

Detecting Viruses in Water

Christon J. Hurst, William H. Benton, and Ronald E. Stetler

Various and divergent approaches that have been used to concentrate and assay viruses from tap water and environmental freshwaters are summarized and briefly explained. The basic principles behind the different methodologies and descriptions of the most recent developments are emphasized. Comparisons help demonstrate the relative sensitivities of different concentration and assay techniques.

Viruses that replicate within and are subsequently shed from the gastrointestinal tract of animals are referred to as enteric viruses. These viruses are principally transmitted by the fecal-oral route, meaning that they cause illness after being ingested by an uninfected individual. The known human enteric viruses are listed in Table 1. Not all of these viruses, however, have been studied with regard to their presence in drinking water. Although some of the viruses listed in Table 1 can also replicate in animals other than humans, their presence in raw waters is presumably related to human fecal pollution.

The human enteric viruses are presumed to present the greatest concern with regard to waterborne viral disease. Although raw waters may also contain related enteric viruses originating from wild and domesticated animals, most of these viruses are considered to have relatively limited pathogenicity for humans. Infectious human enteric viruses can be found in surface freshwaters and groundwaters,¹⁻⁴ where they can originate from septic effluents and wastewater.⁵⁻⁸ Viral contamination of surface water could also result from human recreational activities either in or around bodies of water.⁹ Enteric viruses can survive improperly operated conventional water treatment trains and may then be found in potable water.^{1-4,8,10,11} This cause for public health concern has resulted in the development of many methods for recovering both human enteric viruses and bacteriophages from water. Bacteriophages (viruses that infect bacteria) have been studied in the hope that they may prove useful as indicators of the removal and

destruction of human enteric viruses during water treatment.

The remainder of this article describes and categorizes the various



Viruses were concentrated from river water by directed adsorption onto a pleated membrane cartridge filter.

techniques that have been used for detecting viruses in environmental freshwaters and in treated drinking waters. These techniques fall into two main categories. The first is virus concentration techniques, in which viruses recovered from large volumes of water are concentrated into smaller, more manageable volumes; the second is the viral assay procedures used to examine the concentrated samples.

Virus concentration techniques

Classification of techniques. Five major categories of techniques are used for concentrating viruses contained in water samples (Table 2). Before examining these different concentration techniques, it is helpful to briefly mention the structure of what are termed classical viruses, a category that encompasses all of the viruses listed in Table 1. Classical viruses consist of a nucleic acid core, or genome, which provides their genetic information and is surrounded by a shell of proteins termed a capsid. In some types of viruses this capsid is, in turn, surrounded by a lipid bilayer membrane, or envelope. Of the viruses listed in Table 1, only coronaviruses possess such envelopes. Nonenveloped viruses essentially present themselves to the environment as large protein structures. For this reason, studies like Mizutani's,⁵⁶ which addresses the adsorption of proteins to glass surfaces, help explain the mechanics of virus concentration techniques. The only study found that addressed concentrating enveloped viruses from water samples used the herpes simplex virus and the measles virus,⁵³ neither of which is considered enteric or otherwise of concern with regard to the waterborne transmission of human illness.

Passive adsorption. The first type of concentration technique mentioned in Table 2 is passive adsorption. This technique, originated by MacCallum et al.,⁵⁷ involves the use of pads consisting of either gauze alone or a combination of gauze and cotton batting for the purpose of absorbing a portion of the water sample into which the pads are placed. A small portion of the viruses suspended in the water sample are taken into these pads during the passive absorption process. Any actual adsorption of viruses onto the surface of the gauze or cotton fibers is incidental. The pads may be used in either a stationary or flow-

through configuration.^{14,57} Viruses are recovered from the pads by expression of the absorbed fluid. Additional fluids may be used in kneading the pads to help achieve recovery of any adsorbed viruses. Fluids used for desorbing viruses from surfaces are termed eluants, and the process is termed elution. Following exposure to an adsorbing surface, it is hoped that the fluid, or eluate, will contain any viruses that had been on the surface. The relatively low efficiency of gauze pads for concentrating viruses from water was demonstrated, however, by a study in which such pads were used for prefiltering a water sample to remove large particulate solids, before concentrating the viruses from the same water sample by passage through a layer of virus-adsorbing granular material.³⁷

Directed adsorption. Directed adsorption involves the use of either filters or granular solids as surfaces onto which viruses are deliberately adsorbed and then recovered.^{16,26,27,30,34,37,44,45,58-63} Adsorption is generally facilitated by modifying the water sample, the adsorbent material, or both. The adsorbent material can be modified either during or after its manufacture. Subsequent recovery of the adsorbed viruses can occur either by dissolving the filter material, if it is composed of alginate,⁶⁴ or by exposing the adsorbent to a volume of eluant that is smaller than the original water sample and facilitates a reversal of the virus adsorption process. Most techniques now used for concentrating viruses from large volumes of either tap water or environmental freshwaters belong to the category of directed adsorption. These techniques are discussed in greater detail later in this article. The general mechanisms involved in concentration methods of this type have been presented in publications by Gartner,⁶⁴ England,⁶⁵ Cookson,⁶⁶ Mix,⁶⁷ and Sweet et al.⁶⁸

Ultrafiltration. Ultrafiltration involves selectively removing small molecules from a water sample, including the water molecules themselves, and leaving behind large solutes or suspended particles such as the viruses. This technique uses filters that may be configured either as hollow fibers through which the water sample is passed or sheet filters against which the water is kept in motion by means of a recirculating pump or stirring apparatus.^{46,48,69} The general mechanism used in this type of concentration technique has been discussed by Mix.⁶⁷ The filters may be pretreated before they are used on a water sample to suppress virus adsorption.⁴⁸ Alternatively, a second fluid, that either may or may not serve as an eluant, can be used to flush viruses from the surface of the filters after they have been used to concentrate a water sample.⁴⁶ Ultrafiltration is seldom used for directly concentrating viruses from water samples

TABLE 1
Human enteric viruses

Virus Group	Number of Serotypes*	Illness Caused
Adenovirus	47	Pharyngitis, conjunctivitis respiratory illness, vomiting, diarrhea
Astrovirus	5	Vomiting, diarrhea
Calicivirus	2	Vomiting, diarrhea
Coronavirus	1	Vomiting, diarrhea
Enterically transmitted non-A, non-B hepatitis virus	1	Hepatitis
Enterovirus		
Poliovirus	3	Paralysis, meningitis, fever
Coxsackievirus A	24	Herpangina, respiratory illness, meningitis, fever
Coxsackievirus B	6	Myocarditis, congenital heart anomalies, rash, fever, meningitis, respiratory illness, pleurodynia
Echovirus	34	Meningitis, respiratory disease, rash, diarrhea, fever
"Numbered" enteroviruses (includes enterovirus 72, formerly called hepatitis A virus)	5	Meningitis, encephalitis, respiratory illness, acute hemorrhagic conjunctivitis, fever, hepatitis
Norwalk virus (possibly a calicivirus)	1	Epidemic vomiting and diarrhea
Parvovirus	2	One type possibly associated with enteric infection
Reovirus	3	Not clearly established
Rotavirus	4	Vomiting, diarrhea
"Small round viruses" (possibly enteroviruses)	2	Vomiting, diarrhea

*Total equals 140 viral serotypes.

TABLE 2
Techniques used for concentrating viruses from water samples

Type of Technique	Process Involved	Selected References
Passive adsorption	Unaided entrapment of viruses by adsorption or absorption into pads of gauze or cotton, used in stationary or flow-through configurations. This is followed by expression of the entrapped fluids, with or without the supplemental use of an eluant, to recover the viruses.	1, 12-14
Directed adsorption	Viruses are adsorbed onto the surface of filter materials or granular solids, facilitated in many instances by pretreatment of the water. Processes evaluated as pretreatments include removing soluble organics from the water using charged resins, adjustment of the water sample pH, and addition of salts of metal cations—particularly those that are multivalent such as aluminum and magnesium. Efforts have also been made to pretreat the adsorbent material itself to enhance the efficiency of viral adsorption. Examples of the latter approach include binding metal precipitates or charged polymers to the matrix of microporous filters. Subsequent recovery of the bound viruses from the filter materials or granular solids used as adsorbents is achieved by exposing the adsorbents to an eluant.	12, 15-45
Ultrafiltration	Viruses are retained in the original water sample during a reduction of its volume achieved by pore size exclusion. This generally is accomplished by either recirculating the water under pressure through hollow fiber filters or over the surface of flat sheet filters in either recirculating or nonrecirculating systems. In some cases an eluant is subsequently passed through the same concentration unit to facilitate virus recovery.	46-48
Direct physico-chemical flocculation or phase separation	Viruses are concentrated either by association with a generated precipitate or by selective partitioning of the water sample during processes that yield a polymeric phase separation, or they are retained in the original water sample during a reduction in its volume through hydroextraction.	12, 13, 49-54
Affinity chromatography	Viruses are retained during passage of the water sample through a column of polysaccharide gel particles, which bear covalently linked antibodies that specifically recognize and bind a particular virus type. Viruses are then released from the column by altering the composition of the buffer fluid used for maintaining hydration of the polysaccharide gel.	55

TABLE 3
Solid materials that have been used for concentrating viruses by directed adsorption

Adsorbent	Selected References
Filter materials*	
Acrylonitrile polyvinyl chloride copolymer	31, 71
Alginate	29, 64
Asbestos	72
Asbestos plus cellulose	17, 32, 73
Cellulose plus diatomaceous earth and charged resin	17, 32, 69, 74-76
Mixed cellulose esters	12, 44, 45, 61, 77-80
Nitrocellulose	71
Glass fiber	31, 34, 61, 81
Glass fiber plus acrylic	82
Glass fiber plus epoxy	16, 20, 21, 24, 31, 32, 35, 39, 41, 75, 77, 78, 81, 83-85
Glass fiber plus asbestos and epoxy	31, 32, 61, 75, 76, 83
Charged glass fiber	15, 19, 20, 33, 39, 41, 73
In situ modified microporous filters	23, 42
Charged nylon	41
Polysulfone	71
Polyvinyl chloride	71
Polyvinylidene difluoride	71
Granular solids	
Bituminous coal	18
Charged perlite	40
Charged resin	30, 37
Glass (powdered)	4, 25-27, 70, 86
Hydroxylapatite	36
Magnetic iron oxide	60, 87
Talc plus diatomaceous earth	37, 38, 74, 88

*May be of sheet- or wound-fiber configuration

TABLE 4
Procedures that have been used for secondary concentration of viruses contained in eluates from solid adsorbents*

Category of Eluant	Selected References
Beef extract solution†	
Ammonium sulfate precipitation	70, 73, 104
Hydroextraction	28, 63, 88
Hydroxylapatite adsorption	63
Magnetic iron oxide	28, 87
Organic flocculation (pH adjustment alone)	15, 21, 32, 63, 75-77, 83, 85, 105
Organic flocculation supplemented with diatomaceous earth	106
Organic flocculation supplemented with ferric chloride	81, 107
Organic flocculation supplemented with metal silicates	106
Polyethylene glycol precipitation	63, 108
Ultrafiltration	63, 69, 82
Buffered salt solution‡	
Aluminum hydroxide precipitation	24, 28, 31
Glass (powdered)	27
Magnesium hydroxide precipitation	86
Membrane filter adsorption-elution	28, 34, 61, 84
Polyethylene glycol precipitation	79, 108

*Secondary concentration is also frequently referred to as second-step concentration or reconcentration.

†May be supplemented with materials such as specific amino acids; other protein-derived products have been evaluated as substitutes for beef extract (references for the latter include 76 and 103)

‡These types of eluants may include compounds such as alanine, EDTA, glycine, imidazole, lysine, and polysorbate 80.

TABLE 5
Effect of pH on simultaneous adsorption of three virus types onto different filter materials*

Filter Type	pH	Extent of Viral Adsorption†			
		f2	φX 174	P1	Average‡
A	3.5	81	84	76	80
	7.0	59	63	48	57
	8.0	ND§	ND	ND	
	8.5	40	74	54	56
B	3.5	99	80	84	88
	7.0	100	55	41	65
	8.0	99	66	42	69
	8.5	93	69	48	70
C	3.5	100	99	99	99
	7.0	88	61	98	82
	8.0	100	55	96	84
	8.5	100	56	95	84

*Adapted from reference 41

†Volumes of dechlorinated tap water simultaneously containing all three of the indicated viruses were adjusted to the desired pH and then passed through a sterile 47-mm-diameter filter of the indicated type. The calculated percentage of viral adsorption was based on the difference in titer of water samples before and after filtration. The values given in the table represent the average obtained in three independently conducted trials.

‡Average value for all three virus types

§ND—not done

much larger than 20 L because of the time it takes.

Direct physicochemical flocculation and phase separation. Several techniques fit into the category of direct physicochemical treatment for concentrating viruses from water samples. One general approach is to concentrate viruses through physical association with a flocculant precipitate that is induced by chemical supplementation of the water sample. The types of precipitates produced include aluminum hydroxide, aluminum phosphate, and calcium phosphate.^{53,65} Alternatively, proteinaceous materials may be added to the water sample and then precipitated by means of processes such as adding protamine sulfate⁵⁰ or lowering the pH.⁷⁰ Viruses in the water are concentrated through association with these proteinaceous precipitates.

Polyethylene glycol has been used in two different ways for concentrating viruses from water samples. The first of these, two-phase separation, consists of adding sodium chloride, dextran sulfate, and polyethylene glycol to the water. Ionic strength contributed by the sodium chloride causes polymeric incompatibility, resulting in the formation of two distinct aqueous phases, one containing most of the dextran sulfate and the other containing most of the polyethylene glycol. The densities of these two phases differ sufficiently that they can then separate gravimetrically. Viruses in the water sample undergo a partitioning during the generation and separation of these two phases and are largely concentrated into only one of them.^{51,54,65,66} The second technique involves placing the water sample into a dialysis bag and packing the whole unit in solid polyethylene glycol. The polymer then concentrates the sample by drawing water out of the dialysis bag, a process called hydroextraction.^{51,52}

Like ultrafiltration, the different physicochemical concentration techniques are used seldom, if at all, for directly concentrating viruses from water samples much larger than 20 L. The reason is that the time expended processing water samples by these techniques increases dramatically with sample size. Both techniques are often used, however, in a secondary mode to reduce the volume of eluates generated during the processing of water samples by directed adsorption. This process is variously referred to as secondary concentration, second-step concentration, or reconcentration.

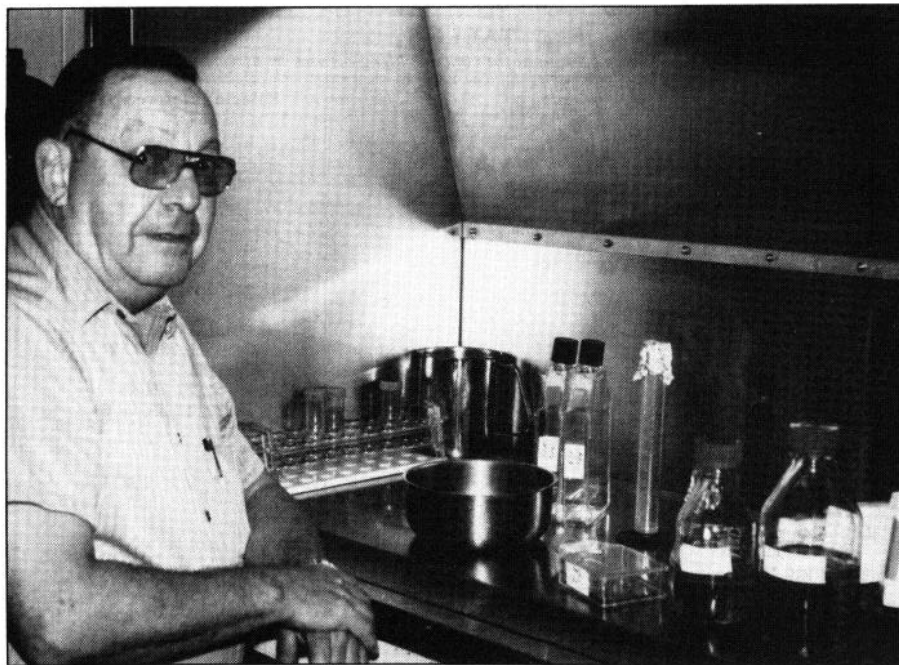
Affinity chromatography. Affinity chromatography consists of passing an aqueous solution through a column of polysaccharide gel material bearing highly specific, purified antibodies that can selectively bind viruses or some other target antigen from the fluid. This binding process is reversible, and the

bound antigens can subsequently be released by passing a smaller volume of a second aqueous solution through the column. The second solution differs from the first in terms of ionic strength and is formulated to reduce the favorability of antigen-antibody binding reactions. Although this technique certainly merits mentioning, its use is limited with regard to recovering viruses from tap water or environmental freshwaters because it is costly and requires a large amount of preparation time and operational skill. In the literature, only one mention could be found of its use for recovering viruses from water samples.⁵⁵

Directed adsorption combined with secondary concentration. Directed adsorption has generally replaced the other types of concentration techniques listed in Table 2 for use in recovering viruses from large volumes of water. The different solid adsorbents used successfully for this purpose are listed in Table 3. These solids are divided into two groups, filter materials and granular solids. The types of filters that have been tested are yarn fiber that is wound around a hollow core to form a depth filtration cartridge,^{31,34,81} sheet filter materials used as flat layers,^{17,29,31,32,34,71,76-79} cartridges prepared as hollow tubes of filter material,^{32,77} and cartridges composed of pleated sheets of filter material.^{28,31} Viruses adsorb onto the filter matrix during the passage of viral-containing water samples through the filters. Recovery of the adsorbed viruses is normally achieved by subsequently passing an eluant through the filters or by dissolving the filter if it is made of alginate.²⁹

Granular solids are generally used in one of three modes: (1) batch utilization by which the granules are mixed into the water sample and then recovered using either filtration³⁰ or magnetic attraction,⁸⁹ the latter requiring that the granules be magnetic; (2) layers of granules either supported on or sandwiched between sheets of nonadsorbing flat filter material through which the water sample is passed;^{18,38} or columns^{26,60} or fluidized beds²⁷ of granules exposed to the water sample using a flow-through configuration. Subsequent desorption of viruses occurs during exposure of the granules to an eluant using one of these same three modes.

Of the many different types and configurations of virus adsorbents, those now preferred for use in recovering viruses from large volumes of water are wound cartridge filters⁸¹ and pleated cartridge filters²⁸ based on either glass fiber or nylon, including types that are positively charged, and columns of glass powder.²⁶ Also of interest is the use of sheet filter material that has been modified in situ either by a precipitation of metal hydroxides within the filter matrix²³ or by coating the filter with cationic



Cultures of mammalian cells were prepared using a laminar-flow filtered air hood.

polymers.⁴² The largest volumes of sample water that have been concentrated by means of cartridge filters and glass powder appear to be, respectively, 1,900 L³¹ and 500 L.²⁷ In comparison, the technique of using a granular adsorbent sandwiched between layers of sheet filter material has been used for concentrating volumes of water as large as 1,000 L.³⁸

In the choice between filters and glass powder columns, the filters seem to offer greater advantage because they are easier to transport and operate in the field. This capability eliminates the need for transporting awkward high-volume water samples back to a laboratory. Following the adsorption of viruses onto the filters, the filters can be transported, including postal shipment, back to the laboratory with little concern about harming either the filter or the adsorbed viruses.⁹⁰⁻⁹² Elution of the filters and subsequent processing of the eluates can then be performed under the more manageable conditions that exist in the laboratory.

Theory of directed adsorption, elution, and reconcentration. The history of virus adsorption to filters dates back to a study published in 1931 by Elford⁹³ who determined that during passage of virus suspensions through collodion (nitrocellulose) filters, viruses were removed from the solution by adsorption onto the surface of the filter matrix as well as by simple sieving action. The adsorption of viruses onto filters, and presumably other solid adsorbents as well, is governed both by electrostatic interactions, which predominate at lower pH levels (≤ 4) and by hydrophobic interactions, which predominate at higher pH levels (≥ 9).⁹⁴⁻⁹⁵ The chemical composition of the filter matrix and the

fluid in which the viruses are suspended influence the extent of attraction or repulsion between the viruses and the filter. Other important factors that relate to virus adsorption onto filters include the chemical composition of the filter matrix,^{71,96} the rate of water flow through the filter material,⁹⁷ and the ratio of filter pore diameter to virus particle diameter.⁹⁶ These factors are important because if the distance between a virus particle and the filter matrix is too great (i.e., the pore size is too large) or the flow rate of the sample through the filter is too great, then the extent of attraction between the virus particle and filter matrix may be insufficient for adsorption to occur.

Cliver⁴⁵ seems to deserve credit for first presenting the application of this adsorption phenomenon to concentrating viruses from water samples. Cliver also discovered that the adsorption of viruses to the membrane matrix was strongly inhibited by the presence of added proteins in the input virus suspension.^{45,96,98} He suggested that this inhibition resulted from a competition of the proteins and viruses for adsorption sites extant on the surface of the filter matrix.⁹⁸ This is indeed a reasonable conclusion in light of the structure of viruses, particularly those that are nonenveloped. Other types of soluble organics, such as humic and fulvic acids, can also interfere with virus adsorption.^{20,32,33,99} Means of facilitating the adsorption of viruses onto solid matrixes include first removing dissolved organic materials from the water samples by passage through a resin column^{34,44,63} and adding salts to the virus suspension.¹⁰⁰ These salts may include chlorides of sodium,¹⁰⁰ magnesium,^{18,20,44,63,92} or,

more effectively, aluminum.^{16,31,81,83,92} Virus adsorption can also be facilitated by adjusting the pH of a water sample, a process that can be performed either in a batching operation or in placing an in-line injector ahead of the filter.^{24,34,101} A pH of approximately 3.5 seems preferable for use with negatively charged filters,^{22,31,78,83} versus the near neutrality (pH = 7) used for the more positively charged filters.^{22,28}

Cliver also discovered that the adsorption process was reversible and was able to recover viruses from filters by eluting them with a proteinaceous solution in the form of diluted blood serum.⁴⁵ Evidence collected from several studies suggests that eluants can be divided into at least two categories based on their mode of action.^{56,95,102} The first category is proteinaceous materials, which simply compete with the proteins of the virus particles for binding sites on the adsorbent.⁵⁶ Beef extract is now the most predominantly used eluant material in this category, although other proteinaceous products may prove to be suitable substitutes.^{75,76,103} The second category consists of compounds that alter the favorability of adsorption.^{94,95,102} These eluants include solutions that contain various active substances. Among them are chaotropic agents like glycine or trichloroacetic acid,⁹⁵ detergents,^{*,102} and EDTA serving as a chelating agent for metal cations.¹⁰²

Both categories of eluant may use elevated pH,^{17,18,24,28,70,104} which would help decrease electrostatic attraction between the viruses and adsorbents.⁹⁴ Table 4 lists the two categories of eluants along with the different approaches that have been used for secondarily concentrating them. The first category, proteinaceous eluants, is represented in Table 4 by beef extract solutions. The second category of eluants is represented under the heading of buffered salt solutions.

With the exception of organic flocculation, the various techniques referenced in Table 4 for secondary concentration of eluates have been discussed earlier in this article. Organic flocculation, introduced by Katzenelson and his co-workers,¹⁰⁵ is performed by lowering the pH of the eluate, during which the proteinaceous material supplied by the beef extract spontaneously precipitates. Viruses contained in the beef extract become associated with this precipitate, which can subsequently be collected by centrifugation and dissolved in a small amount of higher-pH buffer. The process by which viruses are concentrated during this technique may result from a binding of the naturally precipitating material onto the surface of the viruses at higher pH levels where the material is in solution. This bound material would then cause the viruses to aggregate as the pH of the solution is lowered to induce

TABLE 6
Effect of eluant composition on viral recovery by combined adsorption-elution concentration using different filter types*

Filter Type	Eluant‡	Efficiency of Viral Recovery†			
		f2	φX 174	P1	Average§
A	1 M NaCl, pH 7.0	1	36	2	13
	1 M NaCl, 0.1 percent polysorbate 80, pH 7.0	3	44	20	22
	0.5 M EDTA, 0.1 percent polysorbate 80, pH 7.0	40	33	33	35
B	3.0 percent beef extract, pH 9.5	11	6	19	12
	1 M NaCl, pH 7.0	0	3	20	8
	1 M NaCl, 0.1 percent polysorbate 80, pH 7.0	3	4	21	9
C	0.5 M EDTA, 0.1 percent polysorbate 80, pH 7.0	61	17	38	39
	3.0 percent beef extract, pH 9.5	44	2	8	18
	1 M NaCl, pH 7.0	0	5	6	4
	1 M NaCl, 0.1 percent polysorbate 80, pH 7.0	0	32	32	21
	0.5 M EDTA, 0.1 percent polysorbate 80, pH 7.0	16	27	51	31
	3.0 percent beef extract, pH 9.5	14	5	47	22

*Adapted from reference 41

†The NaCl, NaCl-polysorbate 80, and EDTA-polysorbate 80 eluants were buffered, using imidazole added to a final concentration of 0.05 M.

‡Volumes of dechlorinated tap water simultaneously containing all three of the indicated viruses were adjusted to the desired pH (3.5 for type A and 7.0 for types B and C) and then passed through a sterile 47-mm-diameter filter of the indicated type. Adsorbed viruses were recovered by passing 10 mL of the indicated eluant through the filter. Calculation of viral recovery was based on the amount of virus present in the eluant, expressed as a percent of that contained in the water sample before filtration. Values given represent the average of three independently conducted trials.

§Average value for all three virus types

precipitation.¹⁰⁹ This mechanism differs from true coprecipitation, in which neither of the involved materials can precipitate on its own. A pH of approximately 3.5 appears optimal for recovering viruses from beef extract eluates by organic flocculation.^{83,105}

Comparison of techniques for concentrating viruses using membrane filters. The efficiencies of virus adsorption by different filter materials are compared in Table 5. The efficiencies of adsorption in combination with subsequent elution are compared in Table 6. The viruses f2 and φX 174 are bacteriophages that infect *Escherichia coli*. The procedures for their propagation and assay have been described by Ward and Mahler.¹¹⁰ The virus designated P1 is human poliovirus type 1, a human enteric virus. The procedure for propagation and assay of this virus has been described by Benton and Hurst.¹¹¹

Of the different filter types mentioned in Tables 5 and 6, type A† is composed of glass fiber plus an epoxy resin binder, type B‡ is composed of charged glass fiber, and type C§ is composed of charged nylon. The type A material is considered electronegative, whereas the other two types of filter material are considered electropositive. The evaluations represented in these two tables were

conducted using water samples that simultaneously contained all three viruses. The viruses were then differentiated on the basis of selective assay procedures.⁴¹ These results demonstrate that the adsorption process is often far more efficient than the subsequent desorption. The use of these two processes in conjunction is often referred to as adsorption-elution. This process is sometimes called membrane chromatography when filters are used as the adsorbent.⁴⁵ Although the incorporation of EDTA into filter eluants can increase their efficiency, as demonstrated by the results shown in Table 6, a note of caution is important regarding its use for rotaviruses or reoviruses: EDTA can destroy the infectivity of these two virus groups by removing the outer shell layer of their protein capsids.

Virus assay techniques

Stages in the viral assay process. The various procedures that have been used for detecting viruses in concentrates prepared from environmental samples are outlined in Table 7. With the possible

*Such as Tween 80, J. T. Baker Chemical Co., Phillipsburg, N.J.

†Duo-Fine, Filterite, Timonium, Md.

‡Virosorb 1-MDS, AMF Cuno, Meriden, Conn.

§Posidyne N66, Pall Trinity, Glen Cove, N.Y.

exception of direct electron microscopy, each of these can be separated into three parts or stages. The first stage consists of the target material that is recognized by a given technique. In the case of electron microscopy, the target material is virus particles, termed virions, that appear intact. Some assay techniques detect only infectious virions, a category that may differ from those virions recognizable by electron microscopy as visually intact but somehow damaged or deficient so that they lack infectiousness. Still other techniques detect only either viral proteins or nucleic acids. The second stage of the various detection methods consists of target recognition; this is considered a nonvisual step. Target recognition, in turn, facilitates the third stage of the detection methods, visualization of the target material. Target visualization may be direct, as in observing virions by electron microscopy, or indirect, as in observing changes induced within host cells as a result of successful viral infection. Indirect visualization may consist of detecting alterations in cellular morphology, including outright death, or the production of progeny viral proteins or nucleic acids.

In general the infectivity of animal viruses contained in environmental samples is examined using cultures of animal cells that are prepared in the laboratory, rather than inoculating live animals. Figure 1 compares the relative sensitivities and required completion times for the assay techniques used in titrating viruses recovered from environmental samples.

Electron microscopy. The two different approaches for electron microscopy, direct and immune, are presented in Figure 1 in terms of the quantitation of physical viral particles. In contrast, all other viral assay techniques mentioned are represented in terms of their relative sensitivity for detecting viral infectious units. This distinction is important because for many different types of viruses, not all particles in a given preparation may be capable of producing an infection in host cells. For some types of bacteriophages, the ratio of viral particles to infectious units can be as little as between 1 and 2.5. For the various types of animal viruses, the

ratio of viral particles to infectious units can range from 2.6 to more than 100.¹¹²

Both categories of electron microscopy involve the examination of virus preparations after they have been negatively stained using solutions of electron-opaque metals. In the case of immune electron microscopy, the viruses are incubated in preparations of diluted antibodies before they are negatively stained. The virions in the sample normally aggregate during this incubation if a serological identity exists between them and the antibodies. Neither direct examination nor immune electron microscopy is practical for use with environmental samples because both require that extremely large numbers of viral particles be processed into volumes of, typically, less than 1 mL before they are stained for observation. Furthermore, bacterial viruses and other natural materials present in environmental concentrates could interfere with visualizing animal viruses by electron microscopy.

Cytopathogenicity and plaque assays. Cytopathogenicity assays are based on the appearance of visibly observable, characteristically recognizable changes in the morphology of living host cells that result from the process of viral infection. Such changes, termed "cytopathogenic effects," can be assessed by microscopic examination of the infected cells. Plaque assays detect the production of focal areas of death within an infected culture of cells. Detecting these areas of cell death is often facilitated by exposing the culture of cells to a vital stain such as neutral red. At modest concentrations neutral red does not interfere with cellular viability, yet it causes live cells to appear pink and dead cells to appear colorless. Plaques, which represent the focal areas of cell death, are normally detected by visual examination of the cultures without the aid of a microscope.

Explanations of the procedures used in assaying virus preparations by either cytopathogenicity or plaque production, along with specific information on their use with environmental samples and comparisons of their relative effectiveness for this purpose, can be found in references 111 and 113-117. Recent advances for these types of assays include the deliberate use of mixed cell

types within single cultures that are to be inoculated with virus samples. Another example is the treatment of cell cultures with nucleotide analogs such as iododeoxyuridine before the introduction of sample material, both of which can increase assay sensitivity.¹¹¹

Immunological assays. Immunoassays are based on an identity binding between antibodies, used as "probes," and specific "target" antigens of interest to the observer. This identity binding is a natural recognition process and serves as an aid in determining the presence of the target antigens. In the case of viruses, such target antigens may be serologically distinctive portions of either proteins, glycoproteins, or glycolipids. Depending on the laboratory protocol followed, these antigens may be suspended in aqueous solutions, trapped on filters, or associated with infected cells. The antibodies used as probes may first be "tagged" or "labeled" to facilitate subsequently identifying the presence of target antigens with which the antibodies bind. Methods used for the tagging of antibodies include chemically incorporating radioactive isotopes into the molecular structure of the antibodies, which can then be detected by the isotope emissions. Alternatively, the antibodies may be covalently linked to compounds such as fluorescent dyes and functional enzymes. The dyes fluoresce when illuminated at the proper wavelength, normally in the near ultraviolet portion of the spectrum.

When dealing with environmental samples, antibodies conjugated to fluorescent dyes are often used to detect the production of progeny viral proteins within individual cells of an infected host culture. This approach is termed cell immunofluorescence. During performance of the immunofluorescence assay, virally infected cultures of host cells are first exposed to a solution containing fluorescently tagged antibodies, termed probes, whose immunological reactivities are known. During this exposure the probe antibodies bind to the cells wherever the target viral antigen is present. The same cells are later examined using a specially adapted microscope that provides illumination at the proper wavelength for inducing the dye to fluoresce. The resulting fluo-

TABLE 7
Outline of steps involved in viral assay procedures

Stages	Direct Electron Microscopy	Immune Electron Microscopy	Cytopathogenicity or Plaque Assay	Immunological Assay	Nucleic Acid Hybridization
Target material	Virions	Virions	Infectious virions	Viral proteins	Viral nucleic acids
Target recognition	None	Antibody-mediated aggregation	Infection of appropriate cell type	Antigen-antibody binding	Nucleic acid sequence recognition
Target visualization	Morphology of virions	Aggregations of virions	Characteristic cytomorphology or cell death	Radiation count, autoradiogram "spots," fluorescence, or color change	Radiation count, autoradiogram "spots," fluorescence, or color change

rescence allows a recognition of those cells in the culture that were virally infected.^{41,117-119}

Immunological detection assays that use antibodies tagged with enzymes are broadly termed enzyme immunoassays. These enzyme-tagged antibodies can also be said to serve as probes. Horseradish peroxidase is one example of the enzymes used as tags for this purpose. Enzyme-tagged antibodies can be used in assays for the detection of viral antigens in solution, blotted onto filters, or associated with infected cells. Evidence of the desired antigen-antibody binding reaction is subsequently provided by measuring a chemical reaction facilitated by the enzyme. Very often the observed reaction involves conversion of a colorless substrate into a colored product.

The use of enzyme-tagged antibodies for detecting antigens in solution is termed ELISA, an acronym for enzyme-linked immunosorbent assay. References 119 and 120 discuss the use of ELISA to detect viral antigens present in environmental samples. One notable variation of the ELISA technique for detecting viruses in environmental samples relies on first trapping the viral antigens onto filter paper, then performing the ELISA test in situ on that filter.¹²¹ This approach can be termed a blot enzyme immunoassay. The use of enzyme immunoassays for detecting viral antigens directly associated with the infected cells of a host culture is similar to cell immunofluorescence and is, likewise, studied microscopically. In this article, this assay technique is referred to as a cell enzyme immunoassay. Payment and Trudel^{107,122} have provided examples of the use of cell enzyme immunoassays for detecting viruses in environmental samples and compared its sensitivity relative to cytopathogenicity assays. Kurstak et al¹²³ have prepared an excellent review on the use of enzyme immunoassays and other related procedures in virology.

Nucleic acid hybridization. Nucleic acid hybridization assays are based on the use of homologous nucleic acid materials as probes to bind with, or hybridize to, specific target nucleic acids and thereby facilitate the detection of those target nucleic acids. Hybridization, in this sense, is a physical pairing by means of hydrogen bonding between corresponding nucleotide sequences on those nucleic acid strands that respectively represent the probe and target. Depending on the assay protocol, the target viral nucleic acid sequences may variously be freely suspended in an aqueous solution, bound on filter materials, or associated with virally infected cells. Evidence of the desired hybridization (binding) reaction between the probe and targets may be provided by one of

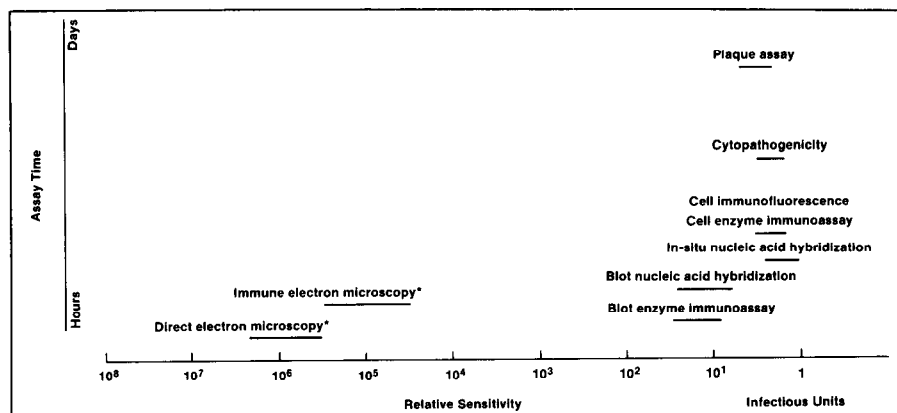


Figure 1. Comparison of viral assay techniques (*—horizontal bars under the various assay techniques represent the approximate range of their sensitivities expressed in terms of physical virus particles for the electron microscopy procedures and in terms of viral infectious units for all other assay techniques)

TABLE 8
Assay titers* for indigenous adenoviruses in concentrates from raw sewage when using HEP-2 cells†

Sample Designation	Cytopathogenicity	Immunofluorescence	In Situ Hybridization
1	33	51	53
2	33	51	107
3	51	80	60
4	46	44	82
5	50	67	83
6	54	47	60
7	46	63	73
8	61	25	70
9	40	25	73
10	58	47	47
Average	47.2	50.0	70.8

*All titers are expressed in terms of infectious units per millilitre of concentrate. Titer values for immunofluorescence and in situ hybridization are directly calculated in terms of infectious units per millilitre. The titer values obtained for cytopathogenicity assays, normally expressed in terms of infectious units per millilitre as a 50 percent endpoint, were divided by a factor of 2 to obtain endpoints expressed in terms of infectious units per millilitre.

†Adapted from reference 117

several methods. The first of these involves the detection of emissions from radioactive isotopes incorporated within the probe nucleic acid material.^{124,125} A second, two-step method involves the use of probe nucleic acids that contain nucleotides tagged, or joined by covalent linkage, to markers such as biotin. In this case, the biotin moieties attached to the probe nucleic acids can, in turn, serve as binding sites for molecules of avidin, which are themselves covalently linked to an enzyme such as horseradish peroxidase. Avidin is a natural compound that binds to biotin with high affinity. In this type of assay, the bound nucleic acid probe material is subsequently detected, as is done for enzyme immunoassays, by measuring the enzyme-mediated conversion of added substrate into products. The use of nucleic acid probes to directly detect viral genomic nucleic acid produced within infected cells is termed in situ nucleic acid hybridization. This process is conceptually similar to using antibodies as probes for detecting viral antigens associated with infected cells. The major difference is whether the target material consists of viral antigens or viral genomic nucleic acids. The use

of nucleic acid probes for detecting viral nucleic acid that has been trapped on filter material is termed blot nucleic acid hybridization. A more detailed discussion on the use of nucleic acid probe hybridization assays for virus detection has been published by Norval and Bingham.¹²⁶

Comparison of viral assay methods. One important point to consider regarding the use of different assay techniques for identifying viruses in environmental samples, and particularly for viral particles recovered from samples subjected to drinking water treatment, is whether the assay result represents infectivity. None of the two types of electron microscopy procedures, the solution ELISA, nor the blot styles of immunoassay and nucleic acid hybridization assay can, by themselves, reveal this most important piece of information. Their results are instead qualitative or semiquantitative. The other methods compared in Figure 1 are directly usable for quantitating viral infectivity because they are based on detecting changes within host cells that are specifically caused by viral replication. The various changes measured are the disruption of normal cytomorphology

(cytopathogenicity), overt cell death (plaque assay), and the production of either viral-specific antigens (immunofluorescence and enzyme immunoassay) or nucleic acid (in situ hybridization). The electron microscopy, solution ELISA, or blotting techniques could be used for estimating the infectivity of viruses present in samples if a part of the sample is examined directly as collected and another part of the same sample is then inoculated into a host cell system supporting the production of viral products that could later be measured by the same type of assay.

Cytopathogenicity, cell immunofluorescence, and in situ hybridization for the detection of indigenous adenoviruses present in concentrates produced from environmental samples are compared in Table 8. These data indicate that the overall numbers of infectious viral units detected by either cytopathogenicity or immunofluorescence were approximately equivalent. The number of viruses detected by in situ hybridization was approximately 40 percent greater than that revealed by the other two techniques. Analysis of these results by means of the paired two-tailed *T*-test revealed that the differences in mean values between the titers obtained by cytopathogenicity and immunofluorescence were not significantly different ($p = 0.67$). The mean value of the titers obtained by in situ hybridization were, however, significantly different from those obtained by either cytopathogenicity ($p = 0.010$) or immunofluorescence ($p = 0.003$). This information suggests that in situ hybridization is superior to either cytopathogenicity or immunofluorescence because of both its greater sensitivity (Table 8) and the speed with which the assay can be completed (Figure 1).

A recent improvement in virus detection methodology, suitable for use in these types of viral assays, relies on the incorporation of selectively acting antiviral compounds such as guanidine into the medium used for maintaining cell culture viability.¹¹⁷ Guanidine suppresses enterovirus replication while not deleteriously affecting the replication of adenoviruses¹²⁷ and thus imparts a measure of specificity into the assays.

Summary

This article, which reviews the subject of detecting viruses in water, encompasses two topics. The first topic consists of methods used for concentrating viruses from large volumes of water into smaller, more manageable volumes. The second topic consists of assay methods used for examining viruses contained in the concentrated samples. Many of the references cited in this article contain flow diagrams that outline the techniques discussed.

Viruses are most suitably concentrated by directed adsorption onto the surface of either a filter material or granular solid. Recovery from the surface is achieved during an elution treatment that reverses the adsorption process. As a result, the viruses are contained in a smaller volume of eluant fluid, which may or may not be further processed by a secondary concentration technique. The choice of a technique for quantitating viruses recovered by the concentration process often involves balancing the relative merits of detection sensitivity versus the time required for assay completion. This article presents information on the actual target materials detected by the various assay techniques and information on evaluating whether a particular type of assay procedure provides a direct measure of viral infectivity. Variations of the nucleic acid hybridization assay may represent the optimum balance for viral assay techniques by simultaneously combining sensitivity, determination of viral infectivity, and rapidity with which the assay can be completed.

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This document has been reviewed and approved in accordance with US Environmental Protection Agency policy. Approval does not signify that the contents reflect the views or policies of the agency, and mention of trade names or products does not constitute endorsement or recommendation for use.

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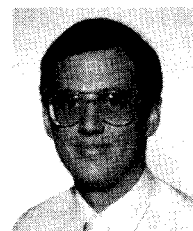
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About the authors:

Christon J. Hurst is a microbiologist with the US Environmental Protection Agency (USEPA), 26 W. Martin Luther King Dr., Cincinnati, OH 45268. He has 15 years of research experience in environmental microbiology, with primary involvement in the development and evaluation of methods for detecting viruses present in water, wastewater sludges, and soils, and the application of modeling techniques in studying the environmental fate of viruses. Hurst is a graduate of the University of Cincinnati (Ohio) with a BS and of the Baylor College of Medicine (Houston, Texas) with a PhD. William H. Benton is a biological laboratory technician and Ronald E. Steller is a microbiologist, both with the USEPA in Cincinnati.