Recovery of Small Quantities of Viruses from Clean Waters on Cellulose Nitrate Membrane Filters

GERALD BERG, DANIEL R. DAHLING, AND DONALD BERMAN
Water Quality Office, Environmental Protection Agency, Cincinnati, Ohio 45226

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A method is described for quantitatively recovering small amounts of viruses from large volumes of buffered, distilled water. Development of the method was motivated by the anticipated need for testing large volumes of renovated sewage for viruses. The method consists of adsorbing viruses onto cellulose nitrate membrane filters (0.45 µm pore size) from water containing sufficient Na₂HPO₄ to produce a molarity of 0.05 and sufficient citric acid to produce a pH of 7, and eluting the adsorbed viruses in 3% beef extract under extended sonic treatment. Complete recovery of poliovirus 1, echovirus 7, and coxsackievirus B3 resulted when less than 100 plaque-forming units were added to 1-liter quantities of water. Recoveries of reovirus 1 were almost as good. Preliminary studies indicate that good recoveries can be made from 25-gal quantities of water. The method described is efficient in waters of high quality and may be useful for recovering viruses in renovated, and perhaps in tap waters, but not in waters containing certain organic matter unless that matter is first removed.

Since viruses do not multiply outside of living susceptible cells, viruses excreted into sewage by man and those that find their way into waterways with rural runoff are always present in small numbers that diminish with natural dieoff and any treatment to which the water in which they occur is subjected. Small numbers of viruses are important, however, because even a single viable virion is capable of producing infection in a susceptible host (10, 13). Although ingestion of a single virion is likely to produce infection and not disease, people infected by small doses may excrete large amounts of viruses, and their contacts, more heavily infected, may suffer overt disease.

It is possible that viruses are introduced into communities by the water route and subsequently transmitted by direct contact. Infection and disease introduced and transmitted in this way, of course, would present to the epidemiologist a classical picture of direct contact transmission, and not the common source transmission that it would be (2).

The importance of small amounts of viruses, detectable at water intakes even with the inefficient techniques available today (G. Berg and D. R. Dahling, unpublished data), dictates the paramount importance of methodology for quantitative recovery of small amounts of viruses from large volumes of water.

Some years ago, Cliver (5) described a method for concentrating viruses from water by adsorbing the viruses onto cellulose nitrate membranes and subsequently eluting the viruses with serum. The method was satisfactory, but both adsorption and elution efficiencies were often poor and varied from test to test. Adsorption was subsequently shown to be complete in the presence of salts (G. Berg and D. R. Dahling, unpublished data; 14, 18). Herein we describe procedures that increase elution efficiency to 100% with several enteroviruses and to near that with a reovirus.

MATERIALS AND METHODS

Viruses. Poliovirus 1 (Mahoney LP), AGKP 43 R, was obtained from M. Ramos Alvarez after 35 passages in cynomolgus monkey kidney cells, and 8 passages in our laboratories in African green monkey kidney cells. Reovirus 1 (Lang), VP 4, was obtained from M. Ramos Alvarez after 11 passages in cynomolgus monkey kidney cells, 5 passages in rhesus monkey kidney cells, and 4 passages in Vero cells in our laboratories. Coxsackievirus B3, VP 3, was obtained from G. D. Hsiung after five passages in mice and four in monkey kidney cells. In our laboratories, the virus was passed three times in African green monkey kidney cells and three times in Vero cells. Echovirus 7 (Wallace), AGKP 8A1, was
obtained from M. Ramos Alvarez after 4 passages in cynomologous monkey kidney cells and 8 passages in African green monkey kidney cells in our laboratories. Viruses were stored in 1-ml amounts at –70°C, thawed shortly before each test, and diluted in sterile distilled water to give the concentrations required.

**Cell cultures.** A continuous line of African green monkey (Cercopithecus aethiops) kidney cells, designated Vero, was obtained in the 137th passage from Microbiological Associates, Inc. Passages 190 through 284 were used for this study. Stocks were maintained in 29-oz screw-capped bottles by weekly passage in minimal essential medium (MEM) containing L-glutamine and nonessential amino acids (Grand Island Biological Co.), and supplemented with 20% fetal calf serum. Cultures for virus assays were prepared from these stocks. Cells were suspended in medium 199 with Hanks salts (Grand Island Biological Co.) supplemented with 10% fetal calf serum, and 20-ml quantities were dispensed into screw-capped prescription bottles (6 oz). The cell concentration in each culture was 10⁸ to 2 × 10⁹. After 3 days, the growth medium was replaced with maintenance medium consisting of Earle's balanced salt solution (EBSS) containing 0.5% lactalbumin hydrolysate, 0.22% NaHCO₃, and 2 to 3% fetal calf serum (pH 7.6). On the day of a test, cultures were washed once with EBSS containing no serum, drained, and inoculated. All media contained 100 units of penicillin, 100 µg of streptomycin, and 1.0 µg of amphotericin B per ml.

All cultures were grown and maintained at 36 to 37°C.

**Virus assays.** Virus titrations were done by the plaque method (7). Titers were expressed as plaque-forming units. Washed, drained cultures were each inoculated with 1 ml of test suspension and incubated at room temperature for 2 hr. The infected cultures were then overlaid with a medium consisting of 83% MEM containing Hanks salts, L-glutamine, nonessential amino acids, 4% fetal calf serum, 0.45% NaHCO₃, 0.02% MgCl₂-6H₂O, 0.003% aqueous neutral red, 2% sterile whole milk (added just before the cultures were overlaid), and an equal volume of 1.8% Isolagar no. 2 (Colab Laboratories, Inc.). This overlay also was supplemented with 100 units of penicillin, 100 µg of streptomycin, and 1.0 µg of amphotericin B per ml of medium. The agar portion of the medium was warmed to 43°C and the liquid portion to 37°C, and the two were mixed immediately before the cells were overlaid. Plaques were counted and marked permanently daily to prevent underestimates that result from overcrowding (4).

**Sonic treatment.** Sonic treatment was applied with a Blackstone Ultrasonic Cleaner manufactured by Blackstone Ultronics, Inc., Sheffield, Pa. The sonic oscillator consisted of a solid state 60-cycle, 1,000-amp, model SS-3 generator with an input voltage of 117 ± 7 v, an output power of 300, and an output frequency of 22 ± 3 kc. The model HT 11.2 tank had piezo-electric transducers, an operating frequency of 23 ± 1 kc, and an active operating surface area of 6 by 13.5 inches (ca. 15.2 by 34.3 cm). Of the 300 w generated, 84°C or about 252 w was the maximum output through the transducers. The tank was cooled by a constant flow of cold tap water maintained at a level of ca. 0.13 to 0.25 inch (ca. 0.32 to 0.64 cm) inside the tank. The apparatus was operated at maximum power and peak activity.

**Beef extract.** Powdered beef extract (lots 1201 and 1372) obtained from Colab Laboratories, Inc., was prepared as a 3% solution in distilled water, and then autoclaved at 121°C for 15 min.

**Pancreatin.** A 1% solution of pancreatin (Colab Laboratories, Inc.), prepared by dissolving 1 tablet in 50 ml of distilled water at 37°C, was made bacteria-free by membrane filtration and further diluted as required for the experimental procedures.

**Buffers.** McIlvaine’s buffer was prepared from reagent grade dibasic sodium phosphate (Na₂HPO₄·7 H₂O) and citric acid (H₃C₆H₅O₇). Borate buffer was prepared from reagent grade boric acid (H₃BO₃), potassium chloride (KCl), and sodium hydroxide (NaOH).

**Membranes and filtration equipment.** Filtration equipment consisted of stainless-steel pressure vessels, stainless-steel pressure filter holders (47 mm), and the regular hydroaluminum stainless-steel filter holders, all manufactured by the Millipore Corp.

The membrane filters used in these tests were type MF (Millipore Corp.), which are composed of mixed esters of cellulose and consist primarily of cellulose nitrates (6). Unless otherwise noted, filters were 47 mm in diameter and had a 0.45 µm pore size. Filters and all related equipment were sterilized by autoclave at 121°C for 15 min.

**Test procedure.** Each virus suspension was prepared by adding 1 ml of a diluted virus stock for each liter of buffer. Each 1-liter quantity of suspension was passed through a cellulose nitrate membrane filter (under suction unless otherwise noted), and each filter was sonically treated in 10 ml of 3% beef extract in a 180-ml beaker for a period of time. Control filters were soaked in 3% beef extract for an appropriate period without sonic treatment. The amount of virus in each liter of suspension was determined by titration of a 10-ml suspension (1 ml per cell culture), consisting of 2 ml of the virus stock suspended in 18 ml of 3% beef extract sonically treated for as long as the test sample was treated.

**RESULTS**

In earlier studies, we tested several eluants with variable success (3). A single lot of undiluted calf serum consistently eluted 100% of poliovirus 1 from membranes, but eluted other viruses inefficiently. Another lot of calf serum tested efficiently with another virus, but eluted poliovirus 1 less effectively. Similar results were obtained with nutrient broth. We quickly found that beef extract was the active component of nutrient broth. The beef extract pastes (Difco) available also produced variable elution patterns.

Subsequently, a dehydrated beef extract preparation (Colab Laboratories, Inc.) became available which made it possible more accurately to
measure its concentration. But elution with this product was also incomplete.

On the assumption that elution was an adsorption-replacement situation, other methods were sought that would facilitate replacement of viruses tenaciously adsorbed on the cellulose nitrate filters. Efforts with homogenization, agitation, and grinding were not successful. Sonic treatment, however, met with considerable success.

Effect of salt concentration and pH on adsorption; effect of sonic treatment on elution. It has been established that salt is required for efficient adsorption of viruses to cellulose nitrate membranes (G. Berg and D. R. Dahling, unpublished data; 14, 18). Our own choice of salts has been the constituents of McIlvaine's buffer, Na₂HPO₄ and citric acid. To determine optimal adsorption conditions for poliovirus 1, we tested this buffer at several concentrations and at several pH levels with the test procedure described above. Simultaneously, we tested for elution of the virus from the membranes in 3% beef extract with and without sonic treatment. Table 1 shows that without sonic treatment complete recovery of the virus from the membranes did not occur in 3% beef extract when treated with modified McIlvaine's buffer for 20 min.

| Eluant treatment | Molarity of buffer* | PFU inoculated* | PFU recovered at pH | 4 | 5 | 6 | 7 | 8 | 9 |
|------------------|---------------------|----------------|---------------------|---|---|---|---|---|---|
| Not sonically treated | 0.005               | 47             | 42                  | 14 | 6 | 19 | 10 | 0  |
|                   | 0.05                | 38             | 25                  | 19 | 20 | 31 | 31 | 11 |
|                   | 0.2                 | 56             | 39                  | 47 | 42 | 39 | 23 | 31 |
| Sonically treated  | 0.005               | 63             | 34                  | 27 | 29 | 22 | 0  |    |
| for 20 min        | 0.05                | 45             | 27                  | 28 | 28 | 33 |    |    |
|                   | 0.2                 | 54             | 50                  | 39 | 52 | 42 | 46 |    |

a McIlvaine's buffer for pH 4 to 7, borate buffer for pH 8 and 9. The buffer concentrations are expressed in terms of the molarity of the Na₂HPO₄ and H₃BO₃. Sufficient citric acid or NaOH was added to adjust pH.

b Controls consisted of 2 ml of virus suspension in 18 ml of 3% beef extract sonically treated for 20 min, or not sonically treated, before inoculation onto cell cultures.

7, recovery efficiency diminished. A higher salt concentration, however, may have improved recovery at pH levels other than 7 also. Since all of the elutions were done at the pH of the 3% beef extract, it is clear that the salt concentration and pH of the water in which the virus was suspended were factors critical for adsorption and not for elution.

In all subsequent experiments, test waters were buffered with McIlvaine's buffer and in final concentration, contained 0.05 M Na₂HPO₄ and sufficient citric acid to bring the pH to 7.

Effect of sonic-treatment period on elution of viruses from cellulose nitrate membrane filters. With the procedure described above, four different viruses were adsorbed from McIlvaine's buffer onto membranes and eluted. The results of these tests follow.

Poliovirus 1. Table 2 shows that all of the
poliovirus 1 eluted from the membranes only after 20 min of sonic treatment in 3% beef extract. This treatment for 40 or 60 min also liberated all of the virus. In one instance, a 45-min soaking of the membrane in 3% beef extract released all of the virus without sonic treatment, but in the other tests shown, and in tests not shown, complete elution did not occur without this treatment.

**Coxsackievirus B3.** Table 3 shows that all of the coxsackievirus B3 eluted also only after 20 min of sonic treatment in 3% beef extract. This treatment for shorter periods brought about incomplete elution of the virus from the membranes. A control membrane soaked in beef extract for 45 min without sonic treatment yielded only about 66% of the filtered virus.

**Echovirus 7.** Elution of echovirus 7 from cellulose nitrate membrane filters by sonic treatment of filters in 3% beef extract is shown in Table 4. Complete elution of virus followed 20 min of sonic treatment in beef extract, and in some instances almost complete elution occurred after 5 min. This treatment of filters for 40 or 60 min also brought about complete elution of virus. About 90% of echovirus 7 was eluted from the filters by soaking them in beef extract for 45 min.

**Reovirus 1.** Elution of reovirus 1 from cellulose nitrate membrane filters by 3% beef extract required a longer period of sonic treatment than did elution of the enteroviruses. Complete elution was often achieved after 40 and 60 min of sonic treatment, but never in less time (Table 5). Pancreatin greatly increases plaque counts of reovirus 1 (20), and addition of pancreatin to the eluant may increase recovery of reovirus 1 from cellulose nitrate filters (Table 5).

**Effect of membrane porosity on the recovery of reovirus 1 on cellulose nitrate membrane filters.** Speed of filtration is related to the pore size of the membrane through which water is filtered. Since we anticipated eventually filtering

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**Table 4. Recovery of echovirus 7 on cellulose nitrate membrane filters**

| PFU inoculated* | Test no. | PFU recovered with sonic treatment for |
|-----------------|---------|---------------------------------------|
|                 |         | 0 min | 2 min | 5 min | 10 min | 20 min | 40 min | 60 min |
| 1,185+          | 1       | 1,010 | 874   | 974   | 1,078  | 1,328  |
| 1,185+          | 2       | 1,010 |       | 1,015 | 1,176  |
| 169+            | 3       | 159   | 53    | 163   | 132    | 204    |
| 169+            | 4       | 159   |       | 133   | 152    |         |
| 74+             | 5       |       |       | 73    |        |
| 45++            | 6       |       |       | 44    |        |
| 93+++           | 7       |       |       | 91    |        |
| 58+++           | 8       |       |       | 56    |        |

* Controls consisted of 2 ml of virus suspension in 18 ml of 3% beef extract sonically treated for 20+, 40++, or 60+++ min before inoculation onto cell cultures.

* Virions eluted from membrane filters for 45 min with 3% beef extract without sonic treatment.

**Table 5. Recovery of reovirus 1 on cellulose nitrate membrane filters**

| Pancreatin conc (%) | PFU inoculated* | Test no. | PFU recovered with sonic treatment for |
|---------------------|-----------------|---------|---------------------------------------|
|                     |                 | 0 min   | 2 min | 5 min | 10 min | 20 min | 40 min | 60 min |
| 0                   | 58              | 1       | 13    | 11    | 7      | 10     | 11     |
| 0                   | 58              | 2       | 13    |       | 11     | 10     | 30     |
| 0.033               | 32              | 3       |       | 4      | 11     | 10     | 67     |
| 0.033               | 44              | 4       | 30    | 13    |       | 67     |
| 0.167               | 48              | 5       | 40    | 8     | 13     |
| 0.167               | 62              | 6       | 67    | 8     | 18     |
| 0.008               | 89              | 7       | 67    | 8     | 18     | 56     |

* Controls consisted of 2 ml of virus suspension in 18 ml of 3% beef extract containing pancreatin in the concentration indicated, sonically treated for 20 min in tests 1 and 2 and for the same period as the membranes in all other tests.

* Virions eluted from membrane filters for 30 min with 3% beef extract and for 15 min with Earle’s balanced salt solution without sonic treatment.
TABLE 6. Influence of membrane porosity on recovery of reovirus 1 on cellulose nitrate membrane filters

| Sonic treatment (min) | PFU inoculated<sup>a</sup> | PFU recovered at various membrane pore sizes (μm) |
|-----------------------|----------------------------|-----------------------------------------------|
|                       |                            | 0.45 | 0.65 | 0.8  | 1.2  | 3.0  | 5.0  | 8.0  |
| 0                     | 59<sup>b</sup>             | 25   | 15   | 18   | 29   |      |      | 1    |
| 20                    | 59                         | 44   | 20   | 28   | 29   |      |      | 0    |
| 40                    | 60                         | 44   | 18   | 27   | 37   | 14   | 9    | 6    |
| 60                    | 81                         | 58   | 68   | 40   | 27   | 16   | 7    | 4    |

<sup>a</sup> Controls consisted of 2 ml of virus suspended in 18 ml of 3% beef extract sonically treated for 20, 40, 60 min.

<sup>b</sup> Virions eluted from membrane filters for 20 min in 3% beef extract without sonic treatment.

TABLE 7. Effect of pressure filtration on the recovery of poliovirus 1 on cellulose nitrate membrane filters

| Volume (liters) | Pressure (psi) | PFU inoculated<sup>a</sup> | PFU recovered with sonic treatment for 20 min | 40 min | 60 min |
|----------------|---------------|-----------------------------|----------------------------------------------|--------|--------|
| 1              | 2             | 73                          | 71                                           |        |        |
| 4              | 4             | 64                          | 64                                           |        |        |
| 6              | 6             | 70                          | 70                                           |        |        |
| 8              | 8             | 71                          | 71                                           |        |        |
| 10             | 10            | 57                          | 57                                           |        |        |
| Vacuum         | 14            | 43                          | 54                                           |        |        |
| 6              |               | 46                          | 47                                           | 41     |        |
| 8              |               | 47                          | 50                                           | 22     |        |
| 10             |               | 47                          | 48                                           | 50     |        |
| 12             |               | 47                          | 41                                           | 59     |        |
| 15             |               | 28                          | 30                                           | 50     |        |
| 4              | 40            | 39                          | 50                                           |        |        |
| 4              | 45            | 50                          |                                              |        |        |
| 4              | 51            | 50                          |                                              |        |        |

<sup>a</sup> Controls consisted of 2 ml of virus suspension in 18 ml of 3% beef extract sonically treated for the same period as the membranes.

For high-volume water sampling, pressure filtration must be used if the samples are to be processed in a reasonable period of time. The procedure for this test was that described above except that the virus test suspension was placed in a stainless-steel pressure vessel and forced through the membrane by compressed nitrogen gas. The results of tests with poliovirus 1 are shown in Table 7. Poliovirus 1 in water filtered through membranes (0.45 μm pore size) at pressures up to 15 psi was usually eluted completely by 20 min of sonic treatment in 3% beef extract.

In one test where 4 liters of water was filtered at 40 psi, complete recovery of virus occurred when the membranes were sonically treated in 3% beef extract for 20, 40, or 60 min (Table 7).

DISCUSSION

The need for sampling water for small amounts of viruses is a complex one, because viruses occur in different quantities in waters of different qualities. Sewage may contain upwards of 1,000 PFU/gal, receiving waters distant from outfalls may contain perhaps a few dozen PFU/100 gal, and renovated waters that have been subjected to good biological treatment and then to advanced chemical and physical treatment may contain less than 1 PFU/1,000 gal even before disinfectants are applied. For a community that consumes a million gallons of water daily, or far less for that matter, even 1 PFU of virus in every 1,000 gal is substantial consumption. Since viruses have been detected at water intakes in only 50-gal samples (G. Berg et al., unpublished data), the viral intake with surface waters must be considerable.

In sewage, which usually contains large amounts of viruses, the major problem is quantitation. Even when only a liter or two is tested, the solid materials which undoubtedly contain much, and perhaps most of the virus, are usually separated and discarded.
The methods that have evolved for recovering viruses from sewage, methods such as adsorption to Al(OH)₃ precipitates (19), protamine sulfate precipitation (B. England, Bacteriol. Proc., p. 194, 1970), and two-phase separation (11, 15), are small-volume methods not readily adaptable to large-volume sampling. None of these systems, fortunately, seems adversely affected by the large amounts of organic matter in sewage. Two large-volume techniques that have been developed, the membrane-filter procedure (5) and the polyelectrolyte method (9, 16), have been used for recovering viruses from sewage (8, 16, 17) but neither is efficient in this application (G. Berg et al., unpublished data; 12).

The viral recovery problem intensifies in receiving waters such as rivers and lakes, however, as dilution and destructive elements in the water environment reduce the numbers of viruses to levels that require analysis of large volumes of water, perhaps 100 gal or more. It is for situations as this that large-volume techniques are being developed.

Unfortunately, the polyelectrolyte technique, which is moderately efficient with poliovirus I in relatively clean waters, is not efficient with other enteroviruses, reoviruses, or adenoviruses (G. Berg, Proc. Nat. Speciality Conf. Disinfec., in press; B. England, personal communication). The effectiveness of the membrane-filter technique is greatly impaired by some kinds of organic matter which apparently react with the adsorption sites on the membranes and allow viruses to pass through.

Thus, the membrane-filter technique can be used only for clean waters, or for waters from which interfering substances have been removed. The need to detect viruses in renovated water has been our prime motivation for developing this technique. Removal of substances that interfere with adsorption of viruses to the membranes should be much easier with renovated water and tap water than with sewage.

A major problem with the membrane-filter method since its inception has been the erratic performance of elution techniques. Complete elution of large amounts of several enteroviruses from cellulose nitrate membrane filters was achieved reportedly by soaking the membrane in serum and then repeatedly circulating the serum through the membranes (18), or by treating the membranes in balanced salt solution containing 10% serum (17). With small amounts of viruses, however (less than 100 PFU/liter), only partial recovery of enteroviruses occurs with calf serum (G. Berg et al., unpublished data) or with beef extract (G. Berg et al., unpublished data; 14), with either of the physical aids used.

It is not clear whether uneluted virions are adsorbed more tenaciously than virions that do elute more readily, or whether the eluant simply does not easily reach all of the interstices in the depths of the membrane where virions may be entrapped. It does seem clear that the virions do not adsorb only to the surface of the membrane, but are trapped and adsorbed inside also (1). High-frequency sonic treatment may increase permeation of the eluant into the narrow channels where the virions are lodged, help break the bonds that hold them to the membrane, and disrupt the membrane, perhaps creating new channels through which virions may be liberated.

Enteroviruses were eluted with less sonic treatment than the reovirus required. It is conceivable that each of the larger reovirions adsorbed to more sites than the smaller enterovirions needed, and that the eluant could not gain access to adsorption sites under the relatively large, flat surface of the reovirus icosahedron that adhered to the membrane as easily as it could to adsorption sites under the smaller adhering, flat surfaces of the enterovirus capsids.

The increased sonic treatment time required for elution brought about by increased filtration pressure suggests that pressure forces virions deep into narrow passages in the membrane.

Increased elution was also brought about without sonic treatment by soaking membranes for long periods in beef extract. It is possible that soaking for a long enough time will elute virions that had not penetrated deeply into the membrane. It seems less likely that deeply penetrating enteroviruses, and perhaps even larger virions superficially imbedded, would be recoverable without application of disruptive energy.

Our studies now extend to volumes of 25 gal and more. By eluting viruses adsorbed to cellulose nitrate membrane filters with sonic treatment in 3% beef extract, we consistently recover less than 100 PFU of virus as efficiently from 25 gal of buffered, distilled water as we do from 1-liter quantities. Data obtained in tests with large volumes of water will be published later.

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