, Chelex 100/Sephadex G-100 columns were used prior to RT-PCR. Chelex 100 resin binds cations including metals and Sephadex G-100 is a size exclusion resin. Treatment of environmental samples with a combination of these resins has been shown to reduce inhibitors (Morteza, 1993). Initial experiments were performed with poliovirus type 1 (LSc) diluted with 1X PBS. Column treatment of poliovirus dilutions resulted in a 2-log decrease of sensitivity as compared to non-treated poliovirus dilutions. The loss of sensitivity may be due to the retention of viruses within the column. Other researchers have also noted a significant decrease of sensitivity after column purification (Ma et al, 1995). Although these columns can be prepared quickly and easily, the reduction of poliovirus detection sensitivity precludes the uses of the columns to diminish RT-PCR inhibition.

Optimization of oligo dT paramagnetic bead capture

The oligo dT paramagnetic beads were designed by the manufacturer for the isolation of mRNA. Enterovirus RNA is of positive polarity and contains a polyA tail at the 3' end and is therefore similar to mRNA. The viral RNA is hybridized to the oligo

dT tract linked to the paramagnetic beads. The beads are washed and resuspended in a small volume of UV treated water.

Two magnetic bead capture procedures were developed. One procedure captures RNA from a 100 μ l sample, while the second uses a larger sample volume of 900 μ l. The manufacturer's protocol suggested using a volume of magnetic beads based on the amount of RNA in the sample with hybridization for 2-3 minutes at room temperature. However, the amount of RNA in a sludge concentrate is not known prior to analysis. Initial experimentation using a 2-3 minute room temperature hybridization resulted in low detection sensitivity. Optimization of these procedures was required. It was determined that magnetic capture of RNA from a 100 μ l sample was optimum with the use of 25 μ l of magnetic beads and hybridization for 5 minutes at room temperature followed by 5 minutes on ice. Specificity during the hybridization step was not necessary because the captured RNA is amplified by RT-PCR using enterovirus specific primers. These conditions resulted in a several-log increase in the detection sensitivity. Five pfu of poliovirus type 1 (LSc) per 100 μ l was detected. This magnetic capture procedure also resulted in a 10-fold concentration of the sample.

In order to evaluate a greater amount of sample per RT-PCR reaction, a 900 μ l sample volume magnetic capture procedure was developed. It was determined that the use of 100 μ l of magnetic beads with a hybridization of 15 minutes at room temperature with rocking, followed by 5 minutes on ice was optimum. The viral RNA was not eluted from the magnetic beads, therefore the magnetic beads were present in the RT-PCR reaction. The increased amount of beads necessary for the capture of RNA from a large volume of sample did not inhibit RT-PCR. Solution hybridization is dependent on

physical contact between the nucleic acid molecules. To increase the likelihood of this occurrence, the hybridization time was lengthened and the reactions were rocked.

Rocking of the tubes kept the magnetic beads in solution where they were available for hybridization. Rocking the tubes dramatically increased the detection sensitivity.

To determine the least amount of poliovirus in a sludge concentrate that would be detected by RT-PCR with the 900 μ l sample volume oligo dT paramagnetic bead capture method, a sludge concentrate was seeded with known amounts of poliovirus. However, prior to seeding with poliovirus type 1 (LSc), the sludge concentrate required a treatment that removed indigenous nucleic acid yet retained RT-PCR inhibitors. Raising the pH of the sludge concentrate to 12 was shown to be an effective and simple method. Five pfu of poliovirus type 1 (LSc) per 1000 μ l of lime stabilized sludge concentrate were detected with this method. A 90-fold concentration of the sample was attained.

Comparison of Chelex 100/Sephadex G-100 columns and oligo dT paramagnetic bead capture methods using a sludge concentrate

A mock sample consisting of a lime stabilized sludge concentrate seeded with 100 pfu/ml of poliovirus type 1 (LSc) was used to compare Chelex 100/Sephadex G-100 columns and 100 μ l and 900 μ l sample volume oligo dT paramagnetic bead capture methods. Sludge concentrates were seeded with poliovirus and the appropriate amounts of sample were analyzed by each method. The 900 μ l magnetic bead capture method consistently achieved a greater poliovirus detection sensitivity than the other methods. This result is primarily due to the increased sample volume that is analyzed per PCR reaction. The number of viruses in an environmental sample is usually low, therefore analysis of a greater portion of the sample leads to a higher sensitivity. As previously

mentioned, viruses have a tendency to form aggregates with each other and particles in solution. Therefore it is difficult to obtain a completely homogeneous sample. Aliquots of identical volume may contain different amounts of virus depending on the degree of viral aggregation. This phenomenon was demonstrated during experimentation that compared the three methods. The sludge concentrate was seeded with poliovirus type 1 (LSc) to a final concentration of 100 pfu/ml. Thus the amount of poliovirus in each portion that was used in the 100 μ l and 900 sample volume magnetic bead capture methods should have been within the detection limits of each assay. However, a PCR product was not formed after the 100 μ l sample volume magnetic bead capture, although previous experimentation demonstrated a poliovirus detection sensitivity of 7.5 pfu/100 μ l. No PCR product was formed after treatment of the seeded sludge concentrate with Chelex 100/Sephadex G-100 columns, thus confirming the inadequacy of this method.

Evaluation of sludge concentrates by RT-PCR with the 900 μ l sample volume oligo dT paramagnetic bead capture method and by plaque assay

Eight lime stabilized sludge concentrates and three raw sludge concentrates were evaluated by plaque assay and RT-PCR with the 900 μ l sample volume oligo dT magnetic bead capture method for the presence of enteroviruses. Enteroviruses were not detected in the lime stabilized sludge concentrates by plaque assay or RT-PCR with magnetic capture. When properly operated, treatment of sludge by lime stabilization reduces viral densities by 2-3 logs (Straub et al., 1993). Therefore, depending on the concentration of enteric viruses in the raw sludge, it is not unusual to find undetectable levels of enteric viruses after lime stabilization. However, the absence of infectious virus does not always indicate the absence of viral RNA.

Inactivation of a virus renders it non-infectious and may result from damage to the capsid, viral RNA, or damage to both. Damage to the capsid that alters the capsid proteins, may prevent the virus from adsorbing and thus infecting host cells. Viruses inactivated in this manner would not be detected by the plaque assay, however, the viral RNA may remain intact and detected by RT-PCR. Damage to viral RNA would prevent the transcription and replication of the viral genome. Extensive damage to the capsid may cause the viral RNA to be released. Liberated viral RNA is rapidly degraded by RNases that are present in environmental samples (Limsawat and Ohgaki, 1997; Tsai et al., 1995). The plaque assay or RT-PCR may not detect viruses inactivated by damage to the RNA. Therefore, under certain circumstances, the results obtained from the plaque assay and RT-PCR may not agree. One study demonstrated that after poliovirus was exposed to various disinfectants, the virus was undetectable by plaque assay, however the viral RNA was still detected by a nucleic acid probe (Moore, 1994). Ma et al. (1994) demonstrated the total loss of poliovirus infectivity following a 5-minute exposure to 1 N HCl, as measured by cell culture. However, poliovirus RNA was still detected by RT-PCR.

Therefore, because enteroviruses were not detected by plaque assay or RT-PCR, lime treatment may have caused the destruction of viral RNA. RNA is known to be very susceptible to degradation by alkali (Freifelder, 1987). In contrast to exposure with HCl, Ma et al. (1994) found that after a 3-minute exposure to 1 N NaOH, poliovirus was undetectable by cell culture and RT-PCR. Proper treatment of sludge by lime stabilization requires the pH to be maintained above 12.0 for 2 hours. Given these conditions, it is likely that viral RNA is destroyed during lime stabilization of sludge.

Due to the rapid degradation of viral RNA, detection of full length viral RNA would be rare and may indicate the presence of infectious virus. The oligo dT magnetic bead method captures enterovirus RNA at the 3' polyA tail and the primers for RT-PCR are located at the 5' noncoding region. Detection of viral RNA by this method suggests that the RNA was full length and may indicate the presence or recent presence of infectious virus

All three raw sludge concentrates were found to contain low numbers of enteroviruses as measured by the plaque assay. Undiluted concentrates were very toxic and resulted in the destruction of the BGM cell monolayer. A 1:10 dilution of the concentrates reduced the sample toxicity and allowed for the development of a few plaques. Four milliliters of sample was evaluated by plaque assay, with the inoculation of 1 ml of sample per 25 cm² flask of BGM cells. Plaques were not evenly distributed among the flasks. Some flasks contained multiple plaques, while plaques were absent on others. Therefore, if only 1 ml of sample had been evaluated, it is possible that plaques would be absent.

Enteroviruses were not detected in the raw sludge concentrates by RT-PCR with the 900 µl sample volume oligo dT magnetic bead capture method. To determine if inhibitors were still present in the samples, sludge concentrates and diluted concentrates that had undergone magnetic bead capture were seeded with poliovirus and amplified by RT-PCR. One concentrate did not yield a RT-PCR product until it was diluted 1:2, while the remaining two concentrates required a 1:10 dilution. This demonstrates the incomplete removal of inhibitors in these raw sludge concentrates by the oligo dT magnetic bead capture method. Other studies have demonstrated the inability of accepted

methods, such as organic solvent extraction (Kopeka et. al., 1993), column chromatography (Abbaszadegan, 1993), and guanidinium isothiocyanate extraction (Shieh et al., 1995) to remove inhibitors from every sludge concentrate, particularly raw sludge concentrates.

After dilution, the concentration of enteroviruses as measured by the plaque assay may have been below the detection limit of RT-PCR with the 900 µl sample volume oligo dT magnetic capture method. The primers used during RT-PCR only amplify human enteroviruses, however plaques on BGM cells may develop from infection with other human enteric viruses, such as reovirus, and viruses of animal origin. It is possible that some of the viruses present in the raw sludge concentrates would not be amplified by RT-PCR.

Conclusions

Due to rapid population growth, many wastewater treatment plants produce more sludge than can be disposed of by conventional methods. Recognizing this problem, the USEPA has encouraged communities to dispose of sludge by applying it to land. Sludge contains nutrients and has been demonstrated to improve the yields of many types of vegetation (USEPA, 1992). At least one third of the sludge generated in the United States is applied to land (USEPA, 1992).

However, sludge may contain pathogens such as enteric viruses. Land application of sludge may expose humans to these pathogens through direct contact or contamination of surface and ground waters. The USEPA has established regulations that reduce the possibility of contact with sludge-derived pathogens. The USEPA has defined two

classes of sludge that may be applied to land, based on the amounts of pathogens that may be present. Class A sludge is presumed to contain undetectable levels of pathogens due to strict adherence to defined treatment regimes. Class B sludge does not receive the same level of treatment and is known to contain pathogens. Some treatments may only reduce the amount of enteric viruses by a factor of ten (Straub et al., 1993).

The direct monitoring of pathogens such as enteric viruses, *Salmonella* sp., and helminth ova are required for some types of Class A sludge. However, for many types of Class A sludge and all Class B sludges, the direct monitoring of pathogens is not required. Instead, the measurement of fecal coliform bacteria is required. Class A sludge must contain fewer than 1,000 mpn of fecal coliforms per gram of total sludge solids and Class B sludge must contain less than 2 million mpn or cfu of fecal coliforms per gram of total sludge solids. Fecal coliform bacteria are often used as indicators of fecal contamination. Treatments that reduce fecal coliform bacteria to below acceptable levels are also presumed to reduce the levels of pathogens, such as enteric viruses. However, enteric viruses are more resistant to some sludge treatments (Straub et al., 1993) and disinfection (Rao and Melnick, 1986). Therefore, sludge may contain unacceptably high levels of enteric viruses, even though the reduction requirements for fecal coliform bacteria were met.

The use of fecal coliform bacteria for monitoring pathogen reduction gained widespread acceptance due to the rapid, simple, and low cost methods used to measure these indicators. It is necessary to use methods for pathogen monitoring that provide results in a short period of time because sludge cannot be applied to land until it is shown to meet pathogen reduction requirements (USEPA, 1992). Low costs ensure that

monitoring can be performed frequently and without burdening communities with higher charges for wastewater treatment.

The standard method for the detection of enteric viruses in sludge is the plaque assay (USEPA, 1992). Results may not be obtained by this method for 30 or more days. Additionally, material and labor costs for the plaque assay are very high. These factors make the plaque assay an impractical method for routine monitoring. The development of methods for the detection of enteric viruses that provide results more quickly and with lower cost may make the direct monitoring of enteric viruses more pragmatic. Detection of enteroviruses in sludge by RT-PCR with the 900 μ l sample volume oligo dT paramagnetic bead capture method resolves both of these problems. Results may be obtained within a few hours and at a cost much lower than the plaque assay. RT-PCR with the 900 μ l sample volume oligo dT paramagnetic bead capture method was found to be most effective for evaluation of lime stabilized sludges. Currently, lime stabilization is favored by eleven of the thirteen New Hampshire wastewater treatment plants that practice land application of sludge. This method would be very useful as a rapid screening technique that would alert handlers to sludges that may contain infectious virus. Officials may then decide if the confirmation by the plaque assay is warranted. The direct monitoring of pathogens, such as enteric viruses, instead of the reliance upon indicator organisms, may further reduce the risks from land application of sludge and make this practice more acceptable to a greater number of communities.

Future experimentation

A combination of methods may be required to remove inhibitors from some sludges. For instance extraction with guanidinium thiocyanate could be performed prior to oligo dT magnetic bead capture. It also possible to further increase the sample volume during oligo dT paramagnetic bead capture. With proper optimization, a volume of 15 ml may be analyzed.

A second round of PCR may increase the detection sensitivity. After PCR with one set of primers is performed, a portion of the reaction is amplified again with a new set of primers positioned within the original amplicon. This process is known as nested PCR. One study demonstrated a detection sensitivity of 1.4 pfu of poliovirus after 30 cycles of PCR and a sensitivity of 0.02 pfu after an additional 30 cycles of nested PCR (Ma et al.,1994).

Finally, evaluation of sludge samples by integrated cell culture-RT-PCR may combine the best attributes from both methods. The sample is first inoculated onto cells and incubated for a few days to allow the growth of any viruses present. The cell culture lysate is then assayed by RT-PCR. Liberated RNA will not be detected by this method because it is rapidly degraded during incubation. After addition of cell culture media, the sample and RT-PCR inhibitors are diluted. The concentration of virus will increase after growth in cell culture and the inhibitors are often diluted enough to allow successful RT-PCR.

BIBLIOGRAPHY

- Abbaszadegan, Morteza, Mary S. Huber, Charles P. Gerba, and Ian L. Pepper.** 1993. Detection of Enteroviruses in Groundwater with the Polymerase Chain Reaction. *AEM* 59:1318-1324.
- Abbaszadegan, Morteza, Peter W. Stewart, Mark W. LeChevallier, Jeffery S. Rosen, and Charles P. Gerba.** 1998. Occurrence of Viruses in Ground Water in the United States. An interim report to the American Water Works Association Research Foundation and the United States Environmental Protection Agency.
- Albert, M., and L. Schwartzbrod.** 1991. Recovery of Enterovirus from Primary Sludge Using Three Elution Concentration Procedures. *Wat. Sci. Tech.* 24:225-228.
- ASTM.** 1992. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington, D.C.
- Atmar, Robert L., Theodore G. Metcalf, Frederick H. Neill, and Mary K. Estes.** 1993. Detection of Enteric Viruses by Using the Polymerase Chain Reaction. *AEM* 59:631-635.
- Barbier, Dominique, Daniel Perrine, Chantal Duhamel, Rene Doublet, and Pierre Georges.** 1990. Parasitic Hazard with Sewage Sludge Applied to Land. *AEM* 56:1420-1422.
- Barron, A.L., C. Olshevsky, and M.M. Cohen.** 1970. Characteristics of the BGM Line of Cells from African Green Monkey Kidney. *Archiv fur die gesamte Virusforschung.* 32:380-392.
- Beaulieux, F., D.M. See, I. Leparc-Goffart, M. Aymard, and B. Lina.** Use of Magnetic Beads Versus Guanidium Thiocynate-Phenol-Chloroform RNA Extraction Followed by Polymerase Chain Reaction for the Rapid, Sensitive Detection of Enterovirus RNA. *Res. Virol.* 148:11-15.
- Belshe, Robert.** 1991. In: Textbook of Human Virology. Mosby Publishing Co. St. Louis, MO.
- Berg, Gerald and Gretchen Sullivan.** 1988. Optimum pH Levels for Eluting Enteroviruses from Sludge Solids with Beef Extract. *AEM* 54:1880-1881.

- Berg, G., E.K. Harris, S.L. Chang, and K.A. Busch.** 1963. Quantitation of Viruses the Plaque Technique. *J. Bacteriol.* 92:155-160.
- Berman, Donald, Gerald Berg, and Robert S. Safferman.** 1981. A Method for Recovering Viruses from Sludges. *J Virol Meth.* 3:283-291.
- Bitton, G., O.C. Pancorbo, and S.R. Farrah.** 1984. Virus Transport and Survival After Land Application of Sewage Sludge. *AEM* 47:905-909.
- Bixby, R.L. and D.J. O'Brien.** 1979. Influence of Fulvic Acid on Bacteriophage Adsorption and Complexation in Soil. *AEM* 38:840-845.
- Brashear, David A. and Richard L. Ward.** 1982. Comparison of Methods for Recovering Indigenous Viruses from Raw Wastewater Sludge. *AEM* 43:1413-1418.
- Brugha R., J. Heptonstall, P. Farrington, S. Andren, K. Perry, and J. Parry.** 1998. Risk of Hepatitis A Infection in Sewage Workers. *Occup Environ Med* 55:567-569.
- Carducci A., S. Arrighi, and A. Ruschi.** 1995. Detection of Coliphages and Enteroviruses in Sewage and Aerosol From an Activated Sludge Wastewater Treatment Plant. *Lett Appl Microbiol* 21:207-209.
- Carlson, G.F., Jr., F.E. Woodard, D. F. Wentworth, and O.J. Sproul.** 1968. Virus Inactivation on Clay Particles in Natural Waters. *J. Water Pollut. Control Fed.* 40:R89-R106.
- Chonmaitree, Tasnee, Craig Ford, Candace Sanders, and Helen L. Lucia.** 1988. Comparison of Cell Cultures for Rapid Isolation of Enteroviruses. *J of Clin Micro.* 26:2576-2580.
- Cooper, P.D.** 1961. The Plaque Assay of Animal Viruses. *Adv. Virus Res.* 8:319-378.
- Craun, G.F. and R. Calderon.** 1996. Microbial Risks in Groundwater Systems: Epidemiology of Waterborne Outbreaks. Under the Microscope, Examining Microbes in Groundwater. *AWWARF.* pp 9-20.
- Current, W.L.** 1987. *Cryptosporidium*: its biology and potential for environmental transmission. *Crit. Rev. Environ. Control* 17:21-51.
- Dahling, D.R. and B.A. Wright.** 1986. Optimization of the BGM Cell Line Culture and Viral Assay Procedure for Monitoring Viruses in the Environment. *AEM* 51:790-812.

- Dahling, Daniel R., Gerald Berg, and Donald Berman.** 1974. BGM, A Continuous Cell Line More Sensitive Than Primary Rhesus and African Green Kidney Cells for the Recovery of Viruses from Water. *Health Laboratory Sciences* 11:275-282
- Damgaard-Larsen, S., K.O. Jensen, E. Lund, and B. Nissen.** 1977. Survival and Movement of Enterovirus in Connection with Land Disposal of Sludges. *Water Res.* 11:503-508.
- Dizer., H., A. Nasser, and J.M. Lopez.** 1984. Penetration of Different Human Pathogenic Viruses into Sand Columns Percolated with Distilled Water, Groundwater, or Wastewater. *AEM* 47:409-415.
- Dubrou, S., H. Kopecka, J.M. Lopez-Pila, J. Marechal, and J. Prevot.** 1991. Detection of Hepatitis A Virus and Other Enteroviruses in Wastewater and Surface Water Samples by Gene Probe Assay. *Water Sci Technol.* 24:267-272.
- Edmonds, Robert L.** 1976. Survival of Coliform Bacteria in Sewage Sludge Applied to a Forest Clearcut and Potential Movement into Groundwater. *AEM* 32:537-546.
- Enders, J.F., Weller, T.H., and Robbins, F.C.** 1949. Cultivation of the Lansing Strain of Poliomyelitis Virus in Cultures of Various Human Embryonic Tissues. *Science* 109:85-87.
- Farrah, S.R., P.R. Scheuerman, and G. Bitton.** 1981. Urea-Lysine Method for Recovery of Enteroviruses from Sludge. *AEM* 41:455-458.
- Geldreich, Edwin E.** 1990. Microbiological Quality of Source Waters for Water Supply pp3-31. In: Drinking Water Microbiology. Gordon A. McFeters (Eds). Springer-Verlag, New York.
- Gerba, C.P., S. M. Goyal, I. Chech, G.F. Bogdan.** 1981. Quantitative Assessment of the Adsorptive Behavior of Viruses to Soils. *Environ. Sci. Tech.* 15:940-944.
- Gerba, C.P. and Haas, C.N.** 1988. Assessment of Risks Associated with Enteric Viruses in Contaminated Drinking Water. Pp489-494. In: Lichtenberg, J.J., Winter, J.A., Wever, C.I., and Fradkin, L. (Eds). Chemical and Biological Characterization of Sludges, Sediments, Dredge Spoils, and Drilling Muds. ASTM STP 976, American Society for Testing and Materials, Philadelphia.
- Gilbert, R.G., C.P. Gerba, R.C. Rice, H. Bouwer, C. Wallis, and J.L. Melnick.** 1976. Virus and Bacteria Removal from Wastewater by Land Treatment. *AEM* 32:333-338.
- Gilgen, M., B. Wegmuller, P. Burkhalter, H.P. Buhler, U. Muller, J. Luthy, and U. Candrian.** 1995. Reverse Transcription PCR to Detect Enteroviruses in Surface Water. *AEM* 61:1226-1231.

- Glass J.S., R.J. Van Sluis, and W.A. Yanko.** 1978. Practical Method for Detecting Poliovirus in Anaerobic Digester Sludge. *AEM* 35:983-985.
- Goyal, Sagar M., and Charles P. Gerba.** 1979. Comparative Adsorption of Human Enteroviruses, Simian Rotavirus, and Selected Bacteriophages to Soils. *AEM* 38:241-247.
- Goddard, Marian R., Janice Bates, and Michael Butler.** 1981. Recovery of Indigenous Enteroviruses from Raw and Digested Sewage Sludges. *AEM* 42:1023-1028.
- Grabow, W.O.K., Irmela G. Middendorff, and Nerine C. Basson.** 1978. Role of Lime Treatment in the Removal of Bacteria, Enteric Viruses, and Coliphages in a Wastewater Reclamation Plant. *AEM* 35:663-669.
- Graff, Judith, John Ticehurst, and Bertram Flehmig.** 1993. Detection of Hepatitis A Virus in Sewage Sludge by Antigen Capture Polymerase Chain Reaction. *AEM* 59:3165-3170.
- Green David, H. and Gillian D. Lewis.** 1995. Enzymatic Amplification of Enteric Viruses from Wastewaters. *Water Sci. Technol.* 31:329-336.
- Hale, A.D., J. Green, and D.W.G. Brown.** Comparison of Four RNA Extraction Methods for the Detection of Small Round Structured Viruses in Faecal Specimens. *J. of Virol. Methods* 57:195-201.
- Harpstead, Milo I., Francis D. Hole, and William F. Bennet.** 1988. Soil Science Simplified. Iowa State University Press. Ames, Iowa.
- Hurst, Christon J., Charles P. Gerba, and Irina Cech.** 1980. Effects of Environmental Variables and Soil Characteristics on Virus Survival in Soil. *AEM* 40:1067-1079.
- Hurst C.J., S.R. Farrah, C.P. Gerba, and J.L. Melnick.** 1978. Development of Quantitative Methods for the Detection of Enteroviruses in Sewage Sludges During Activation and Following Land Disposal. *AEM* 36:81-89.
- Hurst Christon, J., and Tamara Goyke.** 1983. Reduction of Interfering Cytotoxicity Associated with Wastewater Sludge Concentrates Assayed for Indigenous Enteric Viruses. *AEM* 46:133-139.
- Hurst Christon J., and Tamara Goyke.** 1986. Improved Method for Recovery of Enteric Viruses from Wastewater Sludges. *Wat. Res.* 20:1321-1324.
- Hyypia, T., P. Stalhandske, R. Vainionpaa, and V. Pettersson.** 1984. Detection of Enteroviruses by Spot Hybridization. *J. Clin. Microbiol.* 19:436-438.

- Jaykus, L.A., R. DeLeon, and M.D. Sobsey.** 1993. Application of RT-PCR for the Detection of Enteric Viruses in Oysters. *Water Sci. Technol.* 27:49-53.
- Jiang, Xi, Mary K. Estes, and Theodore G. Metcalf.** 1987. Detection of Hepatitis A Virus by Hybridization with Single-Stranded RNA Probes. *AEM* 53:2487-2495.
- Jiang, Xi, Mary K. Estes, T.G. Metcalf, and J.L. Melnick.** 1986. Detection of Hepatitis A Virus in Seeded Estuarine Samples by Hybridization with cDNA Probes. *AEM* 52:711-717.
- Katzenelson, Eliyahu, Badri Fattal, and Tova Hostovesky.** 1976. Organic Flocculation: An Efficient Second-Step Concentration Method for the Detection of Viruses in Tap Water. *AEM* 32:638-639.
- Katzenelson, E., I. Buium, and H.I. Shuval.** 1976. Risk of Communicable Disease Infection Associated with Wastewater Irrigation in Agricultural Settlements. *Science* 194:944-946.
- Keswick, B.H. and C.P. Gerba.** 1980. Viruses in Groundwater. *Environ. Sci. Tech.* 14:1290-1297.
- Kopecka, H., S. Dubrou, J. Prevot, J. Marechal, and J.M. Lopez-Pila.** 1993. Detection of Naturally Occuring Enteroviruses in Waters by Reverse Transcription, Polymerase Chain Reaction, and Hybridization. *AEM* 59:1213-1219.
- Kopecka, H., J. Prevot, M. Girard, F. Fuchs, and M. Aymard.** 1988. cRNA Probes (Riboprobes) Synthesized in vitro for Detecting Enterovirus by Molecular Hybridization. *Ann. Inst. Pasteur. Virol.* 139:217-225.
- Kulski, J.K. and M. Norval.** 1985. Nucleic Acid Probes in Diagnosis of Viral Diseases of Man. *Arch. Virol.* 83:3-15.
- Lance, J.C., and Charles P. Gerba.** 1984. Virus Movement in Soil During Saturated and Unsaturated Flow. *AEM* 47:335-337.
- Lance, J.C., Charles P. Gerba, and Joseph L. Melnick.** 1979. Virus Movement in Soil Columns Flooded with Secondary Sewage Effluent. *AEM* 32:520-526.
- Landry, Edward, F., James M. Vaughn, McHarrell Z. Thomas, and Cheryl A. Beckwith.** 1979. Adsorption of Enteroviruses to Soil Cores and Their Subsequent Elution by Artificial Rain Water. *AEM* 38:680-687.
- Lednar, W.M., S.M. Lemon, and J.W. Kirkpatrick.** 1985. Frequency of Illness Associated with Epidemic Hepatitis A Virus Infection in Adults. *Am J Epidemiol.* 122:226-233.

- Le Guyader, F., V. Apaire-Marchais, J. Brillet, and S. Billaudel.** 1993. Use of Genomic Probes to Detect Hepatitis A Virus and Enterovirus RNAs in Wild Shellfish and Relationship of Viral Contamination to Bacterial Contamination. *AEM* 59:3969-3968.
- Le Guyader, F., E. Dubois, D. Menard, and M. Pommepuy.** 1994. Detection of Hepatitis A Virus, Rotavirus, and Enterovirus in Naturally Contaminated Shellfish and Sediment by Reverse Transcription-Seminested PCR. *AEM* 60:3665-3671.
- Limsawat, Sunun and Shinichiro Ohgaki.** 1997. Fate of Liberated Viral RNA in Wastewater Determined by PCR. *AEM* 63:2932-2933.
- Lin, M., M. Imai, A.R. Bellamy, N. Ikegami, Y. Furuichi, D. Summers, D.L. Nuss, and R. Deibel.** 1985. Diagnosis of Rotavirus Infection with Cloned cDNA Copies of Viral Genome Segments. *J. Virol.* 55:509-512.
- Lipson, Steven M. and G. Stotzky.** 1983. Adsorption of Reovirus to Clay Minerals: Effects of Cation-Exchange Capacity, Cation Saturation, and Surface Area. *AEM* 46:673-682.
- Lund, E.** 1970. Observation on the Virus Binding Capacity of Sludge. In: S.H. Jenkens (eds), *Advances in Water Pollution Research, Proceedings of the Fifth International Conference*, San Francisco. Pergamon Press, Inc., Elmsford, N.Y
- Lund, E., and V. Ronne.** 1973. On the Isolation of Virus from Sewage Treatment Plant Sludges. *Water Res.* 7:863-871.
- Mack, W.N., Y.S. Lu, and D.S. Coohon.** 1972. Isolation of poliomyelitis virus from a contaminated well. *Hlth. Sci. Rep.* 87:271-274.
- Margolin, Aaron B.** 1999. Personal communication.
- Margolin, Aaron B., Martinez J. Hewlett, and Charles P. Gerba.** 1991. The Application of a Poliovirus cDNA Probe for the Detection of Enteroviruses in Water. *Wat. Sci. Tech.* 24:277-280.
- McCabe, Karen J.** 1996. Master's Thesis: Detection of Hepatitis A Virus in Shellfish with a ³²P-labeled cDNA probe.
- Melnick, J.L.** 1947. Poliomyelitis virus in Urban Sewage in Epidemic and Non-epidemic Times. *Am. J. Hyg.* 45:240-253.

- Melnick J.L., D.M. Horstmann, and R. Ward.** 1946. The Isolation of Poliomyelitis Virus from Human Extranatural Sources. II. Comparison of Virus Content of Blood, Oropharyngeal Washings, and Stools of Contacts. *J. Clin. Investig.* 25:275-277.
- Melton, D.A., P.A. Krieg, M.R. Rebagliati, T. Maniatis, K. Zinn, and M.R. Green.** 1984. Efficient *in vitro* Synthesis of Biologically Active RNA and RNA Hybridization Probes from Plasmids Containing a Bacteriophage SP6 Promoter. *Nuc. Acids Res.* 12:7035-7056.
- Metcalf, T.G., J.L. Melnick, and M.K. Estes.** 1995. Environmental Virology: From Detection of Virus in Sewage and Water by Isolation to Identification by Molecular Biology-A Trip of Over 50 Years. *Annu. Rev. Microbiol.* 49:461-487.
- Metcalf, T.G., R. Comeau, R. Mooney, and J.H. Ryther.** 1977. Opportunities for Virus Transport Within Aquatic and Terrestrial Environments. In: Proceedings of Risk Assessment and Health Effects of Land Application of Municipal Wastewater and Sludge. C.A. Sorber and B. Sagik (eds). University of Texas, San Antonio, Texas.
- Monceyron C. and B. Grinde.** 1994. Detection of Hepatitis A Virus in Clinical and Environmental Samples by Immunomagnetic Separation and PCR. *J. Virol. Methods.* 46:157-166.
- Moore, B.E., B.P. Sagik, and J.F. Malina, Jr.** 1975. Viral Association with Suspended Solids. *Water Res.* 9:197-203.
- Moore, Norman J.** 1994. PhD Dissertation: The Detection of Enteric Viruses by Nylon-Based and *in situ* Nonisotopic probe Assays and Their use in Treated and Untreated Waters.
- Moore, Norman J. and Aaron B. Margolin.** 1993. Evaluation of Radioactive and Nonradioactive Gene Probes and Cell Culture for Detection of Poliovirus in Water Samples. *AEM* 59:3145-3146.
- Moore, Rebecca S., Dene H. Taylor, Lawrence S. Sturman, Michael M. Reddy, and G. Wolfgang Fuhs.** 1981. Poliovirus Adsorption by 34 Minerals and Soils. *AEM* 42:963-975.
- Moore, Rebecca S., Dene H. Taylor, Michael M. Reddy, and Lawrence S. Sturman.** 1982. Adsorption of Reovirus by Minerals and Soils. *AEM* 44:852-859.
- Muir, Peter, Felicity Nicholson, Mahmood Jhetam, Sheila Neogi, and Jangu E. Banatvala.** 1993. Rapid Diagnosis of Enterovirus Infection by Magnetic Bead Extraction and Polymerase Chain Reaction Detection of Enterovirus RNA in Clinical Specimens. *J. of Clin. Microbiol.* 31:31-38.

- Pancorbo, O.C., G. Bitton, S.R. Farrah, G.E. Gifford, and A.R. Overman.** 1988. Poliovirus Retention in Soil Columns After Application of Chemical and Polyelectrolyte-conditioned Dewatered Sludges. *AEM* 54:118-123.
- Pancorbo, Oscar C., Phillip R. Scheuerman, Samuel R. Farrah, and Gabriel Bitton.** 1981. Effect of Sludge Type on Poliovirus Association with and Recovery from Sludge Solids. *Can. J. Microbiol.* 27:279-287.
- Powelson, D.K., J.R. Simpson, and C.P. Gerba.** 1991. Effects of Organic Matter on Virus Transport in Unsaturated Flow. *AEM* 57:2283-2286.
- Racaniello, Vincent R. and David Baltimore.** 1981. Molecular Cloning of Poliovirus cDNA and Determination of the Complete Nucleotide Sequence of the Viral Genome. *Proc. Natl. Acad. Sci. USA.* 78:4887-4891.
- Rao, V.C. and Joseph L. Melnick.** 1986. Environmental Virology. Van Nostrand Reinhold, England.
- Reynolds, Kelly A., Kimberly Roll, Roger S. Fujioka, Charles P. Gerba, and Ian L. Pepper.** 1998. Incidence of Enteroviruses in Mamala Bay, Hawaii Using Cell Culture and Direct Polymerase Chain Reaction Methodologies. *Can J. Microbiol.* 44:598-604.
- Richards, G.P. and D.A. Weinheimer.** 1985. Influence of Adsorption Time, Rocking, and Soluble Proteins on the Plaque Assay of Monodispersed Poliovirus. *AEM* 49:744-748.
- Richardson, K.J.** 1989. Use of Nucleic Acid Probes and a Nonradioactive Labeling System for the Detection of Enteroviruses in Water. Ph.D. dissertation. University of Arizona, Tucson.
- Richardson K.J., A.B. Margolin, and C.P. Gerba.** 1988. A Novel Method for Liberating Viral Nucleic Acid for Assay of Water Samples with cDNA Probes. *J. Virol. Methods* 22:13-21.
- Sambrook, J.E., F. Fritsch, and T. Maniatis.** 1989. *Molecular Cloning.* Cold Spring Harbor Press.
- Sattar, S.A.** 1983. Viruses and Land Disposal of Sewage Sludge: A Literature Review. In: Biological Health Risks of Sludge Disposal to Land in Cold Climates. pp 271-289. P.M. Wallis and D.L. Lehman (eds). University of Calgary Press, Calgary, Alberta, Canada.

- Sattar, Syed A. and J.C.N. Westwood.** 1979. Recovery of Viruses from Field Samples of Raw, Digested, and Lagoon-Dried Sludges. *Bulletin of the World Health Organization.* 57:105-108.
- Sattar, Syed A., Sami Ramia, and J.C.N. Westwood.** 1976. Calcium Hydroxide (Lime) and the Elimination of Human Pathogenic Viruses from Sewage: Studies with Experimentally-Contaminated (Poliovirus type 1, Sabin) and Pilot Plant Samples. *Can. J. of Public Health.* 67:221-225.
- Schaub, S.A., and C.A. Sorber.** 1977. Virus and Bacteria: Removal from Wastewater by Rapid Infiltration Through Soil. *Appl. Environ. Microbiol.* 33:609-619.
- Schwab, K.J., R. De Leon, and M.D. Sobsey.** 1993. Development of PCR Methods for Enteric Virus Detection in Water. *Water Sci. Technol.* 27:211-218.
- Schwab, Kellogg J., Ricardo De Leon, and Mark D. Sobsey.** 1996. Immunoaffinity Concentration and Purification of Waterborne Enteric Viruses for Detection by Reverse Transcriptase PCR. *AEM* 62:2086-2094.
- Schmidt, Nathalie J., Helen H. Ho, John L. Riggs, and Edwin H. Lennette.** 1978. Comparative Sensitivity of Various Cell Culture Systems for Isolation of Viruses from Wastewater and Fecal Samples. *AEM* 36:480-486.
- Shieh, Carol Y.S., Ralph S. Baric, Mark D. Sobsey, John Ticehurst, Thomas A. Miele, Ricardo DeLeon, and Renate Walter.** 1991. Detection of Hepatitis A Virus and Other Enteroviruses in Water by ssRNA Probes. *J of Viro. Meth.* 31:119-136.
- Shieh, C. Y.S., D. Wait, L. Tai, and M.D. Sobsey.** 1995. Methods to Remove Inhibitors in Sewage and Other Fecal Wastes for Enterovirus Detection by the Polymerase Chain Reaction. *J. of Virol. Meth.* 54:51-66.
- Sobsey, Mark D., Cheryl H. Dean, Maurice E. Knuckles, and Ray A. Wagner.** 1980. Interactions and Survival of Enteric Viruses in Soil Materials. *AEM* 40:92-101.
- Sorber C.A., B.E. Moore, D.E. Johnson.** 1984. Microbiological Aerosols from the Application of Sewage Sludge to Land. *J Water Pollut Control Fed* 56:830-836.
- Sposito, Garrison.** 1989. The Chemistry of Soils. Oxford University Press, Inc. New York, New York.
- Stagg, C.H., C. Wallis, and C.H. Ward.** 1977. Inactivation of Clay-Associated Bacteriophage MS-2 by Chlorine. *AEM* 33:385-391.

- Stalhandske, P., T. Hyypia, A. Allard, P. Malonen, and U. Pettersson.** 1985. Detection of Adenoviruses in Stool Specimens by Nucleic Acid Spot Hybridization. *J. Med. Virol.* 16:107-118.
- Straub, Timothy M., Ian L. Pepper, Morteza Abbaszadegan, and Charles P. Gerba.** A Method to Detect Enteroviruses in Sewage Sludge-Amended Soil Using the PCR. *AEM* 60:1014-1017.
- Straub Timothy, Ian L. Pepper, and Charles P. Gerba.** 1992. Persistence of Viruses in Desert Soils Amended with Anaerobically Digested Sewage Sludge. *AEM* 58:636-641.
- Straub, Timothy M., Ian L. Pepper, and Charles P. Gerba.** 1994. Detection of Naturally Occurring Enteroviruses and Hepatitis A Virus in Undigested and Anaerobically Digested Sludge Using the Polymerase Chain Reaction. *Can. J. Microbiol.* 40:884-888.
- Straub, Timothy M., Ian L. Pepper, and Charles P. Gerba.** 1995. Comparison of PCR and Cell Culture for Detection of Enteroviruses in Sludge-Amended Field Soils and Determination of Their Transport. *AEM* 61:2066-2068.
- Straub, Timothy, M., Ian L. Pepper, and Charles P. Gerba.** 1993. Hazards from Pathogenic Microorganisms in Land-Disposed Sewage Sludge. In: Reviews of Environmental Contamination and Toxicology.132:55-91. Springer-Verlag Inc., New York.
- Takiff, H.E., M. Seidlin, P. Krause, J. Rooney, C. Brandt, W. Rodriguez, R. Volken, and S. E. Strauss.** 1985. Detection of Adenoviruses in Stool Specimens by Nucleic Acid Spot Hybridization. *J. Med. Virol.* 16:213-218.
- Teltsch, B. and E. Katzenelson.** 1978. Airborne Enteric Bacteria and Viruses from Spray Irrigation with Wastewater. *AEM* 35:290-295.
- Ticehurst, J., S.M. Feinstone, T. Chestnut, N.C. Tassopoulos, H. Popper, and R.H. Purcell.** 1987. Detection of hepatitis A virus by Extraction of Viral RNA and Molecular Hybridization. *J. Clin. Microbiol.* 25:1822-1829.
- Tierney, John T., Robert Sullivan, and Edward P. Larkin.** 1977. Persistence of Poliovirus 1 in Soil and on Vegetables Grown in Soil Previously Flooded with Inoculated Sewage Sludge or Effluent. *AEM* 33:109-113.
- Tsai, Yu-Li, Bich Tran, and Carol J. Palmer.** 1995. Analysis of Viral RNA Persistence in Seawater by Reverse Transcriptase-PCR. *AEM* 61:363-366.
- Tzipori, S.** 1983. Cryptosporidiosis in Animals and Humans. *Microbiol. Reviews.* 47:84-89.

- USEPA.** 1992. Environmental Regulations and Technology: Control of Pathogens and Vector Attraction in Sewage Sludge Under 40 CFR Part 503. EPA/625/R-92/013.
- USEPA.** 1995. Virus Monitoring Protocol for the Information Collection Requirements Rule. EPA/814-B-95-002.
- Vaughn, J.M., E.F. Landry, L.J. Baronosky, C.A. Beckwith, M.C. Dahl, and N.C. Delihias.** 1978. Survey of Human Virus Occurrence in Wastewater-Recharged Groundwater on Long Island. *AEM* 36:47-51.
- Vaughn, James M., Edward F. Landry, Cheryl A. Beckwith and McHarrell Z. Thomas.** 1981. Virus Removal During Ground Water Recharge: Effects of Infiltration Rate on Adsorption of Poliovirus to Soil. *AEM* 41:139-147.
- Verbeek, A. and P. Tijssen.** 1988. Biotinylated and Radioactive cDNA Probes in the Detection by Hybridization of Bovine Enteric Coronavirus. *Mol. Cell. Probes* 2:209-223.
- Wang, D.S., Charles P. Gerba, and J.C. Lance.** 1981. Effect of Soil Permeability on Virus Removal Through Soil Columns. *AEM* 42:83-88.
- Ward, Richard L. and Carol S. Ashley.** 1977a. Identification of the Virucidal Agent in Wastewater Sludge. *AEM* 33:860-864.
- Ward, Richard L. and Carol S. Ashley.** 1977b. Inactivation of Enteric Viruses in Wastewater Sludge Through Dewatering by Evaporation. *AEM* 34:564-570.
- Ward, Richard L. and Richard J. Mahler.** 1982. Uptake of Bacteriophage f2 Through Plant Roots. *AEM* 43:1098-110
- Wellings, Flora Mae, A.L. Lewis, and C.W. Mountain.** 1978. Assessment of Health Risks Associated with Land Disposal of Municipal Effluents and Sludge. Pp 168-179. In: Risk Assessment and Health Effects of Land Application of Municipal Wastewater and Sludges. Sagik, B.P. and C.A. Sorber (Eds). University of Texas, San Antonio, Texas.
- Wellings, Flora Mae, Arthur L. Lewis, and Carrol W. Mountain.** 1976. Demonstration of Solids-Associated Virus in Wastewater and Sludge. *AEM* 31:354-358.
- Wellings, Flora Mae, Arthur L. Lewis, Carrol W. Mountain, and Virginia Pierce.** 1975. Demonstration of Virus in Groundwater after Effluent Discharge onto Soil. *AEM* 29:751-757.
- Yates, Marylynn V., Charles P. Gerba, and Lee M. Kelley.** 1985. Virus Persistence in Groundwater. *AEM* 49:778-781.

APPENDIX A

PREPARATION OF MEDIA

Flake agar (2%)

2 g flake agar (Difco, Detroit, MI)

Bring up to 100 ml with reagent grade water. Autoclave 15 minutes. Leave at 56 °C until use.

Medium 199 (2X)

1.96 g Medium 199 (Sigma)

0.13 g NaHCO₃

0.95 g hepes

0.06 g L-glutamine

2 ml antibiotic/antimycotic (Gibco)

1.2 ml neutral red solution (Sigma)

1 ml 1% MgCl₂

Bring up to 100 ml with reagent grade water. Filter sterilize.

Minimal Essential Media/L-15 Growth Medium

2.65 g MEM (Sigma)

3.45 g L-15 Medium Leibovitz (Sigma)

5 ml non-essential amino acids (Sigma)

2 g hepes

0.5 g NaHCO₃

5 ml antibiotic/antimycotic

5 ml kanamycin (Gibco)

40 ml heat inactivated iron supplemented fetal calf serum

Adjust pH to 7.0. Bring up to 500 ml with reagent grade water. Filter sterilize.

APPENDIX B

BUFFERS AND SOLUTIONS

Phosphate buffered saline (1X PBS)

9.785 g of PBS powder (Sigma)

Bring up to 1000 ml with reagent grade water. Autoclave for 15 minutes. Store at 4 °C. Final pH 7.0.

Tris-Borate-EDTA (5X)

54.0 g Tris base (Sigma)

27.5 g boric acid (Sigma)

20 ml 0.5 M EDTA (pH 8.0)

Bring volume up to 1000 ml with reagent grade water. Store at room temperature.