The detection of enteric viruses by nylon-based and in situ nonisotopic probe assays and their use in treated and untreated waters

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University of New Hampshire, Durham

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The detection of enteric viruses by nylon-based and \textit{in situ} nonisotopic probe assays and their use in treated and untreated waters

Moore, Norman James, Ph.D.

University of New Hampshire, 1994
THE DETECTION OF ENTERIC VIRUSES BY NYLON-BASED AND *IN SITU* NONISOTOPIC PROBE ASSAYS AND THEIR USE IN TREATED AND UNTREATED WATERS

BY

NORMAN MOORE

B.A. Dartmouth College, 1988

DISSERTATION

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

Doctor of Philosophy in Microbiology

May, 1994
This dissertation has been examined and approved.

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Dr. John Collins, Assistant Professor of Biochemistry and Genetics

Date: May 6, 1979
To each of my families.
From my parents and brother Dan
to my wife Amy and child Nick,
how could I go wrong?
ACKNOWLEDGEMENTS

I would like to thank Aaron Margolin for being a friend as well as an advisor. I would also like to thank the other members of my committee; Robert Zsigray, Frank Rodgers, Thomas Pistole, and John Collins for all of their support. I also appreciated the technical support from Robert Mooney and the media help Joseph Danahy.
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ABSTRACT
THE DETECTION OF ENTERIC VIRUSES BY NYLON-BASED AND IN SITU NONISOTOPIC PROBE ASSAYS AND THEIR USE IN TREATED AND UNTREATED WATERS
BY
NORMAN MOORE
University of New Hampshire May, 1994

Potable water is a primary need of humankind. In the interest of public health, the water should be free of infectious agents, such as bacteria, intestinal parasites, and viruses. The current method for assaying water for viruses is cell culture, which is expensive, labor intensive, and time consuming. Nucleic acid probes offer an alternative method which is less costly, more rapid, and requires less training and experience. Consequently, this study provides alternatives to viral monitoring of water sources.

Three nonradioactive nucleic acid probe assays were developed and evaluated by colormetric and chemiluminescent signals. A biotin-tailed probe was able to detect 100 pg of homologous cDNA and $9 \times 10^5$ poliovirus plaque forming units (pfu), while the horseradish peroxidase (HRP) probe was able to detect 10 pg of cDNA and $9 \times 10^4$ pfu, and the digoxigenin probe detected 0.1 pg of cDNA and $9 \times 10^2$ pfu. No differences in sensitivity were seen between colormetric and chemiluminescent detections. Since the digoxigenin probe was the most sensitive of the nonisotopic assays, it was compared to $^{32}$P-cDNA, $^{32}$P-ssRNA, and cell culture for the detection of viruses from environmental samples. The digoxigenin probe and $^{32}$P-ssRNA probes detected a higher number of positive samples than the other two assays.

An in situ assay was developed to detect only infectious viruses. Cells were incubated with viral dilutions, fixed with a 4% solution of formalin, and then probed. The detection limit of in situ hybridizations was 0.9 pfu. The increased sensitivity over cell
culture was most likely due to a subset of polioviruses which can begin the replication
cycle but do not form cytopathic effects.

Poliovirus was then disinfected with chlorine and chlorine dioxide at pH 6, pH 7,
and pH 9 as well as ozone, and ultraviolet light to determine the correlation between cell
culture and digoxigenin probe on disinfected samples. No correlation existed using any
disinfectant as the number of plaque forming units was reduced to zero and the
digoxigenin probe assay did not lose any signal intensity. The only loss of digoxigenin
probe sensitivity was seen at pH 9.
INTRODUCTION

The etiological agents of many waterborne outbreaks are viruses. Viruses are obligate intracellular parasites which replicate intracellularly. Enteric viruses are viruses which replicate in the intestinal tract and can be transmitted via the fecal-oral or respiratory routes (Horstmann, 1967).

Enteric viruses are responsible for a range of diseases from myocarditis (Leslie et al., 1989) and pericarditis to gastroenteritis (Blacklow and Cukor, 1981). In extreme cases, Coxsackie virus can replicate in heart tissue, destroying the organ (Kibrick, 1964). However, the major disease caused by enteric viruses is gastroenteritis. Five to eighteen million children a year in Africa, Asia, and Latin America die due to gastroenteritis (Steinhoff, 1978). Viruses are assumed to be responsible for approximately 50% of these cases (Steinhoff, 1978).

Initial hypotheses suggested that viruses could not persist in the environment for extended periods of time. Therefore, it was assumed they were not transmitted by water. In 1941, Paul and Trask were able to isolate poliovirus in stool samples and sewage demonstrating the survival of viruses outside of the body (Paul & Trask, 1941).

In 1966, virus was first isolated from drinking water that had undergone only marginal chlorination (Coin, 1966). Other enteric viruses were also isolated in succeeding years, including rotavirus (Bishop et al, 1973) which is a major etiological agent in
childhood gastroenteritis and traveller's diarrhea (Bolivar et al., 1978), and hepatitis A (Hejkal et al., 1982 and Divizia et al., 1989) which is notable for its extended survival time in the environment (Sobsey, 1976) and high resistance to chlorine (McLean, 1963 and Keswick et al., 1981).

In many communities with a moderate to high rate of population growth, the volume of human excreta has exceeded the capacity of many sewage treatment plants. Several million viral particles can be found per gram of feces. Raw sewage can contain upwards of 400,000 viral particles per liter (Buras, 1974). Inadequately treated water may routinely escape a plant and contaminate potable surface and ground waters (Lund, 1977). Even a minute quantity of released virus can pose a health threat. Ingestion of one plaque forming unit may be enough to induce a disease state in an individual (Westwood and Sattar, 1976).

Traditionally, ground water has been considered safe from viral contamination due to the soil column which acts as a biological filter. Nearly half the U.S. population relies on groundwater as part of their potable water (Craun, 1984). Many communities use waste water to recharge aquifers (Vaughn et al., 1978). However, faults and channels in the earth, combined with rainfall and other factors such as leaking septic systems can lead to sewage contamination of groundwater (Keswick and Gerba, 1980). Fifty percent of all waterborne disease outbreaks are due to water that has become fecally contaminated (Yates, 1985). Sixty-five percent of these cases are of viral origin (Yates, 1985).

The importance of potable water has been known for approximately 4000 years. A Sanskrit document dictates that "foul" water be boiled, kept in the sun, have a piece of hot
copper dipped into it, and then cooled in a container in the earth (Baker, 1949).

Hippocrates warned travelers 2400 years ago to note where local inhabitants obtained their water (McDermott, 1973). Modern concern for potable water sources came to light in the mid 19th century with a cholera outbreak in London. Dr. John Snow theorized that cholera was transmitted by the water. He discovered that people who obtained their water from a specific pump were becoming infected with the bacteria. By closing the pump, the incidence of infection decreased and the threat of an epidemic diminished (Manual of British Practices, 1950).

**Indicator organisms for enteric viruses**

Due to the high number of bacteria, viruses, and protozoa that can be transmitted via the water route, it is impractical to assay for them all to ensure the water is not fecally contaminated. Therefore, people began looking for indicator organisms which would suggest that the water is contaminated. Indicator organisms should have the following characteristics:

1. Should be associated with the source of the pathogen and should be absent in unpolluted areas.
2. Should occur in greater numbers than the pathogen.
3. Should not multiply in water and in other environments.
4. Should be at least equally resistant to environmental stresses and to disinfection as the pathogen.
5. Should be detectable by means of easy, rapid, and inexpensive methods.
The first indicator organisms were total coliforms (Hoadley and Dutka, 1977). Total coliforms are aerobic and facultative anaerobic gram-negative, nonspore-forming, rod-shaped bacteria that ferment lactose at 37°C to produce gas within 48 hours. These indicators were replaced by fecal coliforms because several coliforms were found to be associated with soils and vegetation, rather than strictly the enteric tract (Hoadley and Dutka, 1977). Fecal coliforms, unlike other coliforms, are able to ferment lactose at 44.5°C.

Fecal contamination may be demonstrated by detecting fecal coliforms (Hoadley and Dutka, 1977). However, no accurate correlation exists between the presence of enteric viruses and fecal coliforms (Payment, 1989 and Keswick and Gerba, 1984). This can be exemplified by the New Delhi hepatitis A epidemic in which 30,000 people contracted infectious hepatitis by drinking fecally contaminated water which had tested negative for fecal coliforms (Viswanathan, 1957). In a second example, nearly 20% of water sampled from 3-meter deep wells in Israel had infectious viruses but lacked fecal coliforms (Marzouk et al., 1980).

In the United States, the U.S. Environmental Protection Agency (EPA) initially assumed that enteric viruses were not present in the environment. The general belief was that once outside the body, environmental stresses would lead to the inactivation of the viruses (Bitton, 1980). This premise was proved incorrect by the work of Metcalf et al. in the 1960's and 1970's at the University of New Hampshire (Metcalf et al., 1974).
collecting samples at different sites, such as Great Bay in New Hampshire, they were able
to demonstrate the presence of human enteric viruses in water (Fields, 1974). This work
showed that under proper environmental conditions, viruses could survive for extended
periods of time (Slanetz et al., 1964). They also documented the recovery of enteric
viruses from shellfish and showed that viruses could still be infectious after a winter inside
the shellfish (Slanetz et al., 1964). Another significant discovery was the potential health
threat of viral transmission along the marine and terrestrial foodchains (Metcalf et al.,
1977).

Enteric viruses do undergo inactivation due to environmental stresses. One of the
most important factors is temperature. Increasing temperature decreases survivability
(Morris and Darlow, 1971). Ultraviolet light from the sun also has virucidal properties
(Morris and Darlow, 1971). Other physical factors include interaction at the air-water
interface and desiccation (Walker, 1970). Chemicals present in the water may also
inactivate viruses. An increased pH is detrimental to viral survivability (Bitton, 1980).
Formaldehyde, nitrous acid, and ammonia will all alter nucleic acids, while phenol can
destroy the protein coat (Bitton, 1980). The organisms present in the water will also play
a part in viral survivability. Bacteria, algae, protozoa, and animals may secrete antiviral
agents or may directly uptake viruses (Bitton, 1978). Byproducts of bacteria anaerobic
digestion, such as ammonia, have been shown to have a virucidal effect at pH values
higher than eight (Ward, 1977).
Concentration of Viruses from the Environment

Water sources that are contaminated by fecal sources usually contain low concentrations of viruses (Bitton, 1980). Therefore, assays were developed to concentrate viruses into a workable volume. One of the first methods was putting a sanitary napkin in the water for a period of two days. It was theorized that viruses would adsorb to the napkin and then subsequently could be eluted from the napkin and assayed (Melnick and Gerba, 1954). However, this method suffered from inefficiency of viral adsorption and inability to determine the amount of water assayed.

Ultracentrifugation and ultrafiltration have also been used to concentrate viral samples. In ultracentrifugation, the sample is placed in centrifuge tubes and spun to create a pellet (Bitton, 1980). This procedure has limitations, such as the small volume that can be centrifuged and the expense of operating such equipment. In ultrafiltration, a sample is passed through a membrane which retains viruses due to size exclusion (Bitton, 1980). This procedure is also limited to small volumes due to the inevitable clogging of the filter.

The method currently in use for collection of viruses from water is known as microporous filtration and elution (Bitton, 1980). Viruses can adsorb to solid surfaces due to electrostatic charges. At a neutral pH, enteric viral coat proteins usually exhibit a net negative charge. If the pH of the surrounding medium drops below the viral isoelectric point, the virus becomes net positively charged. Therefore, viruses can be adsorbed to a positive (Sobsey and Jones, 1979 and Chang et al., 1981) or negative filter (Farrah et al., 1977) depending upon the pH of the surrounding medium. Since most virus
filters have a nominal pore size of 1 μm, the viruses are not retained due to size unless they are already associated with a larger solid. Hydrogen bonding and hydrophobic bonding are two other forces which may work to increase the adherence of viruses to filters (Bitton, 1980).

The electrostatic forces between the viruses and filters are influenced by several factors. Cellulose nitrate and epoxy fiberglass have been shown to have a greater binding efficiency than cellulose triacetate (Goyal and Gerba, 1979). Secondly, by adding cations, the repulsion between virus and filter is minimized, increasing adsorption. Thirdly, by decreasing the flow rate of the water being pumped through the filter, viral retention increases. Finally, waters with high organic loads tend to coat the filter, blocking the binding sites of the filter (Bitton, 1980). Prefilters can be used to remove a majority of the organic material.

Detection of human enteric viruses

Animal inoculation

Animal inoculation was the first method available for the detection of viruses (Hsiung, 1973). Following this method, the appropriate animal host is injected with the sample. The animal must be susceptible to the particular virus or no effects will be seen. Genetically, the closest animals to humans are monkeys, so they are the most susceptible to human viruses. This method is no longer readily used due to the expense, the availability of other procedures of equal sensitivity such as cell culture (Bitton, 1980), and
Cell Culture

Cell cultures can be either primary or continuous. A primary cell culture is initiated with the sacrifice of an animal and the appropriate cells are passed a finite number of times (Bitton, 1980). The cells of a continuous culture have been transformed so they can be passed many times (Bitton, 1980).

The most common cell line used in environmental sampling is Buffalo Green Monkey (BGM) kidney cells (Dahling and Wright, 1986). They have been shown to be more sensitive than primary rhesus and African green monkey kidney cells (Dahling et al., 1974). In the cell culture assay, the cells are grown into a continuous monolayer. The sample to be assayed is incubated on the cells, allowing any potential viruses to adsorb to the cells (Dahling and Wright, 1986). If the appropriate viruses are present, the cells may exhibit cytopathic effects. Positive samples should be reevaluated with other confluent cell monolayers. BGM cells, however, lose sensitivity with increasing passage number and are not highly sensitive to adenoviruses and some echoviruses (Dahling and Wright, 1986).

Cell culture is a sensitive assay, detecting as little as 1 plaque forming unit. However, many problems exist with the technique. No one cell line is able to be infected with all enteric viruses (Schmidt et al., 1978). Secondly, not all viruses cause cytopathic effects and some do so very slowly, such as hepatitis A virus. Some viruses, such as Norwalk virus, have yet to be grown in cell culture. Thirdly, cell culture is time-
consuming and labor-extensive. It can take up to twenty-eight days from the start to final confirmation for a negative sample, during which time, the flasks have to be monitored. Even with antibiotics, the extended time may allow fungi or bacteria to grow, destroying the cell monolayer.

Another problem associated with cell culture is that toxic substances from the environment may be concentrated in the collection process and destroy the cell monolayer. Severe toxicity may necessitate dilution of the sample to minimize destruction of the cell monolayer, which dilutes the low concentration of virus in the sample. Finally, cell culture is expensive since all samples require a minimum of twenty nonreusable cell culture flasks, other disposable material and technician time (Margolin, 1986).

**Antibody assays**

Antibodies specific for viral coat proteins can be labeled with a molecule that is fluorescent, radioactive, or enzymatic. Detection can either be direct or indirect. Direct detection uses a labeled antibody specific for a section of the protein coat. With the indirect antibody techniques, the primary antibody, which is specific for the virus, does not have a label. Instead, a secondary antibody specific for a portion of the first is conjugated to the label. Indirect detection has been shown to increase sensitivity by one order of magnitude (Hurst, 1983).

Fluorescent antibody assays conjugate an antibody with a fluorescent label, such as fluorescein isothiocyanate (Harris et al., 1979). Once the antibody attaches either directly or indirectly, the fluorescent label is excited by the ultra-violet light of an epifluorescent
microscope. One limitation of this technique is that it requires a minimum of $10^4$ to $10^6$ antigens for detection (Gerdes et al., 1982). Also, any nonspecific binding of the antibody can lead to a false positive or high background which can obscure a positive signal. A third problem is that a different primary antibody, either labeled or unlabeled, is needed for every virus (Margolin, 1986).

Isotopic antibody assays attach an isotope, such as $^{125}$I. The radiolabeled antibody is allowed to bind either directly or indirectly and the unbound antibody is washed away (Lemmon et al., 1983). The activity can then be measured using a gamma counter or x-ray sensitive film. Isotopic antibody assays are subject to the same criticisms of fluorescent antibodies.

Enzyme-linked immunosorbent assays (ELISA) involve conjugating an enzyme to an antibody. The enzyme can produce a color change if the appropriate substrate is added. This color change can be seen visually or numerically with the use of a spectrophotometer. The sensitivity of this system lies between $10^1$ and $10^4$ plaque-forming units when working with poliovirus (Hurst, 1983).

**Nucleic acid probe assays on nylon membranes**

Nucleic acid probes are labeled segments of DNA or RNA that bind to complementary nucleic acids of the target virus or organism. They can be made by incorporating isotopic bases, fluorescent dyes, haptens, or enzymes directly or indirectly into the nucleic acid strands. Large quantities of DNA can be made either by cloning the desired piece using a plasmid vector inserted into an appropriate bacterial host or by the
polymerase chain reaction (PCR).

The two most common methods for incorporation of radioactive or hapten-conjugated bases into nucleic acid are nick translation and random priming (Sambrook et al., 1989). During nick translation, *E. Coli* DNA polymerase 1 adds bases to both strand of nicked DNA. Due to the exonuclease activity of the enzyme, it is able to remove each successive base and replace it with the altered bases. In random priming, a heterogeneous pool of oligonucleotides bind to single-stranded DNA and act as primers for DNA polymerases to initiate and begin transcription. Isotopic bases are detected using x-ray sensitive film, while haptens are targeted by antibodies carrying enzymes or any other label.

A variety of nonisotopic nucleic acid probes exist. A common nonisotopic probe is made by conjugating biotin to DNA (Sambrook et al., 1989). An avidin-enzyme complex can then attach due to the inherent affinity between biotin and avidin. The enzyme can break down a substrate to produce either color or light. This protocol is associated with a moderate to high degree of background which can obscure results (Higgs et al., 1990). Horseradish peroxidase can be conjugated to a nucleic acid probe sequence through the use of glutaraldehyde and then used to detect a variety of viruses (Taub et al., 1988). It has the benefits of reduced background as well as reduced assay time as compared to biotinylated probes (Higgs et al., 1990). A disadvantage is that horseradish peroxidase is sensitive to higher temperatures so the probe cannot be exposed to temperatures above 37°C.
Nucleic acids immobilized on nylon membranes

Nylon-backed nitrocellulose membranes can be used to immobilize viral nucleic acids (Margolin, 1986). Nitrocellulose membranes were originally used in probe research, but they were prone to cracking and tearing, and inefficient binding of RNA (Thomas, 1980). Nylon membranes increased the retention of RNA, possibly due to the attraction to the amine and/or carboxyl active groups of the paper (Margolin, 1986).

Nucleic acids can be spotted directly onto nylon membranes or they can be applied through the use of a dot blot apparatus. The dot blot apparatus was originally developed for use with DNA filter hybridizations (Kafatos et al., 1979) and was later adapted for RNA (Thomas, 1980). Many different viruses have been detected with this procedure, including herpes simplex virus (Stalhandske and Pettersson, 1982), Varicella-Zoster virus (Serdlin, 1984), Epstein-Barr virus (Brandsma and Miller, 1981), and hepatitis B virus (Berninger et al., 1982). The dot blot assay was adapted to the detection of enteric viruses for the detection of rotavirus in stool samples and showed an increased sensitivity of between 10 to 100 times as compared to ELISA (Flores et al., 1982).

In situ hybridization

Techniques that detect viral proteins or nucleic acid inside cells show that the virus is not only present but at least partially infectious (Taub et al., 1988). Significant amounts of viral proteins can only be present if the viral nucleic acid is being translated. Unless a nucleic acid probe assay is sensitive enough to detect one viral copy, any viral nucleic acid detected is being actively replicated by the cell.
An example of viral capsid detection is the radioimmuno focus assay (RIFA). With this assay, an antibody labeled with $^{125}$I is used to intracellularly probe a fixed monolayer of cells that have been infected with viruses. Any unbound antibody is washed away and then the sample is exposed to x-ray sensitive film for one week. Positive signals will show up as black dots (Lemmon et al., 1983). This assay was developed for the quantification of HAV. However, the disadvantages of this system include long incubations of up to three weeks, high nonspecific background, and the possibility of other viruses forming CPE and destroying the cell monolayer before HAV concentrations have reached detectable limits (Musial, 1985).

Nucleic acid probes can also be used for in situ detection of viruses. Viruses can be detected in cell smears, formalin-fixed sections, and cell culture. Some of the viruses that have been detected by in situ include human papilloma virus, herpes simplex virus, Epstein-Barr virus, and cytomegalovirus (Taub et al., 1988). Detection of viruses, such as human papilloma, can precede the manifestation of the disease state (Higgs et al., 1988). Hepatitis A virus has been detected using Primary African green monkey kidney (AGMK) cells after infection with a seeded water sample (Jiang et al., 1989). In another study, in situ hybridization was shown to be 40% more sensitive than immunofluorescence and cytopathogenicity for the detection of adenoviruses in sludge (Hurst et al., 1988).
Disinfectants

History

In 1835, the use of chlorine was adapted for use in the environment by Dr. Robley Dunlingser who wrote "to make the water of the marshes potable, it has been proposed to add a small quantity of chlorine or one of the chlorides in small but sufficient amounts to destroy the foulness of the fluid (Baker, 1930)." At that time, it had not yet been determined that water was even a means of transmitting disease. The next decade, Semmelweis proposed that doctors should clean their hands as well as their utensils in chlorine before operating on patients, especially between patients (White, 1986).

The use of chlorine as a routine treatment came in 1854, when the Royal Sewage Commission used chloride of lime in London sewage (White, 1986). The original purpose was not to kill microorganisms, but to reduce the odor. William Soper realized the potential of chlorine to prevent the spread of waterborne disease in 1879 when he used chlorinated lime to treat the feces of typhoid patients before it was dumped into the sewers (White, 1986). The definitive proof of chlorine's effectiveness for disinfection was in 1881 by Koch. He showed that bacterial cultures were destroyed by hypochlorites (White, 1986). In 1902, chlorine was first used as a continual treatment for potable water in Middelkerke, Belgium (White, 1986).

Chlorine Disinfection

When chlorine is dissolved in water, it reaches the following equilibriums (Bitton,
$\text{Cl}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HOCl} + \text{H}^+ + \text{Cl}^-$

$\text{HOCl} \rightleftharpoons \text{H}^+ + \text{OCl}^-$

The species of chlorine compound present is dependent upon the pH of the water. Hypochlorous acid is present in higher concentrations than hypochlorite ion at pH<6. Hypochlorite ions dominate in water above pH 9.5. pH values between pH 6 and pH 9.5 contain a mixture of these two compounds (Bitton, 1980).

$\text{HOCl}$ and $\text{OCl}^-$ are referred to as free available chlorine. Nitrogen compounds rapidly combine with chlorine to form chloramines depending on the pH of the water as follows:

$\text{NH}_3 + \text{HOCl} \rightleftharpoons \text{NH}_2\text{Cl} + \text{H}_2\text{O}, \text{pH} > 8.5$

$\text{NH}_2\text{Cl} + \text{HOCl} \rightleftharpoons \text{NHCl}_2 + \text{H}_2\text{O}, 4.5 < \text{pH} < 8.5$

$\text{NHCl}_2 + \text{HOCl} \rightleftharpoons \text{NCl}_3 + \text{H}_2\text{O}, \text{pH} < 4.5$

(Bitton, 1980)

Chloramines have less virucidal capacity than free available chlorine (Bitton, 1980). Therefore, the presence of free available chlorine is desired. If the chlorine is added to the water, nitrogen compounds are oxidized to nitrogen gas. The point at which this happens is known as breakpoint chlorination. Chlorine is added above the breakpoint to give a residual which can still deactivate microorganisms.

Chlorination is the most popular method of disinfection due to the low cost, ease of use, and effectiveness (Jolley et al., 1985). It is a powerful oxidant that has other positive water treatment qualities, such as "taste and odor control, prevention of algal
growths, maintaining clean filter media, removal of iron and manganese, destruction of
hydrogen sulfide, color removal by bleaching of certain organic colors, maintenance of
distribution system water quality by controlling slime growths, restoration and
preservation of pipeline capacity, restoration of well capacity, main sterilization, and
improved coagulation by activated silica (White, 1986). Concentrations from 0.5 to 1
ppm of hypochlorous acid have been shown to inactivate 99.99% of many enteric viruses
within 30 minutes (Bitton, 1980). One study on partially disinfected viruses suggest that
chlorine destroys nucleic acid even before the nucleic acid is extruded into the
environment and before proteins associated with cell adhesion are altered (O'Brien and
Newman, 1979). Some enteric viruses are more resistant to chlorine than other viruses.
Norwalk agent was exposed to free chlorine levels of 3.75 to 6.25 mg/L for 30 minutes
and was still not completely inactivated (Keswick et al., 1985). Problems exist with
the use of high levels of chlorine. It can be toxic to humans as well as aquatic life. High
levels of chlorine residuals can lead to the formation of trihalomethanes, such as
chloroform. Chloroform has been shown to be carcinogenic to laboratory animals. Humic
substances and fulvic acid are believed to be the precursors to trihalomethanes during
chlorination (Christman et al., 1983). Trihalomethane formation can occur even before
breakpoint chlorination. Coagulation may be used as a pretreatment to remove humic
substances, but the effectiveness varies with the physical properties of the water, such as
organic load, temperature, and pH (Amy and Chadik, 1983).

Environmentally, chlorine may be linked to making oysters more susceptible to the
protistan parasite Perkinsus marinus (Roberts et al., 1975), as well as increasing larval
mortality (Bellanca and Bailey, 1977). Chlorinated discharge has also been linked to increased fish and invertebrate mortality and mutagenic activity (Osborne et al., 1981 and Larson and Schlesinger, 1978). The fish population may also be indirectly affected by a loss in mayflies or phytoplankton, two major fish foods. Chlorine was implicated in altering the tracheal gills of insects, such as mayflies (Simpson, 1980). Phytoplankton were shown to be extremely sensitive to chlorine, showing adverse effects in doses as low as 0.05 mg/L (Hirayama and Hirano, 1970).

**Chlorine dioxide disinfection**

Chlorine dioxide has been proposed as an alternative to chlorine due to its inability to form trihalomethanes and inability to interact with ammonia. It does not have the taste and odor associated with chlorine-disinfected water. Chlorine dioxide has been shown to have a higher virucidal efficiency than chlorine and ozone (Hettche and Ehlbeck, 1953) and its efficiency is independent of pH (White, 1986). It is hypothesized that chlorine dioxide adsorbs to the peptone in the protein coat of the virus, creating a local high concentration of chlorine dioxide (Dowling, 1973). The overall viral capsid shape may then be altered. Evidence for this is that poliovirus 1 inactivated with chlorine dioxide changes its isoelectric point from pH 7.0 to pH 5.8 (Alvarez and O'Brien, 1982).

Chlorine dioxide is an unstable gas and so must be made on-site by combining sodium chlorite "with the aqueous solution of a conventional chlorinator injector discharge (White, 1986)." It is used in an aqueous form due to its explosive quality. Most plants in the U.S. use it as a supplement to reduce the taste and odor of water due to phenolic
A problem with chlorine dioxide is that it leads to the formation of chlorite. At levels as low as 50 mg/L, chlorite has been associated with hemolytic anemia. Chlorine dioxide alone may produce this result in mammals due to the gastrointestinal tract converting chlorine dioxide to chlorite (Abdel-Rahman et al., 1980). Chlorite levels above 100 mg/L have been associated with lower sperm motility in rats, although no teratogenic effect has been seen (Carlton and Smith, 1985). Chlorine dioxide also directly impacts thyroid metabolism in primates without forming chlorites (Bercz et al., 1982).

**Ozone disinfection**

Ozone is a powerful oxidant. It attacks organic compounds by adding oxygen atoms to the unsaturated carbon-carbon bonds, yielding carboxylic acids, ketones, and aldehydes (Bailey, 1975). Ozonation does not impart a taste or odor to water and can remove unwanted color as well as phenolic compounds which give taste to water (McCarthy and Smith, 1974). It also removes precursors of trihalomethanes (Jolley et al., 1986). However, ozonation is more expensive than chlorination and does not have residual protection (White, 1986). Direct exposure to ozone concentrations exceeding 0.25 ppm are considered dangerous to health (White, 1986) and has been shown to destroy plant life (Jolley et al., 1986).

Ozone can be formed by passing air through an electrical discharge. The concentration of ozone can be increased by passing purer oxygen. The germicidal efficiency is not as dependent upon pH as chlorine, but it does lose efficacy with increasing
temperature. Ozone has been used in the United States in water treatment for the past fifty years (White, 1986) and in Europe for sixty years (McCarthy and Smith, 1974). However, it is always followed by another disinfectant, such as chlorine (Jolley et al., 1986).

Ozonolysis of some organic compounds can lead to the formation of hydroperoxides, which have been demonstrated to have a mutagenic effect (White, 1986). When ozone reacts with pesticides, carcinogenic epoxides may be formed (White, 1986). However, the potential public health threat from ozonation is not known (White, 1986).

**Ultraviolet light disinfection**

During ultraviolet radiation, organic molecules absorb the energy. This energy is then dissipated, disrupting unsaturated bonds. Ultraviolet exposure time and intensity have a direct correlation with virucidal activity (White, 1986). However, it is more expensive than chlorine, leaves no residual protection, cannot penetrate turbid water, and lacks an efficient method to monitor the efficacy.

UV light is generated by electron flow in mercury vapor such as in germicidal lamps. The radiation must directly hit the target to destroy it. Therefore, high concentrations of suspended solids can act as a shield, decreasing the disinfecting ability. UV light has also been shown to be effective in destroying phenols, improving the taste and odor of water. Currently, UV irradiated is used in wastewater treatment, but not to a large degree for the production of potable water (Huff et al., 1965). UV light does not lead to the known formation of mutagenic or carcinogenic agents (White, 1986).
However, surviving organisms from UV disinfection show chromosomal aberrations and an increase in the mutation rate (Lawrence and Block, 1968).
METHODOLOGY

The Development of Nylon-based Nucleic Acid Probe Assays for the Detection of Poliovirus DNA and RNA

Poliovirus propagation

Buffalo Green Monkey (BGM) kidney cells (BioWhittaker, Walkersville, MD) were used to propagate poliovirus type 1 (LSc). A closed 75 cm² cell culture flask (Corning, Corning, NY) of BGM cells was grown to confluency with MEM (Autopow, Irvine Sc. Santa Ana, CA) containing 8% fetal bovine serum (Gibco, Grand Island, NY). Once confluent, the growth medium was removed and replaced with maintenance medium. Five hundred microliters of poliovirus stock (9 x 10⁷ plaque forming units/ml) was added giving a multiplicity of infection of approximately 10 to 1. The cells were allowed to incubate 48 hours at 37°C until all cells exhibited cytopathic effects (CPE). The flasks were placed in a freezer and then in an incubator so as to freeze and thaw the cells three times to lyse cells and liberate the viruses. The lysate was poured into a sterile Oakridge tube (Nalgene, Rochester, NY) and centrifuged for 10 minutes at 12,100 x g to remove cellular debris. The supernate was transferred to a second sterile tube and divided into 500 µl microliter aliquots which were placed in sterile 1.5 ml microfuge tubes and stored at -80°C until use.
Poliovirus quantification

Following the Dahling procedure (Dahling, 1986), ten closed 25 cm² flasks (Corning) of BGM cells were grown to confluency with 8% MEM. The cells were rinsed with 1X PBS prior to incubation with the viral dilution. Virus stored at -80°C was thawed and 10 fold dilutions were made from 10⁰ to 10⁻⁸ in 1X PBS. Flasks were labeled and then inoculated with 100μl of the appropriate viral dilution. Two control flasks were used; one inoculated at the beginning and one at the end of the series of flasks. The control flasks received 100μl of 1X PBS, rather than virus.

All flasks were left at room temperature (RT) for 80 minutes, gently rocking the flasks every 10 minutes to ensure even distribution of the inoculum. At the end of 80 minutes, cells were rinsed with 1X PBS. Two percent flake agar was poured into 2X medium 199 and the pH was brought to 6.9 with 1 N HCl. Ten milliliters of the overlay was put into the flasks by pipetting the overlay onto the side and then allowing it to cover the bottom. The agar was allowed to harden in the dark for 30 minutes. Flasks were then inverted and incubated at 37°C. Plaques were counted after 48 hours.

Sample sites

Four sample sites were chosen for this study to collect water that might contain poliovirus. The first was the Durham, NH Wastewater Treatment Plant. Water was obtained from the effluent prior to discharge into the Oyster River. The second site was from Painted Rock, a site downstream of the Durham Wastewater Treatment Plant. This site is under tidal influence. The third site was Durham Town Landing, which is upstream.
from the Durham Wastewater Treatment Plant and is also under tidal influence. The fourth site was Mill Pond. Mill Pond is a freshwater site that empties into Durham Town Landing. The site is separated from the others by a waterfall so that there is no possibility of contamination from the other sites.

**Sample collection**

Two 44-gallon barrels were rinsed with a 10% solution of bleach to inactivate any viruses present prior to the sample collection. The bleach was deactivated with 10% sodium thiosulfate. Three hundred liters of water were collected in the two large barrels. The pH of the water was reduced to 3.5 with 1 N HCl, making the poliovirus net positively charged. Two hundred milliliters of 1 M AlCl₃ was added to act as a salt bridge. The sample was pumped through an 8 inch Filterite® negatively charged filter with a nominal pore size of 1 μm (Memtec America Corporation, Timonium, MD) for viral adsorption.

**Viral concentration**

Viruses were eluted from the filter with 800 ml of 3% beef extract V (Becton Dickinson Microbiology Systems, Cockeysville, MD) in 90 mM glycine pH 9.5. The pH of the beef extract was lowered to 3.5 using 1 N HCl to create a flocculent to which viruses adsorb. The sample was mixed for 30 minutes followed by collection of the flocculent by centrifugation at 15300 x g for 10 minutes in 4 sterile centrifuge tubes. The flocculent was rapidly resuspended in 30 ml of 0.1 M Na₂HPO₄ solution @ pH 9.5 one
tube at a time by vortexing. The pH of the suspension was returned to 7.0 with 1 N HCl.
Non-resuspended debris was removed by centrifuging at 12100 x g for 10 minutes.
Samples were then divided into three aliquots. Three 1 ml volumes from the first aliquot
were spotted on to nylon-backed hybridization membranes for gene probe analysis, the
second 10 ml aliquot was assayed by cell culture analysis, and the third 10 ml aliquot was
stored at -70°C for future use. GeneScreen Plus (Dupont, Boston, MA) was used for all
probe assays except digoxigenin, in which GeneScreen (Dupont) was substituted.

**Cell culture for environmental testing**

Ten 25 cm² flasks were grown to confluency with BGM cells using growth media.
Ten milliliters of the sample to be assayed was incubated with 1 ml of 100X antibiotic
antimycotic solution (Sigma, St. Louis, MO) at 37°C for 60 minutes. Five milliliters of
PBS were pipetted into each flask, rinsed, and poured off. One milliliter of sample was
then placed into each flask. The flasks were rocked every 15 minutes for 2 hours at room
temperature. Five milliliters of 2% MEM was put into the flasks and the flasks were
examined daily for 14 days.

If no cytopathic effects (CPE) were observed after 14 days, two flasks of cells
were frozen and thawed. The medium for each was placed into two sterile Oakridge tubes
and centrifuged at 12,000 x g for 10 minutes. One milliliter of the supernates was used to
inoculate recently confluent BGM monolayers in another two 25 cm² flasks. If no CPE
were seen after 14 days, the sample was considered negative. If CPE were seen in either
the first set of flasks or in the confirmation flasks, the samples were reinoculated into new
25 cm² flasks. Any subsequent CPE caused from the inoculation of the supernatant from the first set of flasks or the confirmation flasks was interpreted as being caused by virus and the original sample was considered positive.

**Preparation of the dot blot apparatus**

The dot blot apparatus (BioRad, Hercules, CA) was autoclaved for 20 minutes, followed by being submerged in a 10% solution of sodium dodecyl sulfate (SDS) for 30 minutes. Using gloves to prevent RNase contamination, each piece was picked up and repeatedly rinsed in distilled water and shaken until all SDS was removed. The apparatus was placed in an oven and baked until dry.

**Spotting environmental samples onto nylon-backed membranes**

The release of viral nucleic acid from the protein coat was by the methods of Richardson et al., 1981. In this procedure, 3 ml of the concentrated sample was digested at 55°C for 1 hour with 240 μl of 20 mg/ml protease K (Sigma). The sample was then placed on ice to bring it back to room temperature quickly. For nonradioactive probe analysis, 1 ml of the sample was passed through a Bio-Rad bio-dot™ apparatus onto a GeneScreen™ or GeneScreen Plus™ hybridization membrane. Samples were done in triplicate, using 1 ml per well. Membranes were baked for two hours at 80°C and then covered in a plastic wrap until use.
Plasmid preparation

*E. coli* cells containing the plasmid PV104 were streaked out onto LB agar plates containing 5 μg/ml tetracycline (Sigma), inverted, and incubated overnight in a 37°C incubator. The next day, a colony was inoculated into two 250 ml Erlenmeyer flasks each containing 100 ml of sterile LB media with 5 μg/ml tetracycline. The flasks were incubated at 37°C for six hours. The contents of each flask were poured into separate Ferrenbocker flasks, each containing 1 liter of LB media with 5 μg/ml tetracycline. The Ferrenbocker flasks were placed in a shaker and agitated for 16 hours at 37°C.

The culture was poured into sterile centrifuge tubes and centrifuged at 15300 x g for 15 minutes. The cells were resuspended in 12 ml of lysis buffer and placed on ice for 10 minutes. Twenty-four milliliters of freshly made 0.2 N NaOH and 1% sodium dodecyl sulfate were added, mixed by inversion, and placed on ice for 10 minutes. Fifteen milliliters of 3 M NaAc, pH 4.6 were added, mixed by inversion, and returned to the ice for 20 minutes. The suspension was centrifuged at 15,300 x g for 10 minutes. The supernate was transferred to a sterile Oakridge tube, spun at 12,100 x g for 10 minutes and the pellet was discarded. DNase-free RNase A (Sigma) was added to give a final concentration of 20 μg/ml and allowed to incubate at 37°C for 1 hour.

An equal volume of phenol/chloroform was added and vortexed for 1 minute. The tubes were spun at 12,100 x g for 5 minutes. The aqueous (top) layers were removed and placed into two other sterile Oakridge tubes. The phenol/chloroform extraction was then repeated. An equal volume of chloroform was added to the DNA preparation and vortexed for 1 minute. The layers were separated by centrifuging at 12,100 x g for 5
minutes. The aqueous layer was removed and placed into another sterile Oakridge tube. Two equal volumes of 95% ethanol were added and the mixture was placed in a -20°C freezer overnight.

The DNA was pelleted in a centrifuge at 12100 x g for 20 minutes. The supernate was removed and the pellet was washed in 70% ethanol. The tube was inverted until dry. The DNA was dissolved in 1.6 ml of MilliQ water (MilliQ Water Purification System, Millipore, Ann Arbor, MI) and then mixed with 400 μl of 4 M NaCl and 2 ml of 13% polyethylene glycol, molecular weight 8000. This solution was incubated in ice water for a minimum of 1 hour. DNA could be recovered by placing aliquots into sterile microfuge tubes and microfuging for 20 minutes, followed by a 70% ethanol wash. The tubes were allowed to dry and then the DNA was resuspended in distilled water.

**Preparation of nylon membrane sensitivity strips**

Serial dilutions of pVR104 were made from 100 ng/100 μl to 1 fg/100 μl. The DNA was denatured by boiling in water for 10 minutes and then the tubes were immersed into an ice water bath for 5 minutes. One hundred microliters of each denatured DNA dilution was spotted onto GeneScreen (Dupont, Boston, MA) or GeneScreen Plus (Dupont) using the dot blot apparatus.

Serial dilutions of poliovirus were also spotted. Dilutions from $10^0$ to $10^{-7}$ were made in DEPC-treated water. Proteinase K was added to a final concentration of 400 μg/ml and the samples were incubated for 1 hour at 55°C. Five hundred microliter volumes of each dilution were adsorbed onto GeneScreen or GeneScreen Plus (Dupont)
nylon membranes wetted with DEPC-treated water with the aid of the dot blot apparatus.

Once all dilutions were spotted onto GeenScreen membranes, they were baked at 80°C for two hours to crosslink the nucleic acids to the membranes. The membranes were covered with a plastic wrap until use.

\textit{\textsuperscript{32}P-ssRNA probe procedure}

\textit{\textsuperscript{32}P-ssRNA probe} was generously provided by A. E. Moore (Moore and Margolin, 1992). The GeneScreen Plus (Dupont) nylon membrane was placed in a 50 ml (Corning) polypropylene centrifuge tube. Five milliliters of prehybridization fluid containing 6 X SSPE, 0.5% SDS, 10% dextran sulfate, and 50% deionized formamide were added to the tube and allowed to rotate for two hours at 50°C in a rotisserie-type hybridization oven. The tube was removed and 5 x 10\textsuperscript{6} counts/minute of probe was added per milliliter of prehybridization fluid. The tube was returned to the oven and rotated at 50°C for 12 hours. Once hybridization was complete, the hybridization fluid was decanted and replaced with 10 ml of 2 X SSPE and rotated \textit{at RT} for 5 minutes. A second wash under the same conditions followed. Two washes with 5 ml of 2 X SSPE/0.5% SDS at 65°C for 30 minutes each were then performed. The final two washes were with 10 ml of 0.1 X SSPE \textit{at RT} for 30 minutes each. The membrane was then air-dried on filter paper. The membrane was wrapped in plastic wrap and placed in a X-ray cassette with a Dupont intensifying screen. It was exposed to XAR-5 x-ray film (Eastman Kodak Company, Rochester, NY) for 48 hours at -70°C. The autoradiograph was then developed by submerging the film for 5 minutes in Kodak D-11 (Eastman Kodak Company), followed
by a 30 second water wash, followed by another 5 minutes in Kodak fixer (Eastman Kodak Company). The film was then washed in water for 15 minutes and hung to dry.

**Biotin-tailed probe production**

In a sterile microfuge tube, 8.3 μl of pVR104, 1 μl of DNase I (Sigma), 0.5 μl dH₂O, and a final concentration of 10 mM MgCl₂ were added. The mixture was incubated for 5 minutes at 37°C, followed by 10 minutes at 65°C. A 0.7% agarose gel was run to ensure that the DNA has been cut into pieces ranging from 100 to 2000 base pairs in length. An equal volume of phenol/chloroform was added and the mixture was vortexed for 1 minute. The aqueous layer was pipetted off and placed into a sterile microfuge tube. The extraction was repeated with chloroform.

One microliter of terminal deoxynucleotide transferase (Enzo Diagnostics, New York, NY) was mixed with 4 μl of terminal transferase dilution buffer. In another sterile microfuge tube, 15 μl of terminal transferase reaction buffer, 10 μl of TTP, 5 μl of the diluted terminal deoxynucleotide transferase, and 1 μg of the sheared plasmid, were added. The volume was brought to 45 μl with sterile dH₂O, followed by 5 μl of 10 mM CoCl₂. The mixture was incubated for 60 minutes at 37°C and then placed in an ice-water bath. Five microliters of 100 mM EDTA were then added to stop the reaction.

**Determining sensitivity of biotin-tailed probe**

Three GeneScreen Plus (Dupont) nylon-membrane sensitivity strips were soaked for 10 minutes in 2 X SSC. The membranes were placed in Seal-A-Meal sealable bags
(Sears Roebuck and Co., Chicago, IL) using a Seal-A-Meal (Dazey, Industrial Airport, KS) with enough biotin prehybridization solution to coat the membrane. Prehybridization occurred for 6 hours at 42°C and then the solution was removed and replaced by the biotin hybridization solution. The first membrane received 0.5 μg/ml probe, while the second received 2.5 μg/ml probe, and the third had 5 μg/ml probe.

Hybridization occurred for 16 hours at 42°C. The membranes were removed and washed 3 times for 10 minutes each in 2 X SSC/0.1% SDS at 42°C. Three subsequent 15 minute washes were performed with 0.1 X SSC/0.1% SDS at 65°C followed by three rinses in 1 X SSC/0.1% SDS. Two microliters of the Biobridge poly A/biotin conjugate (Enzo) was added for every milliliter of a fresh solution of 1 X SSC/0.1% SDS. Enough Biobridge solution was added to adequately coat the membrane and incubated for 2 minutes at RT. An equal volume of 1X SSC/0.1% SDS was added to the Biobridge solution and allowed to stand for 2 more minutes. The solution was removed and the membranes rinsed in 1 X SSC/0.1% SDS for 5 minutes. The membranes were washed once for 5 minutes at 48°C in biotin wash solution 1, followed by two washes for 5 minutes at 48°C in biotin wash solution 2, followed by three washes for 5 minutes at 48°C in biotin wash solution 3, followed by three 30 second rinses in 1 X SSC at RT.

The membranes were transferred to seal-a-meal bags and blocked for 15 minutes at RT in biotin blocking buffer. Once the blocking buffer was removed by squeezing it out of the bags, a 1:250 dilution of Detek 1-hrp complex (Enzo) in 1X Detek dilution buffer was added to barely coat the membranes. The bags were resealed and incubated for an additional 60 minutes. Membranes were removed from the bag and rinsed with the biotin
high salt washing buffer four times for 5 minutes each. After washing, the membranes were placed in biotin predetection buffer for two 5 minute rinses. Membranes were incubated in the substrate mixture for 60 minutes and then rinsed in 1X PBS and allowed to dry. Membranes were photocopied to record the darkest results before the colormetric signal faded.

**HRP probe production**

Five hundred micrograms of pVR104 was added to a sterile microfuge tube and sterile MilliQ water was added to a final volume of 1.4 ml. The tube was placed in a ice water bath and taped in place so no movement was permitted. A sonicator tip was placed as deep as possible into the tube without touching the sides. A W-375 sonicator (Heat Systems-Ultrasonics, Inc., Plainview, NY) was put on an output level of 5, set at 50% duty and the sample was sonicated for 15 minutes. The size range of the sonicated DNA was determined by running 5 μl in a 0.7% agarose gel. The microfuge tube was placed in a vacuum centrifuge and centrifuged until 50 μl of the solution remained. Another gel was run to determine concentrations of the DNA. The DNA was adjusted to a final concentration of 4 mg/ml with MilliQ water.

Thirty microliters of the 4 mg/ml sonicated DNA was denatured in boiling water for 10 minutes and then placed in an ice water bath for another 10 minutes. Fifteen microliters of dH₂O was put into a microfuge tube followed by 40 μl of 20 mg/ml HRP (Digene Diagnostics, Silver Spring, MD). Ten microliters of glutaraldehyde (Digene) was added and gently mixed, followed by 25 μl of denatured DNA and another mixing. Ten
microliters of a Digene Diagnostics proprietary reagent was added. The tube was heated at 37°C for 45 minutes and then placed on ice. Three hundred microliters of glycerol was added to prevent freezing and the probe was then stored at -20°C. The average HRP:DNA ratio was 4:1.

Determining optimum HRP probe concentration

Two sets of three GeneScreen Plus (Dupont) sensitivity strips were prehybridized for 1 hour at room temperature (RT) in a seal-a-meal bag with HRP hybridization buffer. A corner of the first bag from each set was opened and 2.5 μl of probe per 1 ml of HRP hybridization buffer was added and the bag resealed. The same procedure was repeated for the second and third bags with 5 μl and 10 μl of probe added respectively. The membranes were allowed to incubate for 2 hours at RT. After the membranes were removed from the bag, they were rinsed in 0.2% sacrocy1 followed by three 5 minute washes in 0.2% sacrocy1 at 37°C.

For a colormetric result, the membranes were allowed to incubate for 15 minutes in DAB reaction buffer. Membranes were then rinsed 3 times in MilliQ H2O and allowed to dry. For chemiluminescent results, the membranes were incubated for 1 minute in luminol provided in the ECL Western blotting detection kit (Amersham, UK). The membranes were resealed in a seal-a-meal bag and allowed to expose Hyperfilm™-ECL (Amersham) high performance luminescence detection film for 1 minute. The film was developed under safe light conditions. First, it was submersed in Kodak D-11 (Eastman Kodak Company, Rochester, NY) for 5 minutes, followed by a 30 second rinse in water,
followed by immersion for another 5 minutes in Kodak fixer (Eastman Kodak Company).

The film was then washed in water for 15 minutes and then hung to dry.

**Determining optimum HRP hybridization time**

Six sensitivity strips were treated as above using 5 μl of probe, with two being hybridized for 1 hour, two for 2 hours, and two for 16 hours. One set of membranes was developed colorimetrically and the other set luminescently.

**Increasing HRP molecules on probe**

The HRP probe was made as previously described, but with 80 μl of HRP and no water added during the conjugation. The average number of HRP:DNA molecules for this reaction is approximately 8:1. Four sensitivity strips were then made. Two were probed with the 4:1 probe and the other two with the 8:1 probe. One from each set was developed colorimetrically and the other two chemiluminescently.

**Plasmid digoxigenin probe production**

Five micrograms of pVR104 was linearized by incubating it for 2 hours at 37°C with 2 microliters of EcoR1 (10 units/μl) and 1X incubation buffer (Boehringer Mannheim, Indianapolis, IN). A 0.7% agarose gel was run to confirm that the plasmid was completely linearized. One volume of phenol/chloroform was added and vortexed for 1 minute, followed by microfuging for 5 minutes. The top layer was then removed and placed into a second sterile microfuge tube. One volume of chloroform was added and
vortexed for 1 minute, followed by microfuging for 5 minutes. The top layer was removed and placed into a third sterile microfuge tube. Two volumes of ethanol were added and the tube was placed at -20°C overnight.

One microgram of the DNA was boiled for 5 minutes and cooled for 10 minutes in an ice water bath. Two microliters of digoxigenin hexanucleotide mixture (Boehringer Mannheim) and 2 µl of digoxigenin dNTP labeling mixture (Boehringer Mannheim) were added, followed by enough MilliQ water to bring the final volume to 19 µl. One microliter of Klenow enzyme (Boehringer, Mannheim) was added last. The reaction was incubated overnight at 37°C.

The reaction was stopped with the addition of 2 µl of 200 mM EDTA, pH 8.0. Two microliters of 4 M LiCl₂ and 60 µl of ethanol was added and the mixture stored at -20°C for a minimum of two hours. The tube was microfuged for 20 minutes and the supernate decanted. Chilled (-20°C) 70% ethanol was poured in and then removed. The tube was inverted and the pellet allowed to dry. The pellet was dissolved in 50 µl of TE.

Determining optimum plasmid digoxigenin probe concentration

Six sensitivity strips were prepared. The membranes were placed in seal-a-meal bags and 20 ml of digoxigenin hybridization buffer per 100 cm² membrane was added and the bags sealed. Bags containing sensitivity strips were placed in a 68°C oven and prehybridized for two hours. A corner of the bag was cut and the hybridization buffer was decanted and replaced with hybridization buffer containing denatured probe. The probe was denatured by boiling for 10 minutes, followed by cooling 5 minutes in an ice-water
bath. Two membranes received 10 ng/ml probe, two had 50 ng/ml, and two 100 ng/ml. All membranes were placed in a 68°C oven.

Sixteen hours later, the membranes were removed from the bags and rinsed twice for 5 minutes each in digoxigenin wash solution 1 at room temperature, followed by two 15 minute washes in digoxigenin wash solution 2 at 68°C. The membranes were equilibrated in digoxigenin buffer 1 for 1 minute, followed by immersion in digoxigenin buffer 2 with constant agitation. The set of membranes to be developed colorimetrically were incubated in buffer 2 for 60 minutes, while those developed chemiluminescently were incubated 180 minutes. The anti-DIG-alkaline phosphatase antibody was diluted 1:5,000 in buffer 2. Membranes were placed in the antibody dilution for 30 minutes. Excess antibody was then removed with two 15 minute washes with digoxigenin buffer 1. The membranes were then equilibrated for 2 minutes in digoxigenin buffer 3.

For colorimetric detection, the membranes were put into seal-a-meal bags and the digoxigenin colorimetric solution was added. Sixteen hours later, the membranes were removed and washed 5 minutes in TE. For chemiluminescent detection, the membranes were barely covered with Lumi-Phos 530 (Lumigen, Inc., Detroit, MI). The membranes were sealed in seal-a-meal bags and incubated for 30 minutes at 37°C. They were taped to the inside of an X-ray cassette, brought into a darkroom and allowed to expose Kodak X-omat film (Sigma) for 15 minutes. The film was developed by submerging it into Kodak D-11 (Eastman Kodak Company) for 5 minutes, followed by a 30 second water rinse, followed by another 5 minutes in Kodak fixer (Eastman Kodak Company). The film was then washed for 15 minutes in water and then hung to dry.
Analysis of environmental samples with cell culture, $^{32}$P-cDNA, $^{32}$P-ssRNA, and digoxigenin probe

Twenty-three environmental samples with cell culture and $^{32}$P-cDNA results already known were randomly picked and spotted onto GeneScreen Plus and GeneScreen membranes as previously described. The GeneScreen Plus membrane was analyzed by $^{32}$P-ssRNA probe and the GeneScreen membranes by plasmid digoxigenin probe. The results of the latter membranes were visualized by chemiluminescence.

PCR digoxigenin probe production

To prevent vector contamination, digoxigenin probes were made by the polymerase chain reaction (PCR) from the 5' end of the viral nucleic acid using the cDNA in the pV104 plasmid. Poliovirus 1, 2, and 3, as well as Coxsackie virus B1, B3, and B4 were computer analyzed to determine the optimum piece of homologous DNA. A 149 base-pair sequence was chosen from the 5' noncoding region to amplify. The antisense upstream primer was 445-465 (5'-TCCGGCCCCTGAATGCGGCT-3') and the downstream primer was 577-594 (5'-TGTCACCATAAGCAGCC-3') (Abbaszadegan et al., 1993).

In a 0.5 ml microfuge tube, 10 $\mu$l of 10X PCR buffer (Perkin Elmer Cetus, Norwalk, CT), 8 $\mu$l of MgCl$_2$ (Perkin Elmer Cetus), 50 ng of plasmid template, 50 pm of each primer, and 20 $\mu$l of the dNTP labeling mixture (Boehringer Mannheim) was added together and brought up to a total of 99 $\mu$l with MilliQ water. A drop of Nujol mineral oil (Perkin Elmer) was gently layered on top.
The mixture was heated to 95°C for 5 minutes before 1 µl of AmpliTaq DNA polymerase (Perkin Elmer Cetus) was added underneath the mineral oil. The reaction proceeded for 40 cycles. Denaturation occurred at 95°C for 1.5 minutes, annealing occurred at 55°C for 1.5 minutes, and extension occurred at 75°C for 1.5 minutes. The mineral oil was then pipetted off. Two volumes of 95% EtOH were added and allowed to incubate overnight at -20°C. The tube was spun for 20 minutes in a microfuge and the supernate poured off. Chilled (-20°C) 70% EtOH was used to wash the pellet. The pellet was then air-dried and resuspended in 50 µl of MilliQ water.

To determine purity and concentration of the PCR product, a 0.7% agarose gel was cast. One microliter and 5 microliters of the PCR product was run against a lambda ladder. The probe was stored at -20°C until use and then used in the same fashion as the plasmid digoxigenin probe.

The sensitivity was determined by making four sensitivity strips and probing two with 10 ng/ml digoxigenin plasmid probe and two with 10 ng/ml PCR probe. One set was evaluated luminescently and the other colorimetrically.

**Reducing background of digoxigenin probes**

Four sensitivity strips were prepared. The digoxigenin procedure was repeated as above with the following exceptions; the digoxigenin hybridization buffer contained 3% SDS, it was not cooled after denaturing the probe, and the first rinse in buffer 1 contained 0.3% Tween 20. Two membranes were given 10 ng/ml probe, while two were given 50 ng/ml probe. One set was developed luminescently and the other colorimetrically.
In situ Hybridization Study

Preparation of slides

Silane-treated slides with two 4 cm² wells surrounded by teflon borders (DIGENE Diagnostics, Inc., Silver Spring, MD) were placed in a Coplin jar and filled with 95% ethanol so that the level of ethanol was above the top of the slides for a minimum of 10 minutes. The slides were removed with tweezers and individually placed into sterile glass Petri dishes. The dishes were placed under an ultraviolet light source for 60 minutes and then placed in a nonhumid 37°C incubator until the ethanol had completely evaporated.

Cell culture

Each well of the silanated slides was aseptically seeded with 0.5 ml of cells at a concentration per surface area equal to that obtained from the initial flask. For example, a 25 cm² flask can seed 6-4 cm² wells. The media used for the cells was 8% MEM. Throughout the inoculation and after, the slides were kept in the glass Petri dishes at all times.

The slides were kept in a 37°C incubator with 5% CO₂ and a source of humidity. In this case, a container of water was kept in the bottom of the incubator. To keep down the possibility of fungal contamination, 5 g of copper sulfate was added to the water.

After two days, the 8% MEM was pipetted out of each well. Five hundred microliters of 1X PBS was placed on each well and pipetted off. A fresh five hundred
microliters of 8% MEM was then placed into each well. The Petri dishes were then returned to the incubator. Slides were examined daily and used when the monolayer became confluent.

**Cell adhesion with high concentration of formalin**

Seven silanated slides with confluent monolayers of BGM cells were rinsed with 1X PBS. The teflon borders were wiped with a Kimwipe (Kimberly-Clark, Oswell, GA). A 10% neutral-buffered formalin (Sigma Diagnostics) was applied to six slides. Three of these slides were placed into a 37°C incubator and the other three remained on the bench top. The seventh slide had 1X PBS, rather than formalin. After 15 minutes, one slide was taken from the incubator and another from the bench and rinsed with 1X PBS. Two more slides from each group were rinsed with 1X PBS after 60 minutes, and the final two were rinsed after 120 minutes.

All of the teflon borders were again wiped dry and then 500 μl of *in situ* hybridization buffer was added. The slides were heated for 5 minutes at 100°C. The hybridization buffer was again removed, the borders dried, and 500 μl of hybridization buffer containing 25 ng/ml was added to the first well and 500 μl of hybridization buffer without probe was added to the second well. The slides were incubated overnight in a humid chamber at 37°C.

The slides were then washed 10 minutes in 2X SSC at room temperature, 10 minutes in 1X SSC at room temperature, 5 minutes in 0.5 X SSC at 37°C, and 5 minutes in 0.5 X SSC at room temperature. Slides were then dipped in digoxigenin buffer 1.
solution for 1 minute followed by 10 minutes in digoxigenin buffer 1 containing 2% FBS and 0.3% Triton X-100.

The teflon borders were wiped and 500 \( \mu \)l of a 1:500 dilution of antibody conjugate and modified buffer 1 was added. Slides were incubated in a humid chamber 3 hours at room temperature, followed by placement into a solution of digoxigenin buffer 1 for ten minutes, and then 10 minutes in digoxigenin buffer 3. The teflon borders were again wiped and 200 \( \mu \)l of the digoxigenin colorimetric solution was added. After one hour, the slides were rinsed in a solution of TE, followed by a 5 minute wash in a solution of TE. Slides were counterstained 30 seconds in nuclear fast red (NFR), followed by two rinses in distilled water and allowed to air dry. The samples were then coverslipped and examined.

**Cell adhesion with a low concentration of formalin**

Four silanated slides were inoculated with BGM cells and grown to confluency. The slides were fixed as above, but with a 4% formalin solution in PBS. One slide was fixed for 15 minutes, a second for 30 minutes, a third for 60 minutes, and a fourth for 120 minutes. All fixations were done at room temperature. The rest of the procedure followed was exactly the same as with the higher concentration of formalin.

**Digoxigenin probe concentration**

The appropriate probe concentration had to be determined. Four silanated slides were grown to confluency with BGM cells. Each well was rinsed with PBS and the
borders dried. One hundred microliters of virus was diluted in serum-free medium to give a final concentration of 100 pfu/ml. Five hundred microliters of virus was added to one well, while the other well had 500 µl of nonspiked serum-free media. The slides were placed in humid chambers and incubated for 5 hours in a 37°C incubator with 5% CO₂. The slides were then rinsed with PBS and then placed in a solution of 4% formalin diluted with 1X PBS for 60 minutes.

The slides were again rinsed with PBS and the borders wiped. Hybridization buffer was applied to each well and the slides were heated for 10 minutes at 100°C to limit the secondary and tertiary structure of poliovirus (Currey, 1986). Slides were removed from the heat, hybridization buffer blotted off, and 500 µl of hybridization buffer with differing concentrations of probe was then added to each well. The first slide was given 12.5 ng/ml probe, the second 25 ng/ml, the third 50 ng/ml, and the fourth 100 ng/ml. All slides were placed in a humid chamber and incubated overnight at 37°C.

The next day, the slides were washed for one minute in digoxigenin buffer 1, followed by 10 minutes in digoxigenin buffer 1 containing 2% FBS and 0.3% Triton X-100. The borders of the wells were dried and a 1:500 dilution of antibody conjugate in the modified buffer 1 was added. The slides were returned to a room temperature humid chamber and incubated for 3 hours.

The unbound antibody conjugate was removed by washing 10 minutes in digoxigenin buffer 1 followed by 10 minutes in digoxigenin buffer 3. The slide borders were dried and 200 µl of digoxigenin color solution was added for 60 minutes. Slides were then rinsed in TE, followed by a 5 minute wash in TE. The cells were counterstained
in nuclear fast red (Digene Diagnostics, Silver Spring, MD) for 30 seconds followed by
two distilled water rinses. The slides were allowed to dry, then coverslipped and
examined.

**Decreasing antibody time**

To potentially reduce assay time and the persistent level of background, the
antibody incubation time was reduced. BGM were grown to confluency on 4 silanated
slides. The experiment was performed as in the low formalin concentration experiment for
60 minutes with 25 ng/ml probe concentration. One slide was incubated 30 minutes with
the antibody solution, the second for 60 minutes, the third for 120 minutes, and the fourth
for 180 minutes.

**Determining the sensitivity of the *in situ* assay**

The sensitivity of the *in situ* assay needed to be determined and compared to the
plaque-forming unit assay. Ten silanated slides were inoculated with BGM cells and
grown to confluency. After rinsing the media away with PBS, the teflon borders were
dried and each well was inoculated with 100 μl of a poliovirus dilution in serum-free
media. Each well of the first slide was inoculated with 1000 viruses per well. The wells
of the second slide were inoculated with 100 plaque-forming units per well. The
remaining eight slide had 10 plaque-forming units per well. After 5 hours, the viral
solution was rinsed off with 1X PBS.

The assay was performed by fixing the cells for 60 minutes with 4% formalin,
using a probe concentration of 25 ng/ml, and an antibody incubation time of 60 minutes. When the slides were coverslipped, the cells were examined under a compound microscope. Any cells that had purple rather than red cytoplasm were considered positive. The cells in the negative control wells should all contain a red cytoplasm.

Chlorine, Chlorine Dioxide, Ozone, and Ultraviolet Light Disinfection Study

Preparation of chlorine-demand free water

Ten liters of chlorine demand-free water were prepared by spiking MilliQ water with 20 mg/L NaOCl. An ultraviolet light was immersed in the water and the water was stirred until no free chlorine was detectable by the AccuVac DPD Free Chlorine Reagent method (Hach, Loveland, CO) which is read on the DR100 Direct Reading Spectrophotometer (Hach).

Chlorine disinfection

MilliQ water was brought to a pH of 6 using the buffers 500 mM KH₂PO₄, pH 4.4 and 500 mM Na₂HPO₄, pH 9.9. The water was spiked with chlorine to give a final residual of 1 mg/L. Chlorine residuals were read by mixing an aliquot of water with an ampule of AccuVac DPD Free Chlorine Reagent. A 1 ml aliquot of poliovirus was added to a 49 ml aliquot of spiked water. The chlorine residual was measured 5 minutes later. If the residual had fallen below 0.2 mg/L chlorine, the poliovirus dilution was diluted 1:50 in chlorine demand-free water and the residual was measured as before. Dilutions of
poliovirus were made until the residual did not fall below 0.2 mg/L chlorine when 1 ml of the poliovirus dilution was added to 49 ml of the 1 mg/L chlorine-spiked water.

Forty-five milliliters of chlorine-spiked water was added to three separate 50 ml polyethylene tubes followed by 1 ml of the final poliovirus dilution. At two minutes, three 10 ml aliquots from the first 50 ml polyethylene tube were added to each of three 15 ml conical polyethylene tubes, each containing 0.5 ml of 0.05 M Na₂S₂O₃. The chlorine residual was measured as before from the remaining sample in the 50 ml polyethylene tube. The procedure was repeated for the 10 and 60 minute time point.

To ensure that chlorine disinfection did not have an immediate impact upon the viral population before the Na₂S₂O₃ was added, 2.3 ml of Na₂S₂O₃ was added to the polyethylene tube containing 45 ml of the chlorinated water. One milliliter of the virus was then added and three 10 ml aliquots were put into three separate 15 ml polyethylene tubes. All of the samples were brought to pH 7 with 1 M NaOH. The entire procedure was repeated for pH 7 and pH 9. In the case of pH 9, the samples were returned to pH 7 with 1 N HCl. The chlorine study was repeated twice to ensure reproducibility.

**Chlorine dioxide disinfection**

Chlorine dioxide was assayed in a similar fashion as chlorine. The chlorine dioxide was generated on-site by adding 1 g of sodium chlorite to 1 liter of water. In a fume hood, 1.69 g acetic anhydride was added and the solution was brought to a pH of 7 with 5 M NaOH.

An initial dose of 1 mg/L chlorine dioxide was used, followed by the same pH and
time parameters set in the chlorine trial. Residuals were measured in a DR/2000 Direct Reading Spectrophotometer (Hach) using chlorine dioxide reagents (Hach). The chlorine dioxide study was repeated twice to ensure reproducibility.

**Ozone Disinfection**

Three hundred milliliters of MilliQ water containing 2 ml of aliquotted poliovirus was put into a 500 ml Pyrex glass bubbler fitted with a glass frit. One percent ozone was then bubbled through the solution at a rate of 1 liter per minute. The ozone was generated using an Orec Ozonator (Ozone Research & Equipment Corporation, Phoenix, Arizona) set at 0.2 AC amperes.

Three 10 ml aliquots were taken and put into three 15 ml polyethylene tubes before the ozonator was turned on. These samples were time point zero. The ozonator was turned on and stopped after 2 minutes. Three more 10 ml samples were taken, the glass frit replaced, and the ozonator turned on again. Samples were taken again at 10 minutes and 60 minutes. The ozone study was repeated twice to ensure reproducibility.

**Ultraviolet light disinfection**

Two hundred milliliters of MilliQ water were spiked with 1.5 ml of poliovirus stock. Four sterile 15 cm in diameter glass petri dishes were filled with the spiked water so that the depth did not exceed 5 mm. A platform was set up under an ultraviolet light source to a distance that gave a radiation intensity of 5 mW/cm². This intensity was measured using a Blak-Ray Ultraviolet Meter (Ultra-Violet Products, Inc., San Gabriel,
For time zero samples, the contents of one petri dish was poured into two 15 ml polyethylene tubes. The other three petri dishes containing the poliovirus-spiked water were placed on the platform and the ultraviolet source was turned on. After 2 minutes, one of the dishes was removed from the ultraviolet light source and aliquotted into two 15 ml conical tubes. The procedure was repeated at the 10 and 60 minute time points. The UV study was repeated twice to ensure reproducibility.

**Plaque assay for disinfected samples**

One hundred microliter aliquots from each dilution ($10^0 - 10^3$) were taken from each time point assayed. This was done for each disinfection study. All inocula were allowed to infect 25 cm$^2$ flasks of BGM cells, following the technique previously described.

**Digoxigenin probe**

One milliliter aliquots from each time point assayed from each disinfection study was placed into a sterile microfuge tubes. Eighty microliters of a 20 mg/ml stock of proteinase K was added to each microfuge tube, giving a final concentration of 400 $\mu$l/ml. Each tube was incubated for 60 minutes at 55°C.

Dilutions were then made of each time point by removing 70 $\mu$l of each and adding 630 $\mu$l of DEPC-treated water. The dilutions were made from $10^0$ to $10^3$. Five hundred
microliters of all dilutions were then spotted onto GeneScreen using a dot blot apparatus as described previously and baked for 2 hours at 80°C.
RESULTS

Sensitivity of Nonisotopic Nucleic Acid Probes

The use and sensitivity of isotopic nucleic acid probes has already been established. Nonisotopic probes offered the possibilities of being less expensive, less time consuming, and less dangerous. However, the sensitivity of the systems with poliovirus was not known. Three different nonisotopic assays were decided upon and the assays were adapted to use with the nylon membranes. Maximum sensitivity and minimal background were the two goals, since high background may obscure a weak positive. All final protocols for nonisotopic nucleic acid probe assays were evaluated for sensitivity a minimum of three times.

Determining optimum biotin-tailed probe concentration

The probe was prepared as stated in the materials section. The size of the probe fragments were between 100-2000 base pairs as confirmed by gel electrophoresis. Three different concentrations of probe were used on two sets of membranes, including: 0.5 \( \mu g/ml \), 2.5 \( \mu g/ml \), and 5 \( \mu g/ml \) in hybridization buffer. As seen in Table 1, the colormetric assay results did not vary with the concentrations of probe tested. The maximum cDNA seen was 100 pg of cDNA and \( 9 \times 10^5 \) viral plaque forming units. There was a light background on the membranes, possibly interfering with a low intensity signals.
Table 1

Optimization of biotin-tailed probe concentrations for the colormetric detection of homologous cDNA and poliovirus

<table>
<thead>
<tr>
<th>Probe concentration</th>
<th>Lowest cDNA detected</th>
<th>9 x 10^5</th>
<th>9 x 10^4</th>
<th>9 x 10^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 μg/ml</td>
<td>100 pg</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.5 μg/ml</td>
<td>100 pg</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5 μg/ml</td>
<td>100 pg</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
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Table 2

Optimization of the biotin-tailed probe concentrations for the chemiluminescent detection of homologous cDNA and poliovirus

<table>
<thead>
<tr>
<th>Probe concentration</th>
<th>Lowest cDNA detected</th>
<th>Number of PFU detected</th>
<th>9 x 10^5</th>
<th>9 x 10^4</th>
<th>9 x 10^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 µg/ml</td>
<td>100 pg</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.5 µg/ml</td>
<td>100 pg</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5 µg/ml</td>
<td>100 pg</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</table>
Chemiluminescent detection had comparable results as seen in Table 2. Most of the background was minimal, but present. A few dark patches of background were visible. Had they been where the signals were, the signals would have been obscured. In Figure 1, a picture can be seen of the biotin colormetric assay, while Figure 2 demonstrates visualization of any of the chemiluminescent assays.

**Determining optimum HRP probe concentration**

Six sensitivity strips were prepared. The probe concentrations tested included, 0.625 μg/ml, 1.25 μg/ml, and 2.5 μg/ml. One set was detected colormetrically (Table 3) and the other chemiluminescently (Table 4). In the colormetric set, 0.625 μg/ml probe detected as low as 100 pg of cDNA and 9 x 10⁴ poliovirus plaque-forming units. Background was present but minimal. A picture of colormetric HRP signals can be seen in Figure 3. Both 1.25 and 2.5 μg/ml probe concentrations detected down to 10 pg of cDNA and 9 x 10⁴ poliovirus plaque forming units. Background was present but minimal at the 1.25 μg/ml concentration and was beginning to interfere with signal at the 2.5 μg/ml concentration. Approximately the same results were seen chemiluminescently. The difference was that the background began to get too high at the probe concentration of 1.25 μg/ml.

**Determining optimal hybridization time for HRP probe**

Three different hybridization times were tested to determine if sensitivity increased quicker than background. In the colormetric assay (Table 5), no difference was seen
Figure 1

Colormetric detection by biotin-tailed probe

Note: Pink signal intensity has faded over time. Initially, 3 dots were able to be visualized.
Figure 2

Chemiluminescent detection by biotin-tailed, HRP, or digoxigenin probe

Note: Chemiluminescent detection can serve as a permanent record. Signal intensity does not decrease over time.
Figure 3

Colorimetric detection by directly conjugated HRP probe

Note: Signal intensity has decreased over time.
Table 3

Optimization of the HRP probe concentration for colormetric detection of homologous cDNA and poliovirus

<table>
<thead>
<tr>
<th>Probe concentration</th>
<th>Lowest cDNA detected</th>
<th>Number of PFU spotted</th>
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<tr>
<td></td>
<td></td>
<td>$9 \times 10^5$</td>
</tr>
<tr>
<td>0.635 $\mu$g/ml</td>
<td>100 pg</td>
<td>+</td>
</tr>
<tr>
<td>1.25 $\mu$g/ml</td>
<td>10 pg</td>
<td>+</td>
</tr>
<tr>
<td>2.5 $\mu$g/ml</td>
<td>10 pg</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 4

Optimization of the HRP probe concentration for chemiluminescent detection of homologous cDNA and poliovirus

<table>
<thead>
<tr>
<th>Probe concentration</th>
<th>Lowest cDNA detected</th>
<th>9 x 10⁵</th>
<th>9 x 10⁴</th>
<th>9 x 10³</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.625 μg/ml</td>
<td>100 pg</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1.25 μg/ml</td>
<td>10 pg</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2.5 μg/ml</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

*No signal could be seen due to interfering background*
between 1 and 2 hours. Sensitivity did not increase when the hybridization was extended to 16 hours, but background reached an unacceptable level. Even with the background, the positive signals were still visible. Hybridizations that proceeded for 1 and 2 hours gave the same signal intensity chemiluminescently (Table 6), detecting 10 pg of cDNA and $9 \times 10^4$ poliovirus plaque forming units. The sensitivity of the 16 hour hybridization could not be determined due to the high background.

**Increasing HRP molecules on probe**

In an attempt to increase sensitivity, the amount of HRP molecules was doubled from 4 to 8 on average for every piece of DNA. Membranes were run both colorimetrically and chemiluminescently with the two probes. Colorimetrically (Table 7), sensitivity did not increase but background did. The background did not interfere with visualization of the signal. In the chemiluminescent assay (Table 8), sensitivity could not be determined due to the extensive background.

**Determining optimal digoxigenin probe concentration**

Two sets of three sensitivity membranes were assayed using the probe concentrations 10 ng/ml, 50 ng/ml, and 100 ng/ml of hybridization buffer. One set was evaluated colorimetrically and the other by chemiluminescence. In the colorimetric assay (Table 9), 10 ng/ml of probe was able to detect 1 pg of cDNA and $9 \times 10^3$ poliovirus plaque-forming units with some background. At 50 ng/ml of probe, the sensitivity did not increase but background did to a high level, but the signal was still clear. At 100 ng/ml of
Table 5

Optimization of the HRP probe hybridization time for the colormetric detection of homologous cDNA and poliovirus

<table>
<thead>
<tr>
<th>Hybridization time (hours)</th>
<th>Lowest cDNA detected</th>
<th>Number of PFU spotted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9 x 10^5</td>
<td>9 x 10^4</td>
</tr>
<tr>
<td>1</td>
<td>10 pg</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>10 pg</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>10 pg</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 6

Optimization of the HRP probe hybridization time for the chemiluminescent detection of homologous cDNA and poliovirus

<table>
<thead>
<tr>
<th>Hybridization time (hours)</th>
<th>Lowest cDNA detected</th>
<th>$9 \times 10^3$</th>
<th>$9 \times 10^4$</th>
<th>$9 \times 10^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10 pg</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>10 pg</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

*Could not be determined due to interfering background*
Table 7

Optimization of the HRP probe enzyme to DNA ratio for chemiluminescent detection of homologous cDNA and poliovirus

<table>
<thead>
<tr>
<th>HRP:DNA</th>
<th>Lowest cDNA</th>
<th>9 x 10^3</th>
<th>9 x 10^4</th>
<th>9 x 10^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:4</td>
<td>10 pg</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>1:8</td>
<td>10 pg</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 8

Optimization of the HRP probe enzyme to DNA ratio for the chemiluminescent detection of homologous cDNA and poliovirus

<table>
<thead>
<tr>
<th>HRP:DNA</th>
<th>Lowest cDNA detected</th>
<th>9 x 10^5</th>
<th>9 x 10^4</th>
<th>9 x 10^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>4:1</td>
<td>10 pg</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>8:1</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

*Could not be determined due to interfering background
probe, the background was high enough to almost obscure the signal intensity.

Colormetric signal detection can be seen in Figure 4. In the chemiluminescent assay (Table 10), no increase in sensitivity was seen at 10 ng/ml. The background was too high to accurately visualize signal intensity with 50 ng/ml or 100 ng/ml.

**Analysis of environmental samples with cell culture, $^{32}$P-cDNA, $^{32}$P-ssRNA, and digoxigenin probes**

Twenty-three archived environmental samples that had previously been analyzed by $^{32}$P-cDNA probe and cell culture were selected. All environmental samples came from the sites described in the Materials section. Samples were spotted onto nylon membranes and analyzed by $^{32}$P-ssRNA and chemiluminescent digoxigenin probes. All results were then compared (Table 11).

Four samples were tested from the freshwater site. One was positive by plaque assay. The same one was highly positive by all probe methods. $^{32}$P-ssRNA and digoxigenin probes detected a second positive which cell culture and $^{32}$P-cDNA did not. Seven samples were tested from a tidal site upstream from a sewage treatment plant. Cell culture had one positive and the nucleic acid probes had two. The sample positive by cell culture was highly positive by nucleic acid probes. Five samples were assayed from the effluent of the sewage treatment plant. Cell culture and $^{32}$P-cDNA did not detect any positives, while $^{32}$P-ssRNA and digoxigenin probes had two positives. Seven samples were assayed from a salt-water source downstream from the sewage treatment plant. Neither cell culture, $^{32}$P-ssRNA, or digoxigenin probe detected any positives. One sample
Figure 4

Colormetric detection by digoxigenin probe

Note. Signal intensity has faded over time and background has increased.
Table 9

Optimization of the digoxigenin probe concentration for the colorimetric detection of homologous cDNA and poliovirus

<table>
<thead>
<tr>
<th>Probe concentration</th>
<th>Lowest cDNA detected</th>
<th>Number of PFU spotted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>9 x 10^4</td>
</tr>
<tr>
<td>10 ng/ml</td>
<td>1 pg</td>
<td>+</td>
</tr>
<tr>
<td>50 ng/ml</td>
<td>1 pg</td>
<td>+</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>1 pg</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 10

Optimization of the digoxigenin probe concentration for chemiluminescent detection of homologous cDNA and poliovirus

<table>
<thead>
<tr>
<th>Probe concentration</th>
<th>Lowest cDNA detected</th>
<th>$9 \times 10^4$</th>
<th>$9 \times 10^3$</th>
<th>$9 \times 10^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 ng/ml</td>
<td>1 pg</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>50 ng/ml</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

*Could not be determined due to interfering background*
Table 11

Analysis of environmental samples by cell culture for total cultivatable virus and $^{32}$P-ssRNA, $^{32}$P-cDNA, and chemiluminescent digoxigenin probes for poliovirus

<table>
<thead>
<tr>
<th>Sample location</th>
<th>Cell culture</th>
<th>$^{32}$P-cDNA</th>
<th>$^{32}$P-ssRNA</th>
<th>Digoxigenin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1/4</td>
<td>1/4</td>
<td>2/4</td>
<td>2/4</td>
</tr>
<tr>
<td>B</td>
<td>1/7</td>
<td>2/7</td>
<td>2/7</td>
<td>2/7</td>
</tr>
<tr>
<td>C</td>
<td>0/5</td>
<td>0/5</td>
<td>2/5</td>
<td>2/5</td>
</tr>
<tr>
<td>D</td>
<td>0/7</td>
<td>1/7</td>
<td>0/7</td>
<td>0/7</td>
</tr>
</tbody>
</table>

A = freshwater sample

B = saltwater sample taken upstream from water treatment plant and downstream from freshwater location

C = effluent from water treatment plant

D = saltwater sample taken downstream from water treatment plant
was positive by $^{32}$P-cDNA.

**Sensitivity of PCR-generated digoxigenin probe**

Digoxigenin probe was made with PCR using hapten-linked dUTPs. The sensitivity was determined by chemiluminescence and colorimetrically with the same concentration of plasmid derived digoxigenin probe. The results of the PCR generated probe were comparable in signal and sensitivity to the plasmid probe (Table 10).

**Background reduction of digoxigenin probes**

To reduce the background seen with digoxigenin probes, the concentration of SDS was increased in the hybridization buffer from 0.02% to 3% in an effort to nullify the charge of the nylon membrane. Secondly, the detergent Tween 20 was added to buffer 1. Two sets of two sensitivity strips were analyzed. The first set was given 10 ng/ml probe and the second 50 ng/ml. One set was visualized colorimetrically (Table 12) and the other by chemiluminescence (Table 13).

The background was reduced to zero with the 10 ng/ml probe both colorimetrically and with chemiluminescence. Sensitivity increased from 1 pg to 0.1 pg cDNA and from $9 \times 10^2$ to $9 \times 10^1$ poliovirus plaque forming units. The sensitivity did not increase with 50 ng/ml, but a minor degree of background appeared.

**Comparison of nucleic acid probes**

The final sensitivities of the probes assays were compared in Table 14. All work
Table 12

Background-reduced digoxigenin probe for the colormetric detection of homologous cDNA and poliovirus

<table>
<thead>
<tr>
<th>Probe concentration</th>
<th>Lowest cDNA detected</th>
<th>Number of PFU spotted</th>
<th>(9 \times 10^3)</th>
<th>(9 \times 10^2)</th>
<th>(9 \times 10^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 ng/ml</td>
<td>1 pg</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>50 ng/ml</td>
<td>0.1 pg</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
Table 13

Background-reduced digoxigenin probe for the chemiluminescent detection of homologous cDNA and poliovirus

<table>
<thead>
<tr>
<th>Probe concentration</th>
<th>Lowest cDNA detected</th>
<th>$9 \times 10^3$</th>
<th>$9 \times 10^2$</th>
<th>$9 \times 10^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 ng/ml</td>
<td>0.1 pg</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>50 ng/ml</td>
<td>0.1 pg</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 14

Comparison of nucleic acid probe assays for the detection of homologous cDNA and poliovirus

<table>
<thead>
<tr>
<th>Method</th>
<th>Lowest cDNA detected</th>
<th>9 x 10^3</th>
<th>9 x 10^4</th>
<th>9 x 10^5</th>
<th>9 x 10^6</th>
<th>9 x 10^7</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin</td>
<td>100 pg</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HRP</td>
<td>10 pg</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Digoxigenin</td>
<td>0.1 pg</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>^32P-ssRNA</td>
<td>*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

*Not tested
on the $^{32}$P-ssRNA probe optimization was done by Amy Moore (Moore and Margolin, 1992).

**In situ Hybridization Study**

**Cell adhesion with a high concentration of formalin**

Cells were fixed either at room temperature or 37°C for time points from 0 to 120 minutes with a 10% neutral buffered solution of formalin (Table 15). The cells were exposed to 100°C, incubated with or without probe, and developed colorimetrically. Since no virus was added, it was expected that there would be no positive signal. Cells were examined for cell adhesion and background. From previous experiments, it was determined that cells not properly fixed would produce background. When the cells were not fixed, no cells remained on the slide. At the time points, 15, 60, and 120 minutes, approximately 95% of the cells remained. A dark purple background precipitate was on all slides except for the 0 minute time point.

**Cell adhesion with low concentration of formalin**

The cell adhesion experiment was repeated with a 4% concentration of formalin (Table 16). All slides were fixed at room temperature. The zero minute time point had no cell adhesion. The 15 minute time point had an approximate 60% loss in cells. Both the 60 and 120 minute fixations yielded cell losses of about 5%. Background appeared as a light, grainy purple precipitate that was over the cells and on the glass where cells were
Table 15

Cell adhesion with a high concentration of formalin

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Temperature</th>
<th>% cell adhesion</th>
<th>Background with probe</th>
<th>Background w/o probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>RT</td>
<td>95%</td>
<td>C-D</td>
<td>B</td>
</tr>
<tr>
<td>15</td>
<td>RT</td>
<td>95%</td>
<td>C-D</td>
<td>B</td>
</tr>
<tr>
<td>15</td>
<td>37°C</td>
<td>95%</td>
<td>C-D</td>
<td>B</td>
</tr>
<tr>
<td>60</td>
<td>RT</td>
<td>95%</td>
<td>C-D</td>
<td>B</td>
</tr>
<tr>
<td>60</td>
<td>37°C</td>
<td>95%</td>
<td>C-D</td>
<td>B</td>
</tr>
<tr>
<td>120</td>
<td>RT</td>
<td>95%</td>
<td>C-D</td>
<td>B</td>
</tr>
<tr>
<td>120</td>
<td>37°C</td>
<td>95%</td>
<td>C-D</td>
<td>B</td>
</tr>
</tbody>
</table>

RT = room temperature

A = no background

B = light, grainy purple background

C = heavy, grainy purple background

D = heavy purple background
Cell adhesion with a low concentration of formalin

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% Cell adhesion</th>
<th>Background with probe</th>
<th>Background w/o probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>40%</td>
<td>B</td>
<td>A-B</td>
</tr>
<tr>
<td>60</td>
<td>95%</td>
<td>B</td>
<td>A-B</td>
</tr>
<tr>
<td>120</td>
<td>95%</td>
<td>B</td>
<td>A-B</td>
</tr>
</tbody>
</table>

A = no background
B = light, grainy purple background
C = dark, grainy purple background
D = heavy purple background
Determining optimum probe concentration for *in situ* hybridization

Four different probe concentrations were assayed to determine optimal signal vs. low background (Table 17). A dilution of poliovirus was added, allowed to incubate, and then the cells were fixed and probed. The negative control was 1 x PBS incubated in the second well of each slide rather than a poliovirus dilution.

At a probe concentration of 12.5 ng/ml, many cells had a moderate signal intensity while others showed no signal. Background consisted of a light, grainy purple precipitate. At 25 ng/ml probe concentration, the signal intensity of the cells increased without noticeably increasing the general background. Background did increase with 50 ng/ml probe concentration to the point where it started to obscure the difference between infected and noninfected cells. At 100 ng/ml probe, all cells looked positive for virus, regardless if they were in the cells inoculated with poliovirus or the negative control.

Decreasing antibody time for *in situ* assay

To reduce the time of the assay and possibly background, decreased antibody incubation times were assayed (Table 18) on cells infected with poliovirus. Times included 30, 60, 120, and the previous standard of 180 minutes. At 30 minutes, the signal inside cells was decreased along with the background level. The slides from 60, 120, and 180 minutes all looked approximately the same, having high intensity signals and a light, grainy purple background.
Table 17

Optimization of the *in situ* probe concentration for the detection of poliovirus

<table>
<thead>
<tr>
<th>Probe concentration</th>
<th>Well with virus</th>
<th>Well without virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5 ng/ml</td>
<td>3 B</td>
<td>0 B</td>
</tr>
<tr>
<td>25 ng/ml</td>
<td>4 B</td>
<td>0 B</td>
</tr>
<tr>
<td>50 ng/ml</td>
<td>4 C</td>
<td>0 C</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>4 D</td>
<td>0 D</td>
</tr>
</tbody>
</table>

0 = no positive signal  
1 = possible positive cells  
2 = light purple cells  
3 = moderate purple cells  
4 = dark purple signals  
A = no background  
B = light background  
C = moderate background  
D = heavy background
Table 18

Optimization of the antibody time in the *in situ* assay for the detection of poliovirus

<table>
<thead>
<tr>
<th>Antibody incubation (min)</th>
<th>Wells with virus</th>
<th>Wells without virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>3-4 A-B</td>
<td>0 A-B</td>
</tr>
<tr>
<td>60</td>
<td>4 B</td>
<td>0 B</td>
</tr>
<tr>
<td>120</td>
<td>4 B</td>
<td>0 B</td>
</tr>
<tr>
<td>180</td>
<td>4 B</td>
<td>0 B</td>
</tr>
</tbody>
</table>

0 = no signal  
1 = possible positive cells  
2 = light purple cells  
3 = moderate purple cells  
4 = dark purple cells  

A = no background  
B = light background  
C = moderate background  
D = heavy background
Determining the sensitivity of the *in situ* assay

Dilutions of poliovirus were inoculated onto cells, incubated, fixed, probed, and the number of positive cells counted (Table 19). In the first slide, 1000 poliovirus plaque forming units were applied to the first well and 1 x PBS to the second. The second slide received 100 plaque forming units and 1 x PBS to the control. Eight other slides received 10 plaque forming units to the first well and 1 x PBS to the second well.

The slide which received 1000 plaque forming units had a general purple haze to it. Many cells were dark purple. The control well did not have any purple cells. A slight haze was seen in the well which received 100 plaque forming units. The number of positive cells was too high to count. No cells were positive in the negative control. The number of positive cells could be counted in the wells which received 10 plaque forming units. One had 10 cells positive, three had 11 cells positive, and one had 12 cells positive. Three other slides had a greater than 95% cell loss and so were disqualified from the overall results.

**CHLORINE, CHLORINE DIOXIDE, OZONE, AND ULTRAVIOLET LIGHT DISINFECTION STUDY**

**Chlorine disinfection**

MilliQ water with a 1 mg/L chlorine residual was spiked with poliovirus and assayed by cell culture and digoxigenin probe at time points 0, 2, 10, and 60 minutes. The three pH values tested were pH 6 (Table 20), pH 7 (Table 21), and pH 9 (Table 22). Three separate trials were performed. This data is from one representative trial. At pH 6,
Table 19

Determining the sensitivity of the *in situ* assay

<table>
<thead>
<tr>
<th>Number of plaque-forming units applied</th>
<th>Number of positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>TNTC</td>
</tr>
<tr>
<td>100</td>
<td>TNTC</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>10</td>
<td>*</td>
</tr>
<tr>
<td>10</td>
<td>*</td>
</tr>
<tr>
<td>10</td>
<td>*</td>
</tr>
</tbody>
</table>

TNTC = too numerous to count  
* = cell loss
160 viruses were present at the zero time point. After two minutes, only 1 virus was detected by cell culture. No viruses were detected by cell culture at 10 or 60 minutes. The digoxigenin probe initially detected virus at a $10^{1}$ dilution of the original stock solution and remained at this detection level throughout the 60 minutes. At pH 7, the initial starting concentration for poliovirus plaque forming was 120 plaque forming units. By 2 minutes, only 1 plaque forming unit remained. No plaque forming units were present at the 10 and 60 minute marks. Detection by digoxigenin probe remained constant at $10^{1}$. At pH 9, 110 plaque forming units were seen at time 0. By 2 minutes, no plaque forming units were left. The digoxigenin probe detected down to $10^{1}$ of the stock solution again, but the signal intensity was markedly reduced compared to those obtained at pH 6 and pH 7.

**Chlorine dioxide disinfection**

MilliQ water with a 1 mg/L chlorine dioxide residual was spiked with poliovirus and assayed by cell culture and digoxigenin probe at time points 0, 2, 10, and 60. The three pH values tested were pH 6 (Table 23), pH 7 (Table 24) and pH 9 (Table 25). The assay was repeated twice. The data is a representative study. At pH 6, 130 plaque forming units were initially present. By 2 minutes, the number had decreased to 18. No plaque forming units were seen at the 10 and 60 minute time points. Digoxigenin probe detected as low as a $10^{1}$ dilution of the disinfected sample throughout each time point. At pH 7, 220 plaque forming units were present at time 0. No viruses were present at the 2 minute mark. Again, the digoxigenin probe signal intensity did not decrease. At pH 9,
Detection of poliovirus in water samples with chlorine disinfection at pH 6 by pfu and digoxigenin probe

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>pfu</th>
<th>$10^0$</th>
<th>$10^1$</th>
<th>$10^2$</th>
<th>$10^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>160</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>60</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*pfu = plaque forming units*
Detection of poliovirus in water samples with chlorine disinfection at pH 7 by pfu and digoxigenin probe

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>pfu</th>
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<th>$10^{-2}$</th>
<th>$10^{-3}$</th>
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<tbody>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
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<td>60</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

pfu = plaque forming units
Table 22

Detection of poliovirus in water samples with chlorine disinfection at pH 9 by pfu and digoxigenin probe

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>pfu</th>
<th>$10^0$</th>
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</tr>
</thead>
<tbody>
<tr>
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<td>110</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>60</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

pfu = plaque forming units
Table 23

Detection of poliovirus in water samples with chlorine dioxide disinfection at pH 6 by pfu and digoxigenin probe

<table>
<thead>
<tr>
<th>Time (min)</th>
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<th>$10^{-2}$</th>
<th>$10^{-3}$</th>
</tr>
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<tbody>
<tr>
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<td>+</td>
<td>-</td>
<td>-</td>
</tr>
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<td>2</td>
<td>18</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
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<td>60</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$pfu = \text{plaque forming units}$
Table 24

Detection of poliovirus in water samples with chlorine dioxide disinfection at pH 7 by pfu and digoxigenin probe

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>pfu</th>
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<th>10^-1</th>
<th>10^-2</th>
<th>10^-3</th>
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<tbody>
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<td>+</td>
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<td>-</td>
</tr>
<tr>
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<td>60</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

pfu = plaque forming units
Table 25

Detection of poliovirus in water samples with chlorine dioxide disinfection at pH 9 by pfu and digoxigenin probe

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>pfu</th>
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<th>$10^{-1}$</th>
<th>$10^{-2}$</th>
<th>$10^{-3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>180</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>60</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$pfu=\text{plaque forming units}$
180 plaque forming units were obtained initially and had decreased to 0 by 2 minutes. As in the chlorine study, digoxigenin probe intensity did not decrease over time, but the signal intensity was markedly reduced upon comparison to pH 6 and pH 7.

**Ozone disinfection**

MilliQ water was spiked with poliovirus, put into a glass bubbler, and exposed to 1% ozone at a flow rate of 1 liter per minute. At time points of 0, 2, 10, and 60 minutes, samples were taken and analyzed by plaque assay and digoxigenin probe (Table 26). The assay was repeated twice. The data is from one representative study. At 0 minutes, 6400 plaques were seen. By 2 minutes, this number was reduced to 2100. No infectious units were present at 10 minutes. In the digoxigenin probe assay, the initial sensitivity was as low as a $10^{-2}$ dilution and this level did not change over the 60 minutes.

**Ultraviolet light disinfection**

MilliQ water was spiked with poliovirus and removed at time points 0, 2, 10, and 60 minutes. Samples were assayed by cell culture and digoxigenin probe (Table 27). The assay was repeated twice. The data is from one representative study. Initially 5700 plaque forming units were present. After 2 minutes, the number had dropped to 1. One plaque forming unit was still present at 10 minutes, but none were present at the 60 minute mark. The digoxigenin probe detected as low as a $10^{-2}$ dilution throughout the 60 minutes without noticeably decreasing in signal activity.
Table 26

Detection of poliovirus in water samples with ozone disinfection by pfu and digoxigenin probe

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>pfu</th>
<th>$10^0$</th>
<th>$10^{-1}$</th>
<th>$10^{-2}$</th>
<th>$10^{-3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6400</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>2100</td>
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<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>10</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
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<td>60</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

pfu = plaque forming units
Table 27

Detection of poliovirus in water samples with ultraviolet light disinfection by pfu and digoxigenin probe

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>pfu</th>
<th>$10^0$</th>
<th>$10^{-1}$</th>
<th>$10^{-2}$</th>
<th>$10^{-3}$</th>
</tr>
</thead>
<tbody>
<tr>
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<td>+</td>
<td>+</td>
<td>-</td>
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<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>10</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>60</td>
<td>0</td>
<td>+</td>
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</tbody>
</table>

pfu = plaque forming units
DISCUSSION

Introduction to Viral Detection

The Safe Drinking Water Act of 1974 has mandated that potable water sources be free of substances, such as enteric viruses, which may have an adverse effect on public health (Cotruvo and Regelski, 1989). To accomplish this goal, Interim National Primary Drinking Water Regulations (INPDWR) were established within 6 months to set an existing standard while regulations were being finalized. The Environmental Protection Agency (EPA) then published the Advanced Notice of Proposed Rule Making (ANPRM), which included the facts which were known about the contaminant and the existing technology coupled with the potential regulations. After public comments were received, the EPA proposed the maximum containment levels (MCL) and the maximum containment level goals (MCLG). The MCL had enforceable standards, while the MCLG were nonenforceable health goals. After more public comments were received, the EPA finalized the regulations and the methods can be found in Standard Methods for the Examination of Water and Wastewater (1987).

For the detection of enteric viruses, the revised standard method for monitoring potable water quality established in the EPA's information collection rule (ICR) is cell culture. Cell culture, however, has many drawbacks. It is long and labor intensive, requiring a minimum of 4 weeks to pronounce a sample negative for enteric viruses. If a
sample has not produced cytopathic effects (CPE) in a cell line within 14 days, the cells are frozen, thawed, and the supernate is used as an inoculum for cells which must be incubated for another 14 days on fresh cells. In samples that do produce CPE, the procedure followed is as described for negative samples; however, the supernate is first passed through a 0.2 μm filter to ensure that CPE is not caused by a biologic contaminant. This type of labor intensity, along with the expense associated in preparing and processing such samples becomes a critical factor in the number of samples that can actually be assayed on a daily basis.

Another drawback is that many viruses, such as hepatitis A, may infect cells without showing cytopathic effects or may do so only after an extended length of time. Viruses that take an extended period of time to produce CPE may be confused with the cells death occurring from the exhausted supply of nutrients and serum components. Also, there is no universal cell line for all pathogenic viruses that may be found in fecally-contaminated water. Therefore, several different cell lines may be needed to assay for the desired set of viruses. Cell culture will detect the total cultivatable virus, which may even include animal viruses that are not of human origin and would not pose a health risk.

Nucleic acid probes, which detect viruses immobilized on solid support matrices such as nylon membranes, do not suffer from the previously mentioned cell culture drawbacks. Results can be obtained within hours to days depending on the type of assay, which will help limit the needed technician time or allow the technician to increase the number of samples. The need for cell culture flasks is also eliminated. Most dot blot apparatuses contain 96 wells, permitting the evaluation of multiple samples at the same
time. A membrane that has one sample applied is as easy to probe for viruses as a membrane that has 96 samples. All of these advantages work to lower the cost of a nucleic acid probe assay.

Another advantage of the nucleic acid probe assays is that one does not have to worry about other microbiologic organisms interfering with the results. As long as the probe is specific for the viruses, no false positives will occur due to contaminating organisms. The discrepancy which may appear between determining if certain cells are exhibiting CPE or naturally dying is also eliminated. Another advantage is that the homologous sequence used in a nucleic acid probe assay can detect all or some viruses, depending on the sequences used, as opposed to cell culture which needs different cell lines to detect different viruses.

The efficacy of isotopic probes to detect viruses in the environment has previously been demonstrated (Margolin, 1986). Nonradioactive probes offer the advantages of speed, ease of use, and reduction of hazards associated with radioactivity. Once isotopic probes are hybridized to their target and unbound probe washed away, it takes 48 hours for the signal to be visualized on X-ray sensitive film. Isotopic probes also suffer from the disadvantage that as time goes on, they become less sensitive due to their half-life. Therefore, isotopic probes have to be made and used within weeks, while nonisotopic probes can last months to years after they are made. Finally, isotopic probes require proper disposal of all solutions and utensils which come in contact with the radioactivity. The disposal of radioactivity can increase the cost of the probes use as well as present environmental hazards.
**Biotin-tailed probe**

The biotin-tailed probe detected as low as 100 pg of poliovirus cDNA and $9 \times 10^5$ poliovirus plaque forming units. Results were obtained within 24 hours, which is significantly lower than the possibility of 14 days for cell culture and 3 days for isotopic probes. Colormetric detection had a light pink signal which is not as easy to visualize as a darker color. Due to the light signal intensity, minor amounts of background could possibly obscure weak positive signals. Since the color fades over time, the membrane should be photocopied to preserve the results. In chemiluminescent detection, no increase in sensitivity was seen beyond the colormetric results. It does, however, provide a permanent record.

A major deficiency with this assay is that a maximum sensitivity of $9 \times 10^5$ plaque forming units may not be sensitive enough to qualify as an alternative to cell culture. Also when the plasmid is being degraded by DNase, it has to be ensured that the fragments are between 100-2000 base pairs in length. If the DNase treatment is allowed to incubate longer than 10 minutes or if the DNase concentration is too high, the fragment size range could be significantly lower, decreasing the specificity. Although the assay is reduced in time, it does involve a high number of steps and solutions which reduces the ease of use.

**HRP probe**

HRP directly conjugated to DNA has several advantages over the biotin-tailed probe assay. The most important advantage is an increase in sensitivity. The HRP probe
detected 10 pg of poliovirus cDNA and $9 \times 10^4$ plaque forming units, a one log increase over the biotin-tailed probe. A second advantage is that due to the higher concentration of probe in the hybridization mixture, the time of the assay was reduced to 6 hours as opposed to 24 hours. A third advantage is that due to a steric hindrance, two complementary HRP-labeled DNA probes are incapable of binding to each other. Therefore, the probe does not need to be denatured before use. Colormetrically, the signal is purple to brown and can be easily visualized. No increase in signal sensitivity is seen chemiluminescently. Slight background was present in both, but it did not interfere with interpretation. In an attempt to increase the sensitivity of the assay, the number of HRP molecules per DNA fragment was increased from 4 to 8. Sensitivity, however, stayed the same while background increased.

In making the probe, the only difficult step is sonication. Many laboratories are not equipped with a sonicator. Even when one is available, it has to be set up properly so that the sonicating tip is submerged deep enough in the solution to prevent bubbling, but not so deep that it touches the bottom of the eppendorf tube. Sonication generates a lot of heat, so the eppendorf tube must be submerged in an ice-water bath. The bath often heats up, melting the ice which can then allow the eppendorf tube to move. If the DNA is oversonicated, the fragments will be smaller and have less specificity. If the DNA is undersonicated, the fragments will be larger and will not conjugate as well to HRP. Once the probe is made, the assay is easy to perform, involving minimal steps and solutions.
Digoxigenin probe

The digoxigenin probe showed an increase in sensitivity by two logs over HRP and three logs over the biotin-tailed probe. The assay was able to detect 0.1 pg of poliovirus cDNA and $9 \times 10^2$ poliovirus plaque forming units. Assay time was 24 hours as opposed to 6 hours found in the HRP probe. However, it was still significantly faster than cell culture and isotopic probes. Another advantage of this system is that with the increase of SDS in the hybridization buffer from 0.02% to 3% and the addition of Triton X-100 to one of the washes, little background was present. The addition of SDS probably worked by minimizing the charge of the nylon membrane. It may also work as an RNase inhibitor.

Besides the advantage of higher sensitivity and lower background, the PCR digoxigenin probe can be used to detect poliovirus 1, 2, and 3, Coxsackie virus B1, B3, and B4, and echoviruses due to the sequence homology of the selected 149-mer at the 5' end. The identification of the particular enterovirus is less important than the knowledge that the water is contaminated. Another advantage is that the vector sequence is eliminated. When the vector is made into probe, the possibility exists that bacteria containing a plasmid with sequences homologous to the pVR104 vector will give a false positive.

The biotin-tailed and HRP probe assays could also be used to detect a variety of enteroviruses. However, the probe would have to be made by a sequence generated by PCR. If the entire sequence is used, only a small fraction of it would be specific for viral targets other than poliovirus. The concentration of those specific probe fragments would be inadequate for detection. Overall, the digoxigenin assay is more time consuming and
labor intensive than the HRP assay. However, it is less labor intensive than the biotin-tailed assay due to decreased number of steps and solutions.

**Assaying environmental samples with cell culture, $^{32}$P-cDNA, $^{32}$P-ssRNA, and digoxigenin probes**

Environmental samples were randomly chosen from a pool of samples with previously known cell culture and $^{32}$P-cDNA probe results. Samples were assayed by $^{32}$P-ssRNA and digoxigenin probes. The digoxigenin probe assay was chosen over the biotin-tailed probe and HRP probe because it had the highest sensitivity and lowest background. The chemiluminescent detection was performed rather than colorimetric because when environmental samples are applied via a dot blot apparatus to a nylon membrane, they leave a brown spot. This spot can interfere with interpretation of a colorimetric detection.

At the freshwater site, one out of four samples was positive by cell culture and $^{32}$P-cDNA, while two were positive by digoxigenin and $^{32}$P-ssRNA. The positive sample by cell culture gave a high intensity signal with the nucleic acid probes. The second positive was lower intensity but clearly positive. It cannot be proven whether the second positive had any infectious viruses present. However, the freshwater site was separated from the treatment plant by a waterfall, thus the detection of poliovirus cannot be due to sewage that has undergone disinfection. Therefore, the water must have been contaminated by another source containing fecal material. This contamination could be from a nonpoint source, such as runoff from leaking septic systems.

In the samples from the saltwater site downstream from the freshwater site, cell
culture detected one positive out of seven, while all probe assays detected two positives. The sample which was positive by cell culture had a high intensity signal on all probe assays. Viral contamination at this location may be due to the upstream freshwater site or effluent from the wastewater treatment plant. If the viruses are from the plant, it could not be determined whether the viruses have been adequately disinfected so as not to pose a health threat or whether a fraction of the population is still infectious.

No positive samples were detected from the effluent of the treatment plant by cell culture or $^{32}$P-cDNA probe, while two out of five were weakly positive by digoxigenin and $^{32}$P-ssRNA probes. Again, it cannot be determined whether these viruses have been adequately disinfected. In the saltwater site downstream of the plant, cell culture, $^{32}$P-ssRNA, and digoxigenin did not detect any positives, while $^{32}$P-cDNA detected one out of seven positive.

Even though one log of sensitivity separates digoxigenin and $^{32}$P-ssRNA probes, they exhibited a 100% correlation in the environmental samples. The correlation between these two assays with $^{32}$P-cDNA dropped to 82.6%. The correlation with cell culture was also 82.6%. Although nucleic acid hybridization assays lack similar sensitivity to cell culture for detection of laboratory-produced virus, they do possess the sensitivity needed for detection of contaminating enteric viruses in water samples (published in 1993). Noninfectious particles often contain a portion of the genome and so would be detected by nucleic acid probe but not by cell culture. Although noninfectious particles do not pose a health risk, they could serve as an indicator for fecal pollution and can therefore serve as a health warning in water sources considered potable, like groundwater and primary surface
water. One factor to take into consideration is that due to viral clumping, the aliquot assayed by one method may have virus present while a second aliquot assayed by another method may be free of virus. If a significant amount of viral clumping is occurring, the results may be skewed due to the low number of samples assayed.

**In situ Hybridization Assay**

In an *in situ* assay, a sample that may contain virus is allowed to incubate on a cell monolayer. The cell monolayer used must be permissive to the particular viruses being assayed. Once the virus enters the cell, the viral nucleic acid is replicated. Poliovirus RNA replication begins approximately one hour after infection and undergoes exponential growth for the next three hours (Baltimore, 1966). The appropriate incubation time for the viral sample allows adequate time for the adsorption of the virus to the cell, release of the nucleic acid, and highest degree of replication without having the majority of viral nucleic acid packaged in the viral protein coat.

The cells are fixed to stop growth, prevent cell loss, and loss of the viral nucleic acid from the cells during the probe procedure. Cells are then heated to destroy any potential viral nucleic acid secondary and tertiary structure which may interfere with probe binding. In the case of double stranded nucleic acid viruses, heating will denature the two strands. The probe solution is applied with carrier molecules, such as yeast tRNA, sheared salmon sperm DNA, and dimethyl sulfoxide (DMSO), which allow the probe to cross the cell membrane. After hybridization, unbound probe is washed away and then detected. Visualization of the signal often requires the use of a compound microscope.
In this study, poliovirus was the model virus. A period of 5 hours of sample incubation on BGMK cells was chosen by combing a viral adsorption/nucleic acid penetration time of 2 hours with a viral replication time of 3 hours. Different enteric viruses may require more or less incubation time as well as a different cell line, assuming a permissive cell line is known for the virus in question.

**Fixation of cells to a slide**

The first problem faced with the development of the *in situ* assay was to ensure adherence of the cells to a solid surface. Cells had to undergo extensive washings as well as exposure to 100°C temperatures. Early experiments ended unsuccessfully with the loss of all cells from the slide. Commercially-produced silanated slides (Digene) increase cell retention.

The amount of time and concentration of formalin fixation was also examined. When cells were fixed with 10% neutral-buffered formalin, the cell retention level was increased to 95% after 15 minutes of fixation. The cells were heated to 100°C for 5 minutes and probed with the digoxigenin probe as stated in the Methodology section. Even though no poliovirus was added, a dark purple precipitate covered the cells, including the slide where no cells were present. It was assumed that a 10% formalin solution could potentially enter the cell in such a high concentration to burst some cells and produce background.

When a 4% concentration of formalin was used (Jiang et al., 1989), the fixation time required for a 95% cell retention rate rose from 15 minutes to 60 minutes. The
background decreased dramatically compared to the high-concentration formalin experiment. Background consisted of a light, grainy purple precipitate covering the entire well. In a negative control well, probe was not applied. Background was lighter, but still present, indicating that the colormetric reaction itself gives a minor degree of background independent of nonspecific probe binding.

**Optimization of the *in situ* assay**

The optimal probe concentration was determined using the criteria of signal intensity and background level. A light purple precipitate was permitted because it did not interfere with interpretation of positive (infected with replicating poliovirus) versus negative (not infected with replicating poliovirus) cells. When the probe concentration was increased above 25 ng/ml, the level of background became unacceptable. When the concentration was below 12.5 ng/ml, the background did not decrease, but the signal intensity did. Therefore, 25 ng/ml was considered optimal.

Assay time was reduced without decreasing signal sensitivity by reducing the incubation time with the anti-digoxigenin antibody from three hours to one. No visible degree of background reduction was seen. When the antibody incubation was reduced to 30 minutes, a loss in signal intensity and background was seen.

**General consideration for the *in situ* assay**

The *in situ* assay was shown to detect approximately 0.9 plaque forming units. Increased sensitivity compared to the plaque assay may be due to polioviruses that contain
the appropriate sequence to code for early proteins which can aid in nucleic acid replication, but lack the appropriate code for an early or late protein responsible for viral protein coat formation. These viruses would replicate in situ but would not lyse the cell and, therefore, could not be detected by plaque assay or cell culture.

Many problems exist with the in situ assay. The first concern is cell loss. In the experiment to determine sensitivity, three of the ten slides had cell retention rates below 95%. In these cases, the assay would have to be repeated. Secondly, the in situ assay would have to be independently optimized for every virus being assayed for. A nucleic acid probe cocktail would not work because some viruses may require longer incubations for the viral nucleic acids inside of the cells to reach a detectable limit. A third problem is surface area on which the BGM cells adhere to. Each well on the silanated slides is 4 cm². The average amount of surface area assayed by cell culture is 250 cm². Therefore, cell culture has 80X the number of BGM cells than does the in situ assay. Increasing the number of slides assayed by in situ probe is fiscally impractical.

A final problem with the in situ assay is that it has already become obsolete with the introduction of in situ PCR (Bagasra, 1993). Under this procedure, cells could be grown to confluency on a slide, fixed, and amplified using an accessory to a thermocycler. A benefit to this assay is the ability to detect as low as one viral nucleic acid at a fixed time point. Also, panenteroviral primers or a cocktail of primers could be used to detect a variety of viruses at the same time on the same slide.
DISINFECTION STUDY

Chlorine disinfection

MilliQ water with a chlorine residual of 1 mg/L was spiked with poliovirus. At time points of up to one hour, aliquots were taken and the disinfection was stopped by the addition of Na$_2$S$_2$O$_3$. One aliquot was examined by plaque assay and the second by digoxigenin probe colorimetrically. The three pH levels assayed were 6, 7, and 9.

At pH 6 and pH 9, a two-log reduction was seen by 2 minutes. No plaque forming units were present at the 10 minute mark. However, the signal intensity of the digoxigenin probe did not decrease over the 60 minutes. At pH 9, no plaque forming units were present at 2 minutes, showing a minimum of a two-log reduction. In the digoxigenin probe assay, a reduction in signal intensity was seen even when the bleach was inactivated before the addition of virus. It is not known whether the high pH destroyed a portion of the viral nucleic acid or whether a byproduct may be lowering the sensitivity of the probe assay without disrupting viral infectivity.

Chlorine dioxide disinfection

Chlorine dioxide was assayed in a similar fashion as chlorine. At pH 6, a one log reduction was seen by 2 minutes. No infectious viruses were present by 10 minutes. At pH 7 and pH 9, no infectious viruses were present by 2 minutes, corresponding to a minimum of a two log reduction. No reduction in digoxigenin probe intensity was seen
with pH 6 and pH 7 time points over sixty minutes. A reduction in signal intensity was again seen with the pH 9 samples.

**Ozone disinfection**

One percent ozone was bubbled through MilliQ water spiked with poliovirus at a flow rate of 1 liter per minute. After 2 minutes, the number of plaques was reduced by two-thirds. No plaques were witnessed in the 10 minute sample. In the digoxigenin probe assay, the signal intensity did not decrease throughout the 60 minutes. Currently, ozone is not solely used for the production of potable water. Chlorine is usually added to provide residual protection.

**Ultraviolet light disinfection**

Petri dishes contained water spiked with poliovirus was placed under an ultraviolet light source at a distance to give an intensity of 5 mW/cm². A three-log reduction was seen by two minutes in the plaque assay. One infectious unit was present in both the 2 and 10 minute time points and no infectious viruses were present by 60 minutes. No signal intensity reduction was seen with the digoxigenin probe assay over 60 minutes. Similar disinfection rates were seen by Rodgers and coworkers when dealing with poliovirus disinfection at ultraviolet energies between 2 and 9 mW/cm² (Rodgers et al., 1983). Currently, ultraviolet light is only used as a tertiary treatment for potable water in the United States. Another disinfectant, such as chlorine would be added to provide residual protection.
Disinfection discussion

No correlation was seen between viral detection by the plaque assay upon comparison to the digoxigenin probe for any of the disinfectants. Therefore, the viruses are being inactivated but the particular piece of RNA that the probe is specific for is still present. If this assay were used at water treatment plants, adequately processed water could yield positive results by digoxigenin probes while there may not be infectious virus which could pose a health threat. The correlation between plaque assay and digoxigenin probe may be closer in treatment plants as opposed to laboratory experiments due to the uptake or degradation of viral RNA by other organisms. Viral RNA may also be degraded by the chemicals present in the water.

Because of the reduced expense and shorter time as compared to cell culture, nucleic acid probes on nylon membranes can be used as a rapid screening of treated waters. Samples positive by probe with viral nucleic acid would then be assayed by cell culture. Nucleic acid probes are the assay of choice to monitor potable source waters because it should be free of human enteric viral nucleic acid whether it is infectious or noninfectious. Any human enteric viral nucleic acid discovered can be deemed a potential health threat because it indicates a fecal contamination.
BIBLIOGRAPHY


Bailey, P. S. 1975. Reactivity of ozone with various organic functional groups important to water purification. First International Symposium on Ozone for Water and Wastewater Treatment. Proc. 101 Waterbury, CT.


APPENDIX A

PREPARATION OF MEDIA

Flake agar (2%)

2 g agar flake (Difco, Detroit, MI)

Bring up to 100 ml with MilliQ water. Autoclave 20 minutes. Leave at 50 °C until use.

Luria-Bertani (LB) Medium

10 g tryptone
5 g yeast extract
5 g NaCl

Bring to 1000 ml with dH2O and make sure pH is 7. Autoclave 20 minutes.

Luria-Bertain (LB) Agar

Same as medium, but add 15 g agar.

Medium 199 (2X)

1.96 g Medium 199 (Sigma)
0.13 g NaHCO3
0.95 g hepes
0.06 g glutamine
4 ml FBS (Gibco)
1 ml nystatin (Sigma)
1 ml Penicillin/Streptomycin (Sigma)
0.5 ml kanamycin (Sigma)
1.2 ml neutral red solution (Sigma)
1 ml 1% MgCl2

Bring to 100 ml with MilliQ water. Filter sterilize.
Minimal Essential Media (MEM) with 8% Fetal Bovine Serum
(Growth media)
2.65 g MEM (Sigma)
3.45 g L-15 Medium Leibovitz (Sigma)
5 ml Non-essential amino acids (10 mM of each amino acid)
(Whittaker Bioproducts, Inc., Walkersville, MD)
2 g Hapes (Sigma)
0.5 g NaHCO₃
5 ml Nystatin (10,000 units/ml)
(Sigma)
5 ml Penicillin/streptomycin (10,000 units pen. and 10 mg strep./ml)
(Sigma)
2 ml Kanamycin (Sigma)
40 ml Fetal bovine serum (Gibco)
* pH to 7.0. Bring up to 500 ml with MilliQ water and filter sterilize.*

Minimal Essential Media with 2% Fetal Bovine Serum
(Maintenance media)
Same as 8% MEM, except add 10 ml FBS instead of 40 ml.
APPENDIX B

ENZYMES

DNase I (Enzo)
0.5 mg/ml in 0.1 M MgCl₂

Proteinase K (Sigma)
Five milliliters of DEPC-treated water was added to 100 mg of proteinase K. The solution was stored at -20 °C until use.

RNase A (Sigma)
RNase A was dissolved in 10 mM Tris-HCl, pH 7.5 and 15 mM NaCl to a concentration of 10 mg/ml. The solution was boiled for 10 minutes, cooled on ice for 5 minutes, and aliquotted into 500 µl portions in sterile Eppendorf tubes.

Terminal deoxynucleotidyl transferase (Enzo)
80 units/µl in
100 mM K-cacodylate, pH 7.0
0.3 M KCl
5 mM 2-mercaptoethanol
50% glycerol
APPENDIX C

BUFFERS AND SOLUTIONS

BioBridge Labeling Molecule (Enzo Diagnostics, New York, NY)
50X concentrated in
0.15 M NaCl
0.015 M sodium citrate

Biotin blocking buffer
1X PBS
2% BSA
0.1% Triton X-100
5 mM EDTA

Biotin high salt washing buffer
10 mM potassium phosphate, pH 6.5
0.5 M sodium chloride
0.05% Triton X-100
0.1% BSA
1 mM EDTA

Biotin hybridization solution
50% formamide
4X SSPE
5X Denhardt's solution
0.1% sodium dodecyl sulfate (SDS)
0.1 mg/ml denatured salmon testes DNA, sonicated and denatured (Sigma)

Biotin predetection buffer
2X SSC
0.1% BSA
0.05% Triton X-100
1 mM EDTA
Biotin prehybridization solution
- 50% formamide
- 4X SSPE
- 5X Denhardt's solution
- 0.1% sodium dodecyl sulfate (SDS)
- 0.1 mg/ml denatured salmon testes DNA, sonicated and denatured (Sigma)

Biotin substrate mixture
- 25 µl of 1% H₂O₂
- 25 µl AEC solution
- 1 ml of 0.1 M sodium acetate, pH 4.9

Biotin wash solution 1
- 0.5X SSC, 0.1% SDS

Biotin wash solution 2
- 0.2X SSC, 0.1% SDS

Biotin wash solution 3
- 0.1X SSC, 0.1% SDS

Chloroform
Chloroform was made by mixing 24 parts chloroform with 1 part isoamyl alcohol

DAB reaction buffer
- 15 mM Tris-HCl, pH 7.6
- 0.05% NiCl₂
- 0.5 µg/ml diaminobenzidine
- 1 drop per 50 ml of 10% H₂O₂

Denhardt's solution, 100X
- 2% polyvinylpyrrolidone 360
- 2% sodium phosphate buffer, pH 7.4
- 2% bovine serum albumin (BSA)

DEPC-treated water
- 0.1% diethyl pyrocarbonate (Sigma) in MilliQ water
  Allow to stand overnight in 37 °C waterbath.
  Autoclave for 20 minutes.

Detek 1-hrp complex (Enzo)
Detek dilution buffer, 10X
10X PBS
10% BSA
50 mM EDTA

Digoxigenin buffer 1
100 mM Tris-HCl, pH 7.5
150 mM NaCl

Digoxigenin buffer 2
2% bovine serum albumin (Boehringer Mannheim) dissolved in digoxigenin buffer 1

Digoxigenin buffer 3
100 mM Tris-HCl, pH 9.5
100 mM NaCl
50 mM MgCl₂

Digoxigenin colormetric solution
45 µl NBT solution (Boehringer Mannheim)
35 µl X-phosphate (Boehringer Mannheim)
in 10 ml digoxigenin buffer 3

Digoxigenin dNTP labeling mixture (Boehringer Mannheim)
1 mM dATP
1 mM dCTP
1 mM dGTP
1 mM dTTP
0.65 mM Dig-dUTP

Digoxigenin hexanucleotide mixture (Boehringer Mannheim)
10X concentrated hexanucleotide reaction mixture

Digoxigenin hybridization solution
5X SSC
0.5% BSA (Boehringer Mannheim)
0.1% N-lauroylsarcosine, Na-salt
0.02% SDS

Digoxigenin wash solution 1
2X SSC
0.1% SDS
Digoxigenin wash solution 2
  0.1X SSC
  0.1% SDS

DNase I (Enzo)
  0.5 mg/ml DNase I in 0.1 M MgCl₂

DNase I dilution buffer (Enzo)
  10 mM Tris HCl, pH 7.5 and 1 mg/ml BSA

EDTA
  73.05 g disodium ethylenediaminetetraacetate 2H₂O
  NaCl pellets to adjust pH to 8.0 (10 g approximately)
  500 ml total volume in dH₂O

HRP hybridization buffer
  50% formamide
  100 μg/ml salmon testes DNA, sonicated and denatured (Sigma)
  5X Denhardt’s solution
  3.6X SSC
  1% SDS
  10% dextran sulfate, sodium salt

In situ hybridization buffer
  5 ml formamide
  2 ml 20X SSC
  0.2 ml of 50X Denhardt’s solution
  0.5 ml of 10 mg/ml salmon testes DNA, sonicated and denatured (Sigma)
  0.25 ml of 10 mg/ml yeast tRNA (Sigma)

Lysis Buffer
  25 mM Tris-HCL, pH 7.5
  10 mM EDTA
  15% sucrose
  2 mg/ml lysozyme

NBT (Boehringer Mannheim)
  75 mg/ml nitroblue tetrazolium salt in 50% dimethylformamide
Phenol

Phenol was prepared by mixing with 0.1% 8-Hydroxyquinoline. An equal volume of 1 M Tris-HCl, pH 8 was added and mixed for 15 minutes. Once the layers separated, the upper phase was removed and the pH checked. The protocol was repeated until the pH of the phenol was above 7.8. After removal of the last aqueous phase, 0.1 volume of 0.1 M Tris-HCl, pH 8.0 with 0.2% β-mercaptoethanol was added.

Phenol/Chloroform

The phenol and chloroform were prepared as previously described and mixed together 1:1.

SSC, 20X
87.65 g NaCl
44.1 g sodium citrate
Final pH 7.0 and final volume 500 ml with dH2O

SSPE, 20X
3.6 M NaCl
0.2 M sodium phosphate buffer, pH 7.4
0.02 M EDTA

TE
10 mM Tris-HCl, pH 8.0
1 mM EDTA

Terminal deoxynucleotide transferase (Enzo)
80 units of terminal transferase
100 mM K-Cacodylate, pH 7.0
0.3 M KCl
5 mM 2-mercaptoethanol
50% glycerol

Terminal transferase dilution buffer (Enzo)
50 mM K-cacodylate, pH 7.0
5 mM 2-mercaptoethanol
1 mg/ml BSA
1 mM EDTA

Terminal transferase reaction buffer (Enzo)
666 mM K-Cacodylate, pH 7.0
3 mM 2-mercaptoethanol
TTP (Enzo)
   1.0 mM TTP in 50 mM Tris HCl, pH 7.5

X-Phosphate (Boehringer Mannheim)
   50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt