ABSTRACT

Norwalk Virus (NV) and other human enteric viruses in fecally contaminated waters pose a human health risk that is inadequately characterized due to the limitations of current virus detection methods. Standard methods for virus concentration from water using electropositive adsorbent filters have not been tested for NV recovery because there is no convenient infectivity assay system and because the beef extract (BE/G) used to elute the adsorbed viruses inhibits virus detection by reverse transcriptase-polymerase chain reaction (RT-PCR) amplification. Hence, we tested five alternative eluents compatible with RT-PCR (the amino acids arginine, asparagine, glycine, lysine, or threonine) for elution of NV as well as poliovirus type 1 (PV1) and coliphage MS2 (MS2). All three viruses seeded into dechlorinated tap water were adsorbed to Virosorb 1MDS filters and then eluted with either BE/G or an alternative eluent to achieve a 100-fold volume concentration. All three viruses were assayed by endpoint dilution RT-PCR and PV1 and MS2 were assayed by infectivity using the plaque technique as well. With all of the amino acids except threonine, elution efficiency for all three viruses was comparable to or greater than that by BE/G. Virus recovery efficiencies were 50 - 75% over a pH range of 7.0 - 9.8. Viruses were concentrated another 10-fold by polyethylene glycol precipitation, which was also compatible with molecular detection techniques and
gave recovery efficiencies of 10-100%. These results indicate that modified filter adsorption-elution methods using amino acid eluents and subsequent secondary concentration by polyethylene glycol precipitation provide efficient concentration of NV and other viruses in water for subsequent assay by RT-PCR.
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INTRODUCTION

Waterborne outbreaks of acute gastroenteritis are a common and significant threat to public health throughout the world. Many of these outbreaks are caused by fecal contamination harboring enteric viruses, such as Norwalk virus, and enteroviruses such as poliovirus type 1. Furthermore, a viral etiology has been hypothesized for endemic, community-wide waterborne gastroenteritis (58). Because the community water supply implicated in endemic gastroenteritis and many of the water supplies that were implicated in outbreaks contained acceptable levels of coliform bacteria, there is a need for better strategies to assess the virological quality of water. The proposed Information Collection Rule (ICR) proposes the use of an adsorption-elution technique for concentrating viruses from large volumes of water by adsorbing them to and then eluting them from an electropositive filter. A portion of the beef extract filter eluate would be assayed for indicator coliphages such as MS2. Information on the efficiency of recovery of NV and coliphages like MS2 by Virosorb 1MDS filters is limited. Therefore, there is a need for better characterization of Norwalk virus and coliphage MS2 recoveries from these filters. Furthermore, the beef extract used for eluting viruses from 1MDS filters inhibits virus detection by reverse transcriptase-polymerase chain reaction (RT-PCR) amplification. Hence, if efforts are made to detect
NV in water using 1MDS filters, an alternative eluting solution compatible with RT-PCR must be found.

The focus of this research is to develop a rapid, simple, and reliable technique for concentrating viruses from large volumes of water that is compatible with RT-PCR detection. Five alternative filter elution solutions as well as beef extract were tested over a pH range of 7.0-9.8 for virus recovery from Virosorb 1MDS filters, using dechlorinated tap water samples containing three test viruses, Norwalk virus, poliovirus type 1, and coliphage MS2. Viruses within filter eluents were secondarily concentrated another 10-fold by polyethylene glycol (PEG) precipitation. PEG concentrations ranging from 6% to 10% and sodium chloride concentrations ranging from 0.1N to 0.3N were tested in order to identify optimum conditions for virus recovery. Norwalk virus was assayed by RT-PCR, while poliovirus type 1 and coliphage MS2 were assayed by both RT-PCR and infectivity. Virus recovery efficiencies were then computed and compared for the different eluents.
STATEMENT OF RESEARCH OBJECTIVES

The overall objective of this research is to develop a rapid, simple, and reliable method for concentrating viruses from water that is compatible with molecular detection methods for viruses in water. The specific objectives are listed below.

- Assess and optimize conditions for the adsorption-elution concentration of Norwalk Virus (strain 8FIIa), poliovirus type 1, and male-specific RNA coliphage MS2 in tap water using the ViroSorb 1MDS filters

- Identify an alternative eluting solution that will be compatible with reverse transcriptase-polymerase chain reaction (RT-PCR) detection of the enteric viruses recovered from ViroSorb 1MDS electropositive adsorbent filters

- Assess and optimize conditions for secondary concentration of Norwalk virus, poliovirus type 1, and coliphage MS2 in alternative amino acid filter eluents by polyethylene glycol precipitation

- Using virus-seeded laboratory samples of water, demonstrate that low numbers of the three test viruses can be
concentrated by adsorption to electropositive Virosorb 1MDS filters, elution with alternative amino acid eluents, secondary concentration by polyethylene glycol precipitation and detection by RT-PCR amplification of viral RNA and oligoprobe hybridization to confirm virus detection.
LITERATURE REVIEW

A. Public Health Significance of Viruses in Water

Outbreaks of acute gastroenteritis are a significant threat to public health today, even though in many countries there are control measures for disposing of human wastes and treatment of water. Some of these outbreaks can be traced to known bacterial, viral or protozoan pathogens. However, a large number, about one-half of those reported in the United States, can not be linked to specific etiologic agent. Many of these outbreaks of unknown etiology are thought to be attributable to viral agents (8,26,32). Many of the important viral agents of gastroenteritis have been difficult, if not impossible, to detect in water because of the lack of suitable methodology.

Many methods have been proposed and tested for detection of enteric viruses in water, but no single method has been universally accepted or proven effective for all viruses. Problems in virus detection stem directly from the viruses and the water. Waters vary in the types, concentrations, and conditions of viruses present and in water quality characteristics. These factors influence the quantity of water that can be sampled, and the efficiency of virus recovery by a specific concentration method.

Enteric viruses are those whose initial site of attack within the host is the cell lining of the alimentary tract,
resulting in their excretion in large numbers in feces. Enteric viruses are feco-orally transmitted not only by direct and indirect personal contact and by fecally contaminated food, but also by fecally contaminated water. They are responsible for many outbreaks of water-borne gastroenteritis. These viruses lack a lipid envelop, are stable at the low pH levels that are encountered in the stomach, are persistent in the environment, and are generally shed in great numbers in the feces of infected individuals (63). There are more than one hundred different types of enteric viruses, which increases the difficulty of identifying the agent or agents responsible for disease outbreaks.

Enteric viruses are classified into taxonomic groups by differences in their antigenic, morphologic, and chemical characteristics. Of the outbreaks attributed to known enteric viral pathogens, those of the families picornaviridae (genus enterovirus and rhinovirus), reoviridae (genus rotavirus and reovirus), and caliciviridae are documented threats to public health. These viruses and others can exist in fecally contaminated waters for extended periods of time (81) and cause infections through drinking water, bathing water, and contaminated shellfish (3,23,36,40,42,59).

Al. Small Round Structured Viruses

For many years, doctors diagnosed patients with hyperemesis hiemis, or "winter vomiting sickness", without
knowing the agent that was responsible for this generally mild, self-limiting gastroenteritis. In 1969, there was an outbreak showing the symptomatology of this disease in an elementary school in Norwalk, Ohio, and three years later, the causative agent was revealed by electron microscopy in the stool samples associated with the outbreak (37). This virus, the so-called Norwalk virus, is a virion roughly 27 nm in diameter containing a polyadenylated single-stranded RNA genome of about 7.7 kb (31,32). Norwalk and similar viruses, including the Snow Mountain and Hawaii agents, are now generally accepted to be members of the Caliciviridae family (22). They are prototype strains of the small, round structured viruses causing acute gastroenteritis.

Outbreaks of Norwalk virus gastroenteritis are extremely difficult to trace as to the etiologic agent in part because the incubation time is only 15-50 hours, and the symptoms generally persist for only 2-3 days (9). Symptoms of infection include nausea, vomiting, diarrhea, abdominal cramps, and headache lasting between 12 and 60 hours (12). Attack rates of greater than 50% are common in outbreaks of gastroenteritis caused by Norwalk virus and related agents. Such high attack rates are consistent with low infectious doses needed for clinical illness (9). Until recently, viral gastroenteritis outbreaks were confirmed by direct observation of virus particles using electron microscopy. Norwalk and related viruses are shed in relatively low numbers (10⁴ to 10⁶ particles/gram of feces) compared to
rotaviruses, which is close to the detection limits of electron microscopic techniques (12). Detection is improved by reacting the virions with specific antibodies to create immune aggregates that can be concentrated by centrifugation. This also increases the specificity of virus detection. Outbreaks can also be characterized by seroconversion, which increases specific antibody titers of infected individuals within several weeks after the infection (20,21).

Payment, et. al. (59) showed that an overwhelming majority of randomly selected individuals in a prospective household study of water-associated gastroenteritis were positive for IgG antibodies to Norwalk virus. They concluded that at some point in their life, they had been exposed to the virus, possibly from drinking water sources. It was also shown that the percentage of individuals testing positive for antibodies to Norwalk virus increased with increasing age, that there was no age-dependent factor associated with Norwalk, as with rotavirus, and that individuals of all ages are susceptible to infection (59).

Because of the high attack rate, Norwalk virus is particularly threatening to individuals within confined (institutional) settings or living in close quarters. Examples include aboard cruise ships (29) or naval vessels (67), resorts (40), and nursing homes (16). Such individuals are exposed to infectious doses of the virus through drinking water, foods, and contact with other individuals showing clinical symptoms. Many outbreaks have been traced to food
preparation, such as contaminated ice (8,38) and infected food-handlers (29,37,48). Other outbreaks have been associated with shellfish harvested from waters impacted by fecal contamination (15,23,27,39,53). Another, less characterized route of exposure to Norwalk virus, is through aerosolized virus particles coming from the vomitus of individuals showing clinical symptoms of the infection (9,10,37,66). The clinical symptoms produced by Norwalk virus are generally not life-threatening in most healthy individuals, but Norwalk and related viruses cause many outbreaks throughout the world each year.

A2. Enteroviruses

The enteroviruses are thought to cause many outbreaks of acute gastroenteritis each year and they are readily detected in fecally contaminated water and foods. The enterovirus group includes polioviruses (types 1-3), the coxsackie viruses (24 serotypes of type A and 6 serotypes of type B), echoviruses (34 serotypes), and the higher numbered enteroviruses, which include types 68-71 and 72. Symptoms associated with the enterovirus group range from fever, headache, and diarrhea to more serious conditions such as paralytic poliomyelitis, cardiomyopathies, and encephalitis.

A2a. Hepatitis A Virus

Hepatitis A virus, formerly enterovirus 72, is responsible for many outbreaks of enteric illness each year
in the USA as well as the rest of the world. Hepatitis A virus is a member of the picornaviridae family and has been assigned to a genus separate from other enteroviruses due to its genetic differences. Because infected individuals can shed virus particles in great numbers (as high as $10^9$ particles/ gram of feces) and the particles are extremely resistant to inactivation in the environment, HAV is considered a serious risk in fecally contaminated water and food.

Hepatitis A virus is a 27 nm icosahedrally shaped, nonenveloped particle with a single strand of RNA of positive polarity. The virus particles have a very stable protein capsid, allowing it to pass through the stomach and into the small intestine, where it is thought to initiate infection. The virus then spreads via the lymphatic system and bloodstream, targeting the liver, its main site of pathology.

A2b. Poliovirus

Polioviruses are members of the picornaviridae family and the enterovirus genus and is comprised of three antigenic types. The virus particles are roughly 28 nm in diameter, nonenveloped, and contain a single strand of RNA of positive polarity. All three types of poliovirus, as well as the other enteroviruses, are spread through the feco-oral route of transmission. The viruses have been detected in fecally contaminated drinking and bathing waters (61) and
shellfishing areas (3). Their role in epidemic and endemic waterborne disease is poorly documented despite their presence in water and shellfish. Both live attenuated and killed vaccines are available for poliovirus and international efforts are under way to achieve worldwide eradication. However, there are no widely available vaccines against the other enteroviruses.

There are a wide range of clinical symptoms associated with infections by the three types of poliovirus. Generally, infections take one of two courses: the abortive form or the paralytic form. The abortive form, also called aseptic meningitis, is usually very mild with no paralysis and rapid recovery. The paralytic form, or paralytic meningitis, is very serious, where fever, nausea, and vomiting is accompanied by pain in the spine, limbs, and trunk.

There is still debate as to which vaccine is best to eradicate poliovirus throughout the world (65,73,74). There are advantages associated with each. The live attenuated approach allows the virus to replicate within the body so there is a strong immune response producing immunity for life. The other key advantage is that it is inexpensive and administrable orally. The advantage of the killed vaccine is that it is more easily transported and is more stable, allowing it to be administered to people in remote, rural communities. There are suggestions and evidence to support the use of combinations of both the killed and the attenuated vaccines for improved results (77,78).
A3. Other Enteric Viruses

There are many other enteric viruses that are also a risk to public health, such as rotaviruses, reoviruses (reoviridae family), parvoviruses, astroviruses, adenoviruses, and other caliciviruses besides Norwalk virus. As with the previously discussed taxonomic groups, a potential route of exposure to these viruses is through ingestion of fecally contaminated water or shellfish. Many of these viruses are quite different in morphology, composition, and structure; however, clinical symptoms caused by these viruses tend to have some similarities. Most of these viruses are associated with acute gastroenteritis characterized by headache, vomiting, fever, and diarrhea. Some of these viruses are more typically associated with respiratory disease, such as the reoviruses, adenoviruses types 1-39, and several of the parvoviruses. Enteric viruses especially important in many outbreaks of gastrointestinal disease in infants and young children are the rotaviruses and adenoviruses types 40 and 41, but many fewer cases are reported among adults.

B. Indicators of Fecally Contaminated Waters

In order to protect the public health, it is necessary to monitor water supplies for microbial contaminants in a timely and efficient manner in order to prevent the spread of infectious diseases. Ideally, screening would be applied to
sufficiently large volumes of drinking water to assure consistent quality. Because this is not a realistic possibility, the alternative is to examine a more manageable number of representative samples on a regular basis to judge the overall quality of the water. It would be ideal to examine water for human pathogens. This, too, is not realistic because most detection methods for human pathogens are too technically difficult and unreliable, require too much time, and are too costly to use on a frequent basis. Because of this, it is necessary to have indicator organisms that will be present in waters when and where human pathogens are present and that can be detected rapidly, reliably, and inexpensively. Indicators of fecal contamination should be applicable to waters impacted by either human or animal fecal contamination, because human pathogens of concern that are transmitted through the feco-oral route may be present in both types of fecal sources. Ideal fecal indicators would have the following characteristics (64):

1. Applicability to all water types.
2. Absence in unpolluted water.
3. Presence in sewage and polluted waters during pathogenic contamination.
4. Presence in contaminated waters in numbers greater than pathogens of interest.
5. Numbers correlated with the quantity of pollution.
7. Longer survival times in the environment than pathogens of interest.
8. Facile detection by as simple and rapid laboratory analysis as possible consistent with high degree of accuracy.
9. Exhibition of constant biological characteristics.
10. Harmless to man.
Additionally, an ideal indicator would quantitatively predict the risk of waterborne enteric disease by showing a dose-response relationship with the disease.

Bl. Bacterial Indicators

For many years, coliform bacterial indicators have been used to index the quality of water. Coliform bacteria belong to the Enterobacteriaceae which include aerobic and facultative anaerobic gram negative rods which are non-spore forming and ferment lactose with gas production within 48 hours at 35°C. Total coliforms include the genera, Escherichia, Klebsiella, Enterobacter, Serratia, and Citrobacter; however, Klebsiella, Serratia, and Citrobacter are less than ideal indicators because they are not exclusive to the intestinal flora and can be found widely in the environment. Fecal coliforms have proven to be better indicators of fecally contaminated waters, and are separated from total coliforms by elevating the temperature of incubation to 44-45°C. In waters impacted by fecal contamination, fecal coliforms general comprise most of the total coliforms.

Coliform bacteria are still used today to assess the quality of water. However, there have been many outbreaks of non-bacterial gastroenteritis from waters meeting the current coliform bacteria standards. This casts doubt as to whether the current indicators and standards for them have the ability to adequately protect public health from enteric
viral pathogens that may be present in low numbers in drinking waters and other vehicles the environment. Because of this, attention is being focused on alternative indicator organisms such as *Escherichia coli*, *Clostridium perfringens*, *Enterococci*, *Bifidobacterium* spp. and *Bacteroides* spp., and bacteriophages.

B2. FRNA Coliphages

Bacteriophages are viruses that infect and use bacterial cells as their hosts. It has been suggested that bacteriophages of common enteric bacteria would be better indicators of fecal contamination and of the presence of enteric viruses than are indicator bacteria such as coliforms. This is because bacteriophages are themselves viruses and have similar morphology, size, and composition as the viral pathogens of interest.

Coliphages are a heterogeneous group of bacteriophages that use *E. coli* as their host. There are six different groups, or families, of coliphages that are classified as Groups A-E. Groups A-D infect host cells by attaching directly to the cell wall of the bacteria that they infect and are termed somatic coliphage. Groups E and F infect only those bacteria that have sex pili, and are termed male-specific. Sex pili are produced only at certain temperatures by the bacteria and are used for genetic exchange. Group E coliphages are RNA based and Group F are DNA based. The Group E coliphages, or male-specific RNA coliphages, have
been proposed as good indicator organisms. Within the Group E coliphages, there are four different subgroups, or serotypes. Serotypes I and IV are generally associated with animal fecal contamination, while serotypes II and III are generally associated with human fecal contamination (19). Serotype II is an exception because high numbers have been associated with swine fecal contamination.

Coliphage MS2 is a male-specific, RNA based coliphage that belongs to the serogroup I. It is roughly 24 nm in diameter and is comprised of a single strand of RNA. Many studies have been done to compare coliphage MS2 with enteric viruses, and it has compared favorably. The availability of rapid and reliable detection methods for male-specific coliphages also contributes to their potential as indicators for enteric viruses.

C. Concentration of Viruses from Water

Very few environmental waters contain sufficient numbers of pathogens to be directly detected by current methods. Pathogens can generally be detected in raw wastewater, but for most other waters, pathogens that may be present must be concentrated from large volumes. A number of methods have been proposed for concentrating enteric viruses from large-volume environmental samples. The most widely used and accepted method is concentration by adsorption to and elution from microporous filters. Other methods include:

precipitation with salts and organic compounds (55),
ultrafiltration (13), and hydroextraction/dialysis. These methods have proven useful for a second step of virus concentration after initial concentration by adsorption to and elution from filters.

Filters for virus adsorption and elution can be classified into one of two types depending upon the electrochemical properties of the filter used: negatively charged or positively charged. Both methods rely on the fact that virus particles are hydrophilic colloids having both negatively charged and positively charged functional groups on their surfaces. Most viruses that are electrostatically adsorbed to other surfaces can be desorbed or exhibit a negative charge at neutral and basic pH conditions, and a positive charge at acidic pH conditions. Virus particles electrostatically adsorb to other charged surfaces by electrostatic interactions and can be released when the pH of the solution is changed to make both surfaces the same charge, thereby causing electrostatic repulsion. Generally, viruses adsorb to other surfaces at acidic pH where the viruses are either less electronegative or become electropositive, and may desorb from other surfaces at alkaline pH where the viruses and the adsorbent become electropositive. Elution of adsorbed viruses is also facilitated by the presence of an agent that competes with the virus for adsorption sites.
C1. Negatively Charged Filter Method

Negatively charged filter methods for virus concentration rely on an electropositive bridge to efficiently bind the negative virus particle to the negative filter material. Most negatively charged filters are composed of either fiberglass or cellulose esters with or without a polymeric resin binder (71). Wallis, et. al. (80) found that adsorption of viruses to negatively charged nitrocellulose filters could be greatly enhanced by the addition of trivalent and divalent salts after the pH of the sample had been adjusted to 3.5. The salts that they used were AlCl$_3$ as the trivalent salt and MgCl$_2$ as the divalent salt. Later, they decided to use AlCl$_3$ because much less (100-fold) was needed for efficient adsorption (80).

There are several advantages and disadvantages associated with the use of negatively charged filters. Disadvantages for using negatively charged filters include having to precondition the water before filtering the sample. There is the need for additional hardware and chemicals, and there is the possibility of virus inactivation by lowering the pH of the solution before and during filtration. An important advantage in using the negatively charged filters is their low cost. Some researchers have chemically treated negatively charged filter material with cationic polymers in order to enhance their virus adsorbing properties without the need to precondition the sample water (60).
C2. Positively Charged Filter Method

Positively charged filters have been comprised of a number of different materials, having characteristic chemical, hydraulic, and dirt-holding characteristics. Initially, Sobsey and Jones (69) found that asbestos-cellulose filters could adsorb poliovirus over a wide range of pH with no modification of the sample. They also showed that electropositive, cellulose-diatomaceous earth "charge modified" resin filters could concentrate poliovirus over a wide pH range (71). Sobsey and Glass (70) reported an electropositive pleated cartridge filter containing a double layer of thin-sheet fiberglass-surface modified resin filter material that could efficiently and reliably adsorb small numbers of poliovirus from seeded tap water with no additional salts over a wide pH range. This filter was called the Virozorb 1-MDS and is widely accepted and used today for screening of large volumes of water for human viruses.

Positively charged filters have the advantage of being easy to use because they adsorb viruses directly. There is no fear of virus inactivation because the water sample remains at ambient pH. Disadvantages include the high price of the filters and the fact that humic and fulvic acids, which are present in many environmental waters and are negatively charged, reduce the efficiency of these filters to concentrate viruses that may be present in the water samples (25,26,72). There have been studies done with other filters
and adsorbent material in an attempt to find lower cost alternatives to the positively charged Virosorb LMDS filters (50,56,57,68).

C3. Virus Elution from Filters

Many solutions have been evaluated for eluting viruses from both positively and negatively charged membrane filters. Generally, virus elution depends on weakening the electrostatic and hydrophobic interactions between the viruses and the filters so that the viruses will be released into the eluting solution. High pH buffers were first used as eluting solutions, and many others, including basic amino acids, casein, tryptose phosphate broth, nutrient broth, beef extract, and urea, have since been tested for their use as eluents (6,18,34,56,62,68,69,70,71,72). Additives, such as Tween 80, a nonionic detergent, have also been shown to aid in virus elution from membrane filters.

Beef extract, supplemented with 0.05M glycine and adjusted to pH 9.0 to 10, has been the most commonly used solution for eluting viruses from Virosorb LMDS filters in recent years. Beef extract contains proteinaceous constituents which may compete with viruses for adsorption sites on the surface of the filters. This, along with the elevated pH, makes this an effective virus eluent. Beef extract is also compatible with secondary concentration methods such as organic flocculation (55). Two disadvantages to using beef extract as a virus eluent are that the elevated
pH at which it must be used may effect virus infectivity and
the proteinaceous constituents within the beef extract are
inhibitory to molecular detection methods for viruses.

Basic amino acids have shown promise as eluents for
viruses from membrane filters (18). Glycine at pH 10.5 to
11.5 was used as the eluent in early virus concentration
methods. Farrah and Bitton (18) compared several amino
acids, including 3% solutions of glycine, arginine, lysine,
glutamic acid, and aspartic acid, for elution efficiency of
poliovirus type 1 adsorbed to negatively charged membrane
filters. They concluded that neutral and basic amino acids
were relatively good eluents, and acidic amino acid solutions
were poor eluents. Beef extract was included in these
experiments, and arginine, lysine, and glycine gave
comparable virus elution efficiencies.

Many other useful eluting solutions include basic amino
acids in combination with other constituents. The most
commonly used eluting solution contains beef extract along
with glycine. Shields, et. al. (68) used 0.2M sodium
trichloroacetate with 0.2M lysine to elute several
enteroviruses from epoxy-fiber glass filters. Hepatitis E
virus was concentrated and detected by molecular methods
after concentration by adsorption to membrane filters and
elution with urea-arginine phosphate buffer (34).

Amino acid solutions appear to be very promising eluents
for recovery of enteric viruses from the surface of membrane
filters. Several appear to give comparable recovery
efficiencies to the commonly used beef extract solution. Amino acid solutions are well characterized and are compatible with molecular detection methods, allowing for detection of culturable and nonculturable enteric viruses.

C4. Secondary Concentration of Filter Eluents with Polyethylene Glycol

Virus concentrations may still be low in filter eluates after concentration by adsorption to and elution from Virosorb LMDS filters. Therefore, a secondary concentration step should be included for efficient concentration and detection of viruses from large volumes of water. There have been several methods used to further concentrate viruses in filter eluents, including inorganic and organic precipitation (55), ultrafiltration (13), and precipitation by polyethylene glycol (46, 47, 79, 83).

Polyethylene glycol (PEG) is a chemically inert, nontoxic, water-soluble synthetic polymer that can precipitate a number of proteins. PEG is generally used in conjunction with sodium chloride in order to create a phase separation, with the proteins of interest in the precipitate (7). There are two differing theories as to the mechanism of action for PEG, that by Atha and Ingham and that by Lee and Lee.

Atha and Ingham (2) contend that PEG acts as an "inert solvent sponge" that sterically excludes proteins from solution, effectively increasing their concentrations until
precipitation occurs. The dynamics of this process is very similar to "salting out". Protein size, concentration, charge, and the initial ionic strength of the solute will effect precipitate formation.

Lee and Lee (41) contend that unfavorable protein surface charges produce an unfavorable thermodynamic effect with the solubilized PEG which causes it to be excluded from the "protein zone". Because of this, at appropriately high concentrations of polymer, protein precipitation or crystalization occurs. More highly charged or hydrophobic proteins will be more easily precipitated than those of lesser charge.

PEG precipitation is a valuable method for concentrating small amounts of virus that may be present in environmental samples because it will not interfere with the infectivity of the virus and is compatible with molecular detection techniques. Only trace amounts are left in the pellet, which was proven by Yamamoto et. al. (82), when they used \(^{14}\text{C}\) labeled PEG and measured to see if the \(^{14}\text{C}\) was in the pellet. The trace amount of PEG that is left can be readily separated by filtration or by density gradient purification.

Another advantage that is gained from this type of recovery technique is that extensive pH manipulation is not required (46). In a series of experiments by Lewis and Metcalf (46), this method proved to be more effective than organic flocculation techniques for recovery of several important enteric viruses. The flexibility of this procedure
allows for minimal laboratory equipment and a staff with minimal training in order to have efficient virus and bacteriophage recoveries from moderate to large volume samples (4,14,30,49).

D. Detection Methods of Viruses in Water
D1. Infectivity Assay

Historically, a very useful method to detect and quantify viruses in water is by infectivity assay. Infectivity assays give the most useful information relative to human health because viruses are detected through their ability to infect susceptible host cells. Viruses detected by infectivity are those posing a threat to public health because they could potentially infect an individual and cause disease. Virus particles that are not able to infect cells due to a faulty protein coat, inactivation of their genetic material, or some other reason are not detected by this method. A major limitation of this technique is that different viruses attack different cells within the body, so it is necessary to identify host cells that can be grown in culture for detection of the viruses of interest. Many enteric viruses have been tested for their ability to grow in different cell lines that can be isolated and grown in culture. However, for some of the more important enteric viruses, a cell line that will support their growth has not been identified.
There are two cell culture methods for isolating and quantifying enteric viruses in environmental samples. The first involves inoculating a portion of the sample into cells growing in liquid medium culture dishes to see if any viruses present will cause cytopathic effects (CPE) and cell death over several days or weeks of observation. Generally, primary cells that have been recently isolated and that have not been maintained in culture for long periods, are used because they tend to be more sensitive to virus infection. Some continuous cell lines have been found to be nearly as sensitive as primary cells for enteric virus isolation. A second method for virus isolation and assay involves inoculating a portion of the sample into a layer of cells, allowing the viruses to adsorb, and adding an agar overlay. A dye that is taken up by the cells is added to the agar medium. When viruses are present, they will cause localized areas of virus infection. Eventually, these areas will appear as clear zones within the cell monolayer called plaques. These plaques can be counted and the number of infectious viruses present can be calculated. The plaque assay is an extremely useful technique for quantifying infectious virus particles.

A limitation of these techniques is the high cost of the cultures, the need for a high level of technical skill, and the high cost of cell culture facilities and equipment. Another problem encountered when attempting to isolate viruses from environmental samples are many faster growing
bacteria that will invade the culture media and ruin the virus assay. Generally, this can be overcome by the addition of antibiotics or other chemicals that will inhibit the growth of the bacteria.

D2. Molecular Assay

Molecular detection of viruses is another useful approach for monitoring viruses in water. Because there are generally low numbers of enteric viruses present in environmental water samples, direct hybridization (gene probe) assays are rarely acceptable for detection and quantification of these viruses. Direct hybridization assays are not sensitive enough for detection of the low numbers of enteric viruses that may be present in environmental waters.

Another approach to molecular detection is based on amplification and detection of the nucleic acid (genetic material) within the virion. The most widely used nucleic acid amplification technique is polymerase chain reaction (PCR) to amplify the double-stranded viral DNA or RNA. In many cases, the viral genetic material is single-stranded RNA, and a prior step involving reverse transcription must be performed in order to form a double-stranded molecule that can then be amplified by PCR. This combined method for amplifying single-stranded viral genomic RNA is termed reverse transcriptase-polymerase chain reaction, or RT-PCR. Steps for this include the following: (i) heating the virus particles to denature the capsid and expose the viral genomic
RNA, (ii) adding random bases and reverse transcriptase enzyme in order to form a complimentary strand, (iii) the addition of primers complimentary to sequences in each strand of the double-stranded nucleic acid that are separated by several tens to hundreds of nucleotides, and then (iv) a series of heating and cooling steps to successively synthesize a new double strand from each initial double strand, thus amplifying a particular region exponentially. The RT-PCR product that is formed can then be separated by size using agarose gel electrophoresis, and visualized by staining with ethidium bromide.

The RT-PCR method is especially useful for detection of fastidious viruses for which there is no cell culture method available (35,52). It is also less time-consuming and no more costly than cell culture would be. Disadvantages of this method include the need for technical skill in nucleic acid methods and the specialized equipment used to perform the detection methods. The PCR method is very sensitive (75,76), with theoretical detection limits of one virus particle possible, however, this method will detect all virus particles for which the genomic region to be amplified is intact. This amplified region is generally only 100-500 bases within a genome that may be thousands to tens of thousands of bases in length. That means that this method will detect viruses from which the nucleic acid has already been released, that may not be intact, and that may have a damaged. These forms of viruses may no longer be infectious.
A confirmatory test that can be performed to insure that the amplified genetic material, or amplicon, is actually what was expected, is hybridization or gene probing. This is performed by transferring a portion of the amplified DNA to a positively charged membrane filter, fixing the amplicon to the membrane by UV irradiation or baking in a vacuum oven, and adding fragments of a complimentary single stranded nucleic acid that will attach by complimentary base-pairing to the amplicon. The complimentary single stranded nucleic acid, or gene probe, is labeled with a radioactive or other chemically reactive marker, so that it can be visualized. By performing RT-PCR and hybridization (gene probing), the amplified region of the target genetic material can be detected and confirmed. The amplicon will contain the primers, whose sequence is known, and an internal sequence complimentary to the gene probe, also of known sequence. This approach provides confidence that the genetic material of the virus of interest is being detected.

D3. Other Assays

Other potential assays to detect viruses in water rely on direct observation of the virus particles or immuno-detection using antibody interactions with the protein capsid of the virus. One such detection method is simply direct observation by electron microscopy. A second more specific technique than just electron microscopy is immune electron microscopy (IEM), where antibodies to the virus react
specifically with them to make the virus particles easier to identify. IEM is a serological assay for the virus, because only those viruses specific to the antibody will react (1,17,44,45). A limitation of both of these techniques is the detection limit. Most electron microscopes scan a field approximately $10^{-6}$ m wide and therefore $10^5$ to $10^6$ virus particles must be present in order to be detectable (11).

Other immunoassays also rely on attachment of antibody to antigen, the virus capsid. Both radioimmunoassays and enzyme immunoassays have been used to quantify viruses or their antibodies (20,21,24,33,43,51,54). The only difference between the two methods is the basis of detection of the antigen-antibody reaction: one relies on detecting radioactive label and the other uses colorimetric detection of an enzyme label. Limitations of these methods include the need for specialized reagents (specific antibodies to the target), and for RIA, the use of radiolabeled reagents (11). The enzyme immunoassay was developed after the radioimmunoassay in order to alleviate the need for working with radioactive reagents, and both were found to have similar, detection limits (28). Using colorimetric detection, the intensity of the color corresponds to the amount of antigen in the sample. A main limitation of these assays is their detection limit, which is about $10^4$-$10^5$ virus particles.

All of the methods previously discussed aside from infectivity assays and nucleic acid amplification assays have
similar limitations of high detection limits. The numbers of enteric viruses present in environmental water samples are generally very low, which renders these detection and quantification methods of little use for these samples. Only infectivity assays and nucleic acid amplification assays are sensitive enough to detect the low levels of enteric viruses that may be present in environmental water samples.
METHODS AND MATERIALS

A. Viruses and Host Cells

A1. Preparation of Norwalk Virus (8FIIa Strain)

Norwalk virus used in these experiments was obtained from a composite stool sample generated during a Norwalk virus human volunteer study. Small parts of frozen stool were chipped off and resuspended 10% w/v in phosphate-buffered saline (PBS). After approximately two hours of incubation in the PBS at 4°C, virus was extracted from the sample by organic solvent extraction with either chloroform or trichlorotrifluoroethane (Freon). One part organic solvent was added to two parts stool suspension, vigorously vortexed for two minutes, and subjected to centrifugation at 5000 x g for 20 minutes at 5°C. The aqueous supernatant was removed and placed at 4°C until it could be assayed by endpoint dilution reverse transcriptase-polymerase chain reaction (discussed later).

Additional virus was obtained by adding a volume of PBS to the pellet equal to the supernatant removed and subjecting it to vortexing and centrifugation as before. The aqueous supernatant layer was collected and retained. The pellet was serially extracted in this manner six to seven times. Supernatant extracts were saved and stored at -70°C until used for experiments.
A2. Propagation of Poliovirus Type 1 (LSc Strain)

The poliovirus used for these experiments was propagated in Buffalo Green Monkey Kidney (BGMK) cells. Virus stocks were inoculated into >90% confluent layers of BGMK cells in 850 cm² roller bottles at a multiplicity of infection of 0.3 PFU/cell. Viruses were allowed to adsorb for 60 minutes at 37°C on a Belco roller apparatus, after which maintenance media was added to the bottles and they were returned to the roller apparatus. After approximately 48 hours, the cells in the roller bottles exhibited >90% cytopathology and the virus was harvested. The roller bottles were frozen at -70°C and thawed three times to lyse any remaining cells and to release viruses from the cellular matrix.

The cell lysate was mixed with Freon at a ratio of two parts cell lysate to one part solvent. This mixture was homogenized using a Sorval Omnimixer for two minutes and then subjected to centrifugation at 5000 x g for 20 minutes at 5°C. The supernatant was collected and stored at 4°C until titered by plaque assay. Once titered, the virus extracts were frozen at -40°C in 10 mL aliquots until used for experiments.

Additional poliovirus was extracted from the cell debris of the initial cell lysate pellet with a volume of phosphate-buffered saline equal to the volume of the initial supernatant. This mixture was homogenized for two minutes and subjected to centrifugation. The supernatant was collected and stored at 4°C until titered by plaque assay.
Once titered, the virus extract in PBS was frozen at -40°C in 1 mL aliquots until used for experiments.

A3. Propagation of Bacteria Coliphage MS2

Bacteriophage MS-2 used in these experiments was obtained from the American Tissue Culture Collection (ATCC 15597-B1) and was propagated in the bacterial host cell E. coli C-3000 (ATCC 15597) using the standard double agar layer (DAL) method. Top agar tubes with an agar concentration of 0.75% were inoculated with 0.1 mL of host and 0.1 mL of a ten-fold dilution of phage, and poured on top of 1.5% bottom agar plates. The plates were then incubated at 37°C for 24 hours. To those plates showing confluent lysis of the bacterial lawn, 5 mL of phosphate-buffered saline was added and the top layer was scraped from the bottom layer of agar. The bacteriophages in this mixture were then separated from the remaining cell debris and agar by organic solvent extraction. One part chloroform was added to two parts bacteriophage mixture and homogenized in a Sorval Omnimixer for two minutes. This mixture was then subjected to centrifugation at 5000 x g for 20 minutes at 4°C in order to recover the supernatant containing the phage, which was then stored at 4°C until titered. After titering by plaque assay, 2 mL aliquots were frozen at -40°C until needed for experiments.
B. Experimental Design

Bl. Water Samples

Chapel Hill tap water drawn from a faucet in room 3206 McGavran-Greenberg Hall was dechlorinated with 50 mg/L sodium thiosulfate and used for experiments. The water was then pH adjusted to pH 6.0 with constant stirring using 0.1M and 1.0M hydrochloric acid. The experiments were performed under a laminar flow hood. Norwalk virus, poliovirus-1, and coliphage MS2 were added to the water with constant stirring to a final concentration of approximately $10^4$-$10^5$ PFU (PCRU for Norwalk virus)/mL. The seeded water was mixed for approximately five minutes before being filtered.

B2. Filter Material

Double layers of Virosorb 1MDS electropositive microporous filters (Cuno, Meriden, CT) in stainless steel filters holders were used for these experiments. Two series of experiments performed: initial experiments tested the efficiency of the alternative eluents using poliovirus-1 and further experiments tested Norwalk virus, poliovirus-1, and coliphage MS2 for adsorption efficiency and elution efficiency using alternative eluents. Initial poliovirus experiments were performed using 47 mm diameter filters, and subsequent experiments were performed using 25 mm diameter filters in order to conserve limited stocks of Norwalk virus. Filters were placed in the stainless steel filter holders and autoclaved before being used for experiments.
For the initial experiments with poliovirus to test alternative eluents, 500 mL of water was filtered through 47 mm diameter filters. For subsequent experiments with Norwalk virus, 200 mL of water was filtered through 25 mm diameter filters. These volumes are equivalent to 100 L of water filtered through 10-inch long, pleated 1MDS cartridge filters. A flow rate of water through the filters of 1.5 - 3.0 mL/minute/cm², which was equivalent to 25 - 50 mL/minute, was chosen because this flow rate was used in previous experiments with these filters (69).

B3. Alternative Eluting Solutions

Five different alternative eluents were used at three different pH levels to test for recovery of the three test viruses from Virosorb 1MDS filters. The conventional eluent for the Virosorb 1MDS filters is an alkaline beef extract-glycine solution (pH 9.8). As alternatives, 0.5M solutions of arginine, asparagine, glycine, lysine, and threonine were tested at pH 9.8, pH 8.5, and pH 7.0. The pH of each solution was adjusted using 10N and 1N sodium hydroxide and the solutions were autoclaved. Each eluting solution was stored at 4°C until used for experiments. Experiments on poliovirus were performed by filtering 500 mL of water, spiked with approximately $10^4$ PFU/mL of poliovirus through double layers of 47mm diameter Virosorb 1MDS filter material. Viruses were adsorbed at pH 6.0, and eluting solutions were tested at pH 9.8, 8.5, and 7.0. Elution protocol consisted
of 2 successive elutions of 5 mL each. Viruses present in each elution volume and the filtrate were quantified by plaque assay.

**B4. Secondary Concentration by Polyethylene Glycol (PEG) Precipitation**

Virus eluents from previous adsorption/elution experiments were combined and pH adjusted to 7.2. Additional virus (= 10^4 PFU/PCRU for Norwalk) was added to each eluent to compensate for any loss due to storage. Arginine, lysine, and glycine were tested for PEG precipitation using PEG concentrations ranging from 6% to 10% and sodium chloride concentrations ranging from 0.1N to 0.3N.

After eluents were pH adjusted and additional virus was added, the samples were separated into 6 mL volumes. PEG and NaCl were then added and allowed to precipitate at 4°C overnight. Concentrations tested were 6% PEG/0.1N NaCl, 6% PEG/0.3N NaCl, 8% PEG/0.1N NaCl, 8% PEG/0.3N NaCl, 10% PEG/0.1N NaCl, and 10% PEG/0.3N NaCl.

After overnight storage, samples were pelleted at 13,800 x g for 15 minutes. Supernatents were removed and the pelleted virus was resuspended in 1/10 volume phosphate-buffered saline for subsequent plaque assay and/or molecular assay. Both the pellets and the supernatents were assayed and compared to determine PEG precipitation efficiency.
C. Virus Quantification by Infectivity Assay

C1. Poliovirus-1 (LSc Strain)

Plaque assays were performed using BGMK cells in 60 mm dishes grown to >95% confluency at 37°C and 5% carbon dioxide atmosphere. The cell growth media was aspirated from the dishes and 0.1 mL of 10-fold dilutions of sample were added. Viruses were allowed to adsorb to the cells for 60 minutes in the incubator with agitation every 10 minutes. After the adsorption period, cell maintenance medium, supplemented with 0.75% bacto-agar and 1.5% neutral red, was added to the cells and allowed to solidify. The cells were then incubated for approximately 48 hours and plaques were counted; they were re-counted after 72 hours.

C2. Coliphage MS2

Coliphage MS2 titers were determined by plaque assays using the double agar layer method (DAL) and E. coli C-3000 bacterial host cells. Top agar tubes with an agar concentration of 0.9% were inoculated with 0.1 mL of host and 0.1 mL of a ten-fold dilution of phage sample, and poured on top of 1.5% bottom agar plates. The plates were incubated inverted at 37°C, and plaques were counted after 24 hours and 48 hours.

D. Virus Quantification by Molecular Assay

Reverse transcriptase-polymerase chain reaction and oligoprobe hybridization (RT-PCR-OP) was used to quantify
Norwalk virus, poliovirus type 1, and coliphage MS2 in these experiments. RNA-PCR kits from Perkin Elmer-Roche (Alameda, CA) were used throughout these experiments. Manufacturer's instructions were followed, except the reaction volume for reverse transcription (RT) was increased from 20 µL to 30 µL to accommodate a 10 µL virus sample. Temperature cycling was performed in a Perkin Elmer Cetus thermal cycler (model 480, Norwalk, CT). The temperature profile for the RT step consisted of 99°C for 5 minutes to release the RNA from the virions, 25°C for 20 minutes to allow the tubes to be removed from the machine and reverse transcriptase and RNA inhibitor to be added to the tubes (these are not heat stable), 42°C for 60 minutes to allow annealing of random hexamers and to synthesize the first strand of cDNA, and finally 99°C for 5 minutes to inactivate the enzyme. After chilling, Taq polymerase and primers were added to the samples for PCR amplification. The temperature profile for PCR with the primers used for each of the three viruses consisted of 40 cycles, each cycle consisting of 95°C for 1.5 minutes, 55°C for 1.5 minutes, and 72°C for 1.5 minutes. A 15 µL portion of the PCR product was analyzed by electrophoresis on a 1.7% agarose gel and visualized by UV light after being stained with ethidium bromide.

The PCR product was then Southern transferred from agarose gels to positively charged nylon membranes in 0.4M sodium hydroxide. The cDNA was bound to the membrane by UV cross-linking for 3 minutes. Oligoprobes were prepared using
a Genius non-radioactive end-labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, Indiana) to label the 3' terminal end with digoxigenin-dUTP using terminal transferase and purifying by alcohol precipitation. Oligoprobe hybridizations were performed in a Hybaid Mini 6 hybridization oven (model H9360, Woodbridge, NJ) at 55°C according to the instructions provided in the Boehringer Mannheim Biochemicals kit. Immunological detection of PCR-oligoprobe hybrids was performed using an anti-digoxigenin alkaline phosphatase antibody conjugate and an enzyme-catalyzed colorimetric reaction.

The primers and oligoprobes used to detect the 8FIIa strain of Norwalk virus used in these experiments amplified the genomic region encoding the viral polymerase. A PCR product of 260 bases was amplified by the primers and the internal oligoprobe confirmed a 21 base pair region in the amplicon. The 5' primer used was CAAATTATGACAGAATCCTTC, the 3' primer used was GAGAAATATGACATGGATTGC, and the internal oligoprobe used was ATGTCATCAGGGTCAAAGAGG. The 5' primer contained the same sequence as the viral RNA, and the 3' primer contained a sequence complimentary to that of the viral RNA. The internal oligoprobe was synthesized in the (+) sense orientation so that they would hybridize with cDNA or PCR product only, and not with viral genomic (+) sense RNA.

RT-PCR for detection of poliovirus type 1 followed the same protocol and temperature profile as that used for Norwalk
virus, and the PCR product was analyzed by electrophoresis with visualization by UV light. Oligoprobe hybridization was not performed. The primers for poliovirus amplified a 197 base pair region from the highly conserved 5' nontranslating region of enteroviruses. The 5' primer was CCTCCGGCCCCCTGAATG and the 3' primer was ACCGGATGGCCAATCCAA.

Coliphage MS2 was quantified by RT-PCR in the same way as Norwalk virus and poliovirus. PCR product was analyzed by 1.7% agarose gel electrophoresis and visualized with UV light. Oligoprobe hybridizations were not performed. The primers to detect coliphage MS2 amplified a 233 base product within the replicase region of the genomic DNA. The 5' primer used was TAAGCTACGGGAGGCCGAATG and the 3' primer used was GCTTGTTCAGCGAACTTCTTG.
RESULTS

A. Recovery of Poliovirus Type 1 with Alternative Eluents

In order to apply molecular detection methods to viruses concentrated on Virosorb 1MDS filters, alternative eluents to beef extract were explored. Because beef extract has proven to be inhibitory to molecular detection techniques, the alternative eluents must be chemically well characterized and not inhibitory to molecular detection.

Results for poliovirus recovery using beef extract and alternative elution media are summarized in Table 1. Data are combined values from the two elutions. Four alternative amino acid eluents, lysine, arginine, asparagine, and glycine, gave virus recoveries approximately equal to that of beef extract at pH 9.8 (Figure 1), with recovery efficiencies of 60-67%. Threonine was discontinued for testing in subsequent experiments due to less efficient virus recovery at pH 9.8 (45%). As shown in Table 1, the four other alternative eluting solutions gave 2-3 times higher recoveries than BE/G at pH 8.5 and 7.0 (59-90% versus 27-30%) from 47mm diameter filters.

B. Coliphage MS2 Adsorption-Elution Experiments

Male-specific, RNA coliphages, such as MS2, have been proposed as indicators of enteric viruses and the proposed
C. **PCRability of Norwalk Virus in Beef Extract**

Beef extract has proven to be inhibitory to molecular detection methods used for enteric viruses. In order to test the inhibitory effects associated with beef extract, serial dilutions of two beef extract mock eluents were made and each dilution was seeded with equal amounts of Norwalk virus. The one mock eluent was from a filter that previously had water passed through it using the same experimental protocol as previously described for virus adsorption-elution experiments. The other eluent was beef extract-glycine that had not been passed through a filter. Experiments were performed with Norwalk virus seeded at 100 PCRU/sample and 10 PCRU/sample. The diluted, seeded samples were subjected to molecular detection methodology.

Experimental results are shown in Figures 3 and 4 for samples seeded at 100 and 10 PCRU, respectively. With samples seeded at 100 PCRU/sample (Figure 3), the beef extract eluting solution is inhibitory in the undiluted sample and the $10^{-1}$ sample, but there are visible bands present in the $10^{-2}$ and $10^{-3}$ dilutions for both the filtered and the non-filtered samples. With samples seeded at 10 PCRU/sample (Figure 4), the beef extract eluting solution is inhibitory in the undiluted sample, $10^{-1}$, and $10^{-2}$ filtered sample, but there is a visible band present in the $10^{-3}$ dilution. In the non-filtered sample, the beef extract is inhibitory in the undiluted and $10^{-1}$ sample, but there are positive bands in the $10^{-2}$ and $10^{-3}$ dilutions. These results
indicate that beef extract is indeed inhibitory to Norwalk detection by RT-PCR, and that additional inhibitors are eluted from adsorbent filters that have processed tap water.

D. Norwalk Virus Adsorption to and Elution from Virosorb 1MDS Filters

Norwalk virus adsorption-elution experiments were performed using enough added virus so that the inhibitory effects associated with the beef extract could be avoided through dilution of the beef extract filter eluate. These experiments were performed using the same protocol as was used for the coliphage MS2 adsorption-elution experiments, except 25mm diameter filters were used and the volumes were proportionally reduced. This was done to minimize the amount of Norwalk virus that was consumed in each experiment. Virosorb 1MDS, double layer, 25mm diameter filters were used to filter 150 mL of water seeded with Norwalk virus at approximately $10^4$ PCRU/mL. Tap water was adjusted to pH 6.0, 7.5, or 9.0. Viruses adsorbed to the filters were eluted using two successive 2 mL volumes of 1.5% beef extract-glycine at pH 9.8. Recovery efficiencies were computed assuming a detection limit of 1 PCRU as the lowest sample dilution yielding a visible band in agarose gel electrophoresis after PCR amplification.

Results for the Norwalk virus experiments testing adsorption at pH 6.0, 7.5, and 9.0 and elution with beef extract are summarized in Table 3 and represented graphically
in Figure 5. Viruses were adsorbed with efficiencies that ranged from 90 to >98%, with very little virus (<2%) in either the pH 6.0 and 7.5 filtrates. As would be expected, the Virosorb 1MDS filters showed a lower efficiency of virus adsorption at the higher pH of 9.0 (90%) than at the lower pH levels of 7.5 and 6.0 (>98%). Recovery of adsorbed Norwalk virus by elution with beef extract-glycine, pH 9.8, ranged 35-55%. These results indicate that about one-third to one-half of the adsorbed Norwalk viruses are eluted. The fate of the unaccounted for Norwalk viruses that adsorbed to the filters is unclear. It is possible that Norwalk virus elution efficiency is underestimated.

E. Norwalk Recovery with Alternative Eluents

Having established that Norwalk virus could be concentrated from tap water by adsorption to and elution from Virosorb 1MDS filters, alternative amino acid eluents were applied to elute the adsorbed viruses from the filters so that molecular detection methods could be applied more efficiently to Norwalk virus detection. All three test viruses were seeded to dechlorinated tap water at approximately $10^4$ PFU (PCRU for NV)/mL and filtered through 25mm diameter double layers of Virosorb 1MDS filter material. Two successive 2 mL volumes of elution media were collected from the filters and assayed by infectivity (poliovirus and MS2) or molecular detection methods (all three viruses). The data were combined and are reported in Table 4. Infectivity
data and RT-PCR data are compared for poliovirus and coliphage MS2 (Table 4). In general, RT-PCR data are consistent with infectivity data within the precision limits of the endpoint titration method for RT-PCR detection (about 1/2 log10).

Preliminary experiments using alternative amino acid eluting media established that the relative recovery efficiencies of all alternative eluents except for threonine (60-67%) were approximately equal to the efficiency of beef extract (67%) at pH 9.8 (Table 1). Relative virus recovery efficiencies were much higher with the alternative eluents at the lower eluting pH of 8.5 and 7.0 (59-80% and 62-90%, respectively) than with beef extract (27% and 33%, respectively). Arginine, asparagine, lysine, and glycine were chosen as alternative eluting media to beef extract for the experiments involving Norwalk virus, poliovirus, and coliphage MS2.

Lysine gave the highest recovery efficiency and asparagine gave the lowest recovery efficiency for Norwalk virus at all eluting pHs. Recovery efficiency with lysine was approximately 100% at pH 9.8, 8.5, and 7.0. Arginine was the next most efficient giving recoveries of 42%, approximately 100%, and 11% at pH 9.8, 8.5, and 7.0, respectively. Glycine gave recoveries of approximately 100%, 42%, and 2% and asparagine gave recoveries of 32%, 20%, and 20% at pH 9.8, 8.5, and 7.0, respectively.
F. Secondary Concentration of Filter Eluates by Polyethylene Glycol Precipitation

Viruses concentrated by adsorption to and elution from positively charged filters may still be in low concentrations within the filter eluate, and the eluate volume may be too large for efficient and economical virus detection. Therefore, a second concentration step should be applied. Polyethylene glycol (PEG) precipitation is simple, cost-effective, and compatible with molecular virus detection methods. Because the precipitation is carried out at pH 7.2 where most viruses are extremely stable, this method has proven useful for effectively concentrating many enteric viruses from a variety of suspension media (2,7,14,30,46,47,49,79,82,83). Sodium chloride is generally used in addition to PEG to aid with the precipitation by "salting out" viruses that may be present (7).

The objective of these experiments was to determine the optimum concentrations of PEG and NaCl to add to each alternative amino acid eluate in order to achieve efficient precipitation and recovery of viruses. Amino acid eluates from previous experiments were pooled and used for these experiments. Asparagine was not used because of its inability to remain soluble near neutral pH where PEG precipitation takes place. PEG concentrations of 6%, 8%, and 10% and NaCl concentrations of 0.1N and 0.3N were tested. Both the pellet and the supernatent were assayed to determine which combinations were most efficient in precipitating
viruses and recovering them in the resuspended precipitate (Table 5).

For Norwalk virus, there was efficient precipitation with all three combinations of PEG and 0.3N NaCl for arginine and all tested combinations for lysine (Table 5). Precipitation did not appear to be as efficient with glycine because there was detectable virus in the supernatant samples at all conditions tested. From infectivity data for poliovirus type 1 and coliphage MS2, it appears that there are virtually 100% recovery efficiencies when using 10% PEG and 0.3N NaCl. There are poliovirus type 1 and coliphage MS2 infectivity recovery efficiencies ranging from 1-100% with other PEG and NaCl combinations. With all three eluents, there is a trend of increasing virus recoveries with higher PEG concentrations and the higher NaCl concentration of 0.3N.
### TABLE 1. Poliovirus-1 Recovery with Beef Extract and Alternative Eluents at Varying pH

<table>
<thead>
<tr>
<th>Recovery Efficiencies</th>
<th>pH 9.8</th>
<th>pH 8.5</th>
<th>pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5M Threonine (Thr)</td>
<td>45%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.5M Lysine (Lys)</td>
<td>64%</td>
<td>59%</td>
<td>62%</td>
</tr>
<tr>
<td>0.5M Arginine (Arg)</td>
<td>67%</td>
<td>79%</td>
<td>85%</td>
</tr>
<tr>
<td>0.5M Asparagine (Asx)</td>
<td>67%</td>
<td>64%</td>
<td>90%</td>
</tr>
<tr>
<td>0.5M Glycine (Gly)</td>
<td>60%</td>
<td>80%</td>
<td>63%</td>
</tr>
<tr>
<td>beef extract-glycine (BE)</td>
<td>67%</td>
<td>27%</td>
<td>33%</td>
</tr>
</tbody>
</table>

- 1.0% Beef Extract-0.05M Glycine
- viruses adsorbed to Virosorb 1 MDS 47mm filters at pH 6.0
- 500 mL of water filtered; 2 serial elutions of 5 mL each were combined
- data based on plaque assay
- Thr dropped from pH 8.5 and pH 7.0 trials due to lack of efficiency at pH 9.8
- 3 trials for pH 9.8 and 2 trials for pH 8.5 and 7.0
FIGURE 1. Poliovirus Recovery with Alternative Eluents

- ■ 0.5M Threonine (Thr)
- □ 0.5M Lysine (Lys)
- ■ 0.5M Arginine (Arg)
- ■ 0.5M Asparagine (Asx)
- ■ 0.5M Glycine (Gly)
- ■ Beef extract-glycine (BE)

Elution pH:
- pH 9.8
- pH 8.5
- pH 7.0

Pools H9.

Pools H8.

Eltion pH

Simmons 50
Table 2. Coliphage MS-2 Adsorption-Elution Efficiency

<table>
<thead>
<tr>
<th>Adsorption pH</th>
<th>6.00</th>
<th>7.50</th>
<th>9.00</th>
</tr>
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<tbody>
<tr>
<td>Eluent</td>
<td>40%</td>
<td>74%</td>
<td>24%</td>
</tr>
<tr>
<td>Filtrate</td>
<td>1%</td>
<td>1%</td>
<td>12%</td>
</tr>
</tbody>
</table>

- elutions with 1.5% Beef Extract-0.05M Glycine pH 9.8
- viruses adsorbed to Cuno 1MDS 47mm filters at pH 6.0
- 500 mL of water filtered
- 2 serial elutions of 5 mL each were combined
- data based on plaque assay
- 5 trials for pH 6.0 and 2 trials for pH 7.5 and 9.0

![FIGURE 2. Coliphage MS-2 Recovery Efficiency](image)
Figure 3. Norwalk Virus Adsorption-Elution with 300 PCRU/sample
Figure 4. Norwalk Virus Adsorption-Elution with 10 PLOU/sample

60 bp Marker
Table 3. Norwalk Virus Adsorption-Elution Efficiency

<table>
<thead>
<tr>
<th>Adsorption pH</th>
<th>6.00</th>
<th>7.50</th>
<th>9.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eluent</td>
<td>41%</td>
<td>35%</td>
<td>55%</td>
</tr>
<tr>
<td>Filtrate</td>
<td>0%</td>
<td>0%</td>
<td>10%</td>
</tr>
</tbody>
</table>

- elutions with 1.5% Beef Extract-0.05M Glycine pH 9.8
- viruses adsorbed to Cuno 1MDS 25mm filters at pH 6.0
- 150 mL of water filtered
- 2 serial elutions of 2 mL each were combined
- 2 trials for pH 6.0, pH 7.5, and pH 9.0
- RT-PCR data assumes 1 PCRU in lowest sample dilution giving a visible band in agarose gel after RT-PCR amplification

FIGURE 5. Norwalk Virus Recovery Efficiency
Table 4. Norwalk Virus, Poliovirus 1, and Coliphage MS-2 Recoveries with Alternative Eluting Solutions at pH 9.8(A), 8.5(B), and 7.0(C)

<table>
<thead>
<tr>
<th>(A.) pH 9.8</th>
<th>Norwalk Virus</th>
<th>Poliovirus 1</th>
<th>Coliphage MS-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT-PCR</td>
<td>RT-PCR</td>
<td>Infect.</td>
</tr>
<tr>
<td>0.5M Arginine (Arg)</td>
<td>0 (?)</td>
<td>20%</td>
<td>=100%</td>
</tr>
<tr>
<td>0.5M Asparagine (Asx)</td>
<td>32%</td>
<td>=100%</td>
<td>63%</td>
</tr>
<tr>
<td>0.5M Glycine (Gly)</td>
<td>100%</td>
<td>=100%</td>
<td>77%</td>
</tr>
<tr>
<td>0.5M Lysine (Lys)</td>
<td>100%</td>
<td>=100%</td>
<td>=100%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(B.) pH 8.5</th>
<th>Norwalk Virus</th>
<th>Poliovirus 1</th>
<th>Coliphage MS-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT-PCR</td>
<td>RT-PCR</td>
<td>Infect.</td>
</tr>
<tr>
<td>0.5M Arginine (Arg)</td>
<td>&gt;100%</td>
<td>=100%</td>
<td>17%</td>
</tr>
<tr>
<td>0.5M Asparagine (Asx)</td>
<td>20%</td>
<td>20%</td>
<td>35%</td>
</tr>
<tr>
<td>0.5M Glycine (Gly)</td>
<td>42%</td>
<td>=100%</td>
<td>42%</td>
</tr>
<tr>
<td>0.5M Lysine (Lys)</td>
<td>100%</td>
<td>=100%</td>
<td>40%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(C.) pH 7.0</th>
<th>Norwalk Virus</th>
<th>Poliovirus 1</th>
<th>Coliphage MS-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT-PCR</td>
<td>RT-PCR</td>
<td>Infect.</td>
</tr>
<tr>
<td>0.5M Arginine (Arg)</td>
<td>11%</td>
<td>&gt;100%</td>
<td>70%</td>
</tr>
<tr>
<td>0.5M Asparagine (Asx)</td>
<td>20%</td>
<td>100%</td>
<td>60%</td>
</tr>
<tr>
<td>0.5M Glycine (Gly)</td>
<td>2%</td>
<td>=100%</td>
<td>37%</td>
</tr>
<tr>
<td>0.5M Lysine (Lys)</td>
<td>=100%</td>
<td>100%</td>
<td>55%</td>
</tr>
</tbody>
</table>

- All viruses adsorbed to Cuno 1MDS 25mm filters at pH 6.0
- 200 mL of water filtered; 2 serial elutions of 2 mL each were combined
- RT-PCR data assumes 1 PCRU in lowest sample dilution giving a positive result after RT-PCR, Southern transfer, and hybridization and gene probing
Table 5. Norwalk Virus, Poliovirus 1, and Coliphage MS2 Polyethylene Glycol Precipitation Recoveries from Arginine(A), Glycine(B), and Lysine(C)

<table>
<thead>
<tr>
<th>(A) Arginine</th>
<th>Norwalk RT-PCR</th>
<th>Poliovirus 1 Inf.</th>
<th>Poliovirus 1 RT-PCR</th>
<th>Coliphage MS2 Inf.</th>
<th>Coliphage MS2 RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG Conc.</td>
<td>NaCl Conc.</td>
<td>6%</td>
<td>0.1N</td>
<td>10%*</td>
<td>70%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6%</td>
<td>0.3N</td>
<td>100%</td>
<td>47%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8%</td>
<td>0.1N</td>
<td>10%</td>
<td>70%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8%</td>
<td>0.3N</td>
<td>100%</td>
<td>88%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10%</td>
<td>0.1N</td>
<td>10%</td>
<td>88%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10%</td>
<td>0.3N</td>
<td>10%</td>
<td>=100%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(B) Glycine</th>
<th>Norwalk RT-PCR</th>
<th>Poliovirus Inf.</th>
<th>Poliovirus RT-PCR</th>
<th>Coliphage MS2 Inf.</th>
<th>Coliphage MS2 RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG Conc.</td>
<td>NaCl Conc.</td>
<td>6%</td>
<td>0.1N</td>
<td>10%*</td>
<td>35%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6%</td>
<td>0.3N</td>
<td>10%*</td>
<td>82%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8%</td>
<td>0.1N</td>
<td>10%*</td>
<td>24%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8%</td>
<td>0.3N</td>
<td>100%*</td>
<td>71%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10%</td>
<td>0.1N</td>
<td>100%*</td>
<td>59%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10%</td>
<td>0.3N</td>
<td>100%*</td>
<td>100%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(C) Lysine</th>
<th>Norwalk RT-PCR</th>
<th>Poliovirus Inf.</th>
<th>Poliovirus RT-PCR</th>
<th>Coliphage MS2 Inf.</th>
<th>Coliphage MS2 RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG Conc.</td>
<td>NaCl Conc.</td>
<td>6%</td>
<td>0.1N</td>
<td>100%</td>
<td>47%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6%</td>
<td>0.3N</td>
<td>100%</td>
<td>56%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8%</td>
<td>0.1N</td>
<td>100%</td>
<td>81%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8%</td>
<td>0.3N</td>
<td>100%</td>
<td>90%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10%</td>
<td>0.1N</td>
<td>100%</td>
<td>67%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10%</td>
<td>0.3N</td>
<td>&gt;100%</td>
<td>=100%</td>
</tr>
</tbody>
</table>

* virus detectable in supernatent samples

- amino acid eluents pooled, adjusted to pH 7.2, and divided into 6 mL subsamples
- combinations of PEG and NaCl added to 6 mL subsamples
- precipitation overnight at 4°C
- RT-PCR data assumes 1 PCRU in lowest sample dilution giving a positive result after RT-PCR, Southern transfer, and hybridization and gene probing
DISCUSSION

Waterborne transmission of enteric viruses pose a documented threat to public health as is evident by the number of outbreaks that occur each year in the USA as well as the rest of the world. In order to better characterize and control the risks from viruses in water, it is imperative that there be a rapid, efficient, and reliable method for detection of low numbers of viruses which may be present in environmental water samples. Because viruses are generally in low concentrations in environmental samples, concentration methods must be applied to large sample volumes before viruses can be detected. As an ultimate goal, these concentration and detection methods should enable the detection of all waterborne, human enteric viruses, including those that are not detectable by current cell culture assays. The aim of this work was to develop a method for concentrating low numbers of enteric viruses from large volumes of water that is compatible with both conventional and molecular virus detection methodology.

The most accepted virus concentration methods for large volume water samples use filters to electrostatically bind viruses, relying on the fact that most viruses carry a net negative charge in waters at or near neutral pH. Both negatively and positively charged filters have been shown to efficiently concentrate viruses from water of varying
quality. However, positively charged filters are more widely accepted because they are much easier to use, require less technical expertise, and the sample does not need to be manipulated as to pH or by adding divalent or trivalent salts. Also, negatively charged filters adsorb viruses efficiently only at low pH (pH \approx 3.5), which may stress or damage the virus capsid, causing a loss of infectivity. Positively charged filters need no additional equipment or reagents and adsorb viruses directly over a wide pH range. The most commonly used positively charged filter for concentrating viruses from water is the Virosorb 1MDS filter and the most widely accepted eluent is 3% beef extract / 0.05M glycine.

There are several methods by which solutions elute viruses from the surface of positively charged Virosorb 1MDS filters. A change in pH of the eluting solution which surpasses the isoelectric point of the filter will make it appear negatively charged. Because of this, both the filter and the virus particles carry like electrostatic charges that are negative and they will repel one another, allowing the viruses to be suspended within the eluting solution rather than remaining attached to the filter surface. An example of this occurs with elution using beef extract/glycine, because there is efficient elution of poliovirus at pH 9.8 and inefficient elution at lower pHs (Table 1).

Another factor that may effect virus elution from filters are constituents within the eluting solution that may
compete for adsorption sites on the surface of the Virosorb 1MDS filters. Constituents within the eluting solutions carrying a negative charge will compete for the positive adsorption sites. This is enhanced by particles with high isoelectric points that remain negatively charge if the pH of the eluting solution is raised. This may be a contributing factor for elution with beef extract/glycine.

Viruses may be efficiently eluted from positively charged Virosorb 1MDS filters by positively charged eluting solutions that compete with the surface of the filters for the negatively charged virus particles. Examples of this include the amino acid eluting solutions arginine, lysine, and to some extent glycine (Figure 6). Arginine and lysine have high pK values of 12.0 and 10.0, respectively, making them positively charged at pHs used for virus elution (7.0-9.8). This is substantiated by consistently high virus recovery efficiency over a wide pH range (Table 1).

Finally, viruses are adsorbed to filters through not only electrostatic interactions but also through hydrophobic interactions. Regions on the surface of most viruses tend to be hydrophobic and will be captured in hydrophobic regions within the positively charged Virosorb 1MDS filters. If these interactions are weakened, the viruses will be released into the solution surrounding the virus particles.

An important waterborne, human enteric virus that is a documented threat to public health is Norwalk virus. Many waterborne outbreaks of nonbacterial, acute gastroenteritis
Figure 6. Structures of Amino Acids at pH 7.5

- L-threonine (thr)
- L-asparagine (asn)
- L-arginine (arg)
- L-lysine (lys)
have been linked to this pathogen through epidemiological studies. An analytic problem with Norwalk virus is the lack of cell culture assay method for detecting and quantifying the virus. Because of this, RT-PCR is used for detection and quantification of low levels of Norwalk virus. This study was done to characterize Norwalk virus adsorption to and elution from the Virosorb 1MDS filters commonly used for concentrating enteric viruses from water. Because RT-PCR and oligoprobe hybridization was used for Norwalk virus detection, the methods had to be compatible with this analytical approach.

In model experiments on Norwalk virus adsorption to Virosorb 1MDS filters from dechlorinated Chapel Hill drinking water over a pH range of 6.0-9.0, the virus was adsorbed efficiently (90-98% adsorption). However, adsorption efficiency tended to be comparatively lower at the higher pH of 9.0 (90%) than at the lower pH levels of 6.0 and 7.5 (98%), as would be expected from previous experiments done with other test viruses (69,70).

Several solutions were tested to elute Norwalk viruses adsorbed to the Virosorb 1MDS filters. These included a standard beef extract-glycine solution as well as 0.5M concentration of the amino acids, arginine, asparagine, glycine, and lysine. All solution were tested over the pH range of 7.0 to 9.8. Beef extract efficiently eluted Norwalk virus from the filters, with recoveries ranging from 35% to 55%. Norwalk virus recovery with beef extract eluent tended
to be higher at the higher elution pH of 9.8 (55%) rather than at the lower elution pH of 7.0 (41%). However, beef extract proved to be inhibitory to molecular detection methodology. For this reason, there needed to be high initial numbers of viruses in order to dilute the samples beyond the inhibitory effects of the beef extract and detect the virus by RT-PCR. This level is not practical or realistic for the low levels of Norwalk virus expected in water.

Norwalk virus was also efficiently eluted from the filters using the alternative amino acid eluents of arginine, asparagine, glycine, and lysine over a wide pH range. There did not appear to be any inhibitory effects on RT-PCR associated with these alternative eluents as there was with the beef extract. Therefore, low numbers of Norwalk virus should be detectable. Norwalk recovery with alternative eluents ranged from 2% to approximately 100%. In general, there appeared to be somewhat higher Norwalk virus recoveries from the Virosorb 1MDS filters with pH 9.8 and pH 8.5 eluents (42≈100% and 20≈100%, respectively) than with pH 7.0 eluents (2≈100%). Arginine and lysine appear to be the best alternatives to beef extract, with Norwalk virus recoveries of about 11≈100% and ≈100%, respectively. These recoveries are equal to or greater than those with beef extract.

Because virus concentration and detection methods should be universally applicable to all enteric viruses in water, it is also important to characterize the performance of these
methods for viruses other than Norwalk virus. Poliovirus, a widely studied enterovirus, and bacteriophage MS2, a prototype coliphage proposed as an indicator of enteric viruses, were compared to Norwalk virus for adsorption to and elution from Virosorb 1MDS filters. An advantage in using these particular viruses is that they could be quantified by both infectivity assay and by RT-PCR. Both poliovirus and coliphage MS2 were adsorbed efficiently over a wide pH range (>88%), which is what was expected from previous studies (6,69,70). Poliovirus was eluted efficiently at pH 9.8 with beef extract as well as with the alternative eluents (67% and 60-67%, respectively). However, at the lower pH levels of 8.5 and 7.0, the alternative eluents provided much higher recoveries (59-90%) than beef extract (27% and 33%, respectively). Coliphage MS2 was recovered with equal or greater efficiencies using the alternative eluents (24-100%) than by beef extract (24-74%).

These results suggest that Virosorb 1MDS filters will efficiently adsorb many waterborne viruses of significant public health importance over a wide pH range. The Virosorb 1MDS filters tended to adsorb Norwalk virus, poliovirus, and coliphage MS2 more efficiently at the lower pH of 6.0 than at the higher pH levels of 7.5 or 9.0. However, virus adsorption efficiency was greater than or equal to 88% at all pH levels tested, so it is possible to sample waters using these filters for waterborne enteric viruses without adjusting the pH of the water.
The alternative amino acid eluents can be used to elute the adsorbed viruses from the Virosorb 1MDS filters. The alternative eluents proved to be compatible with widely accepted molecular detection techniques and were as effective as beef extract for eluting the three test viruses from the filters. These alternative eluents can be used at lower pH levels than beef extract, reducing the risk of virion damage at high pH levels which might reduce the detection of viruses by infectivity assay. Alternative eluents were compatible with both infectivity assays as well as molecular assays, allowing for detection of both culturable and nonculturable viruses.

Virus elution from the Virosorb 1MDS filters was done using two successive elution volumes. Because several of the alternative eluents were effective, it is possible that these elutions could be done using either the same eluting solution or two different eluents, either separately or in combination.

Three of the alternative eluents were effective in recovering adsorbed viruses and should be further evaluated for elution of viruses from the Virosorb 1MDS filters. These were arginine, glycine, and lysine. Asparagine proved to be inadequate because it precipitated near neutral pH. This may be due to an isoelectric point within the working pH range of 7.0 to 9.8. In order to keep it in solution to perform these experiments, it had to be warmed. The precipitation of asparagine near neutral pH is an important deficiency because
this is the pH range for polyethylene glycol precipitation of viruses from the eluent as a secondary concentration step.

A question that should be addressed regarding recovery efficiencies pertains to values in supernatents and resuspended virus pellets that when added are greater than 100%. There are several possibilities which may lead to this. The precision limits of the molecular assays is not very high, due solely to the nature of the assay method. For most of these experiments, serial \( \log_{10} \) or 1/2 \( \log_{10} \) dilutions were made, and the samples were determined to be either RT-PCR/oligoprobe positive or negative at each dilution. This allows for a great deal a variability within each sample assayed. In addition to this, all of the recovery efficiencies were computed by comparison to an initial sample titer. If this sample titer was not accurate, all of the computed efficiencies could be high or low. Another cause for error within several of the experiments was that they were single trials, rather than multiple trials. Because of the magnitude of the work, it was not feasible to repeat these trials. Recoveries greater than 100% could also be possible due to the viruses that were used for the experiments. Crude virus stocks which were not highly purified were used and could have contained aggregated virus particles. During the experiments, these virus aggregates could have become dispersed; therefore, giving higher than expected virus titers.
Polyethylene glycol precipitation proved be a useful secondary concentration step for further concentrating enteric viruses eluted from Virosorb 1MDS filters by alternative eluents. The precipitation is performed at pH ≈ 7.2, which is a pH where viruses are stable. Polyethylene glycol is not inhibitory to molecular detection techniques, is simple and cost-effective, and does not require specialized equipment or sophisticated technical skills. Also, no acidification or additives, such as ferric chloride are needed as they are with organic flocculation of beef extract as a secondary concentration procedure. Such acidification and additives may interfere with virus stability and detection.

In summary, Virosorb 1MDS filters will effectively concentrate Norwalk virus, as well other human enteric viruses, from large volumes of water over a wide pH range. Three amino acid eluents, arginine, glycine, and lysine, were effective alternatives to beef extract eluent that are compatible with infectivity and molecular detection methodology, and they can be used at neutral pH where concentrated viruses will be more stable. PEG precipitation, using 10% PEG and 0.3N NaCl, efficiently concentrated the viruses in the alternative amino acid eluting solutions another 10-fold, and proved to be compatible with molecular detection of viruses. This concentration and detection methodology can be applied for detection of a variety of enteric viruses, either culturable or not.
Further experiments should include the application of these techniques to other seeded, model waters, such as groundwaters and surface waters and to field samples. Field samples may contain different levels of inhibitors, such as humic and fulvic acids. The extent of interference with virus adsorption to and elution from ViroSorb 1MDS filters by these inhibitors should be characterized. The alternative amino acid eluents should be examined at different concentrations than 0.5M and possibly with additives that might further improve virus elution, such as non-ionic detergents or salts. In addition, these alternative eluents should be tested for recovery of other model viruses such as rotaviruses, which have a double capsid and may respond differently to concentration methods based on adsorption to and elution from adsorbent filters.
CONCLUSIONS

- Norwalk virus (strain 8FIla) in water will adsorbed to electropositive Virosorb 1MDS filters with an efficiency ≥ 90%, as do other enteric viruses.

- Four alternative amino acid eluting solutions (0.5M arginine, asparagine, lysine, and glycine) that are compatible with RT-PCR detection will elute Norwalk virus, poliovirus-1, and bacteria coliphage MS2 adsorbed to Virosorb 1MDS filters.

- The amino acid eluting solutions eluted the three test viruses with equal or greater efficiencies than beef extract-glycine (BE/G) over an eluting pH range of 7.0 to 9.8.

- Polyethylene glycol precipitation using 10% PEG and 0.3N NaCl provided an efficient means to secondarily concentrate viruses another 10-fold in the alternative amino acid filter eluates.

- Overall virus recovery efficiency by adsorption to Virosorb 1MDS filters, elution with amino acid alternative eluting solutions, and secondary concentration by polyethylene glycol precipitation was good, ranging 2--=100%.
References:


