APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1984, p. 144–150
0099-2240/84/010144-07$02.00/0

Vol. 47, No. 1

Round Robin Investigation of Methods for the Recovery of Poliovirus from Drinking Water

J. L. MELNICK,[1]* R. SAFFERMAN,[2] V. C. RAO,[1] S. GOYAL,[1]† G. BERG,[2] D. R. DAHLING,[2] B. A. WRIGHT,[2] E. AKIN,[3] R. STETLER,[3] C. SORBER,[4] B. MOORE,[4] M. D. SOBSEY,[5] R. MOORE,[5] A. L. LEWIS,[6] AND F. M. WELLINGS[6]

Baylor College of Medicine, Houston, Texas 77030[1]; U.S. Environmental Protection Agency, EMSL[2] and HERL,[3] Cincinnati, Ohio 45268; University of Texas, Austin, Texas 78712[4]; University of North Carolina, Chapel Hill, North Carolina 27514[5]; and Epidemiology Research Center, Tampa, Florida 33614[6]

Received 25 April 1983/Accepted 20 October 1983

Six laboratories actively involved in water virology research participated in a methods evaluation study, conducted under the auspices of the American Society for Testing and Materials Committee on Viruses in the Aquatic Environment, Task Force on Drinking Water. Each participant was asked to examine the Viradel (virus adsorption-elution) method with cartridge-type Filterite filters for virus adsorption and organic flocculation and aluminum hydroxide-hydroextraction for reconcentration. Virus was adsorbed to filter media at pH 3.5 and eluted with either glycine buffer (pH 10.5) or beef extract-glycine (pH 9.0). Considerable variation was noted in the quantity of virus recovered from four 100-liter samples of dechlorinated tapwater seeded with low (350 to 860 PFU) and high (1,837 to 4,689 PFU) doses of poliovirus type 1. To have a more uniform standard of comparison, all the test samples were reassayed in one laboratory, where titers were also determined for the virus seed. Test results of the Viradel-organic flocculation method indicated that the average percentage of virus recovery for low-input experiments was 66%, with a range of 8 to 20% in two laboratories, 49 to 63% in three laboratories, and 198% in one laboratory. For the high-input experiments, two laboratories reported recoveries of 6 to 12%, and four laboratories reported recoveries of 26 to 46%. For the Viradel aluminum hydroxide-hydroextraction procedure, two laboratories recovered 9 to 11%, whereas four obtained 17 to 34% for low-input experiments. For the high-input tests, two laboratories reported a recovery of 3 to 5%, and four recovered 11 to 18% of the seeded virus. Each laboratory was also encouraged to test other methods reported to be useful and reliable, but which had not been widely used. The methods tested included magnetic iron oxide, "Virozorb" electropositive filters, Virozorb filters in combination with membrane filters, and membrane disks alone. The average virus recoveries for the four methods were 36, 20, 0.4, and 5%, respectively. Possible differences in quality of the water in which the virus was diluted and in the quality of the eluents are some of the variables suspected for the wide differences in virus recovery results from different laboratories. Nevertheless, there was sufficient consistency among four of the six laboratories for the task force to recommend the Viradel-organic flocculation procedure as a provisional method for the recovery of human enteroviruses from drinking water.

The need for a more thorough and systematic evaluation of the public health problem of virus transmission via drinking water has been emphasized (2, 16, 17, 30). Such an evaluation requires the availability and utilization of a simple and reliable method to quantitatively detect small numbers of viruses in large volumes of raw and finished drinking water.

In 1972 Wallis, Melnick, and their colleagues introduced the virus adsorption-elution (Viradel) method for concentrating low levels of virus from water (25, 26), and the method has been widely used since then (6, 8, 9, 11–14, 24). A modification of this procedure was introduced as a tentative method in the 14th edition of *Standard Methods for the Examination of Water and Wastewater* (1).

To establish the reliability of the proposed tentative standard method, the American Society for Testing and Materials Committee on Viruses in the Aquatic Environment initiated "round robin" comparative testing of the Viradel procedure as described by the Virology Section, Environmental Monitoring and Support Laboratory (EMSL) Cincinnati, U.S. Environmental Protection Agency (U.S. Environmental Protection Agency *Manual of Methods for Virology*, in preparation). During the first phase, the precision of the test procedure in the different laboratories was examined, rather than the sensitivity of the procedure. Thus, the virus load was intentionally high so that significant amounts of virus could be measured. The six laboratories participating in the testing program were encouraged to examine other methods that had been reported to be useful and reliable.

Uniformity was maintained in regard to two aspects: (i) seed virus was produced and distributed from a single pool of monodispersed poliovirus type 1 by the Department of Virology and Epidemiology of Baylor College of Medicine, and (ii) one-third volume of the sample concentrates from each participating laboratory and seed virus from Baylor were sent to the Environmental Protection Agency (EPA) EMSL Cincinnati virus laboratories for assay on BGM cell cultures. Differences existed with respect to (i) the quality of the tapwater in which the seed virus was diluted, (ii) the sources of beef extract and glycine eluents, (iii) the batch numbers of Filterite filters (Filterite Corp., Timonium, Md.), (iv) the BGM cultures used in the different laboratories, and (v) the plaque assay and cell culture procedures.

* Corresponding author.
† Present address: Veterinary Diagnostic Laboratory, College of Veterinary Medicine, University of Minnesota, St. Paul, MN 55108.

MATERIALS AND METHODS

Virus. The attenuated vaccine strain of poliovirus type 1 was used as the test virus. The virus was grown in BGM cells, concentrated, and partially purified by membrane chromatography (7, 12). It was filtered to remove viral aggregates and was considered to be monodispersed (27). The stock virus was prepared as pools and diluted in Tris-buffered saline containing 2% fetal calf serum and approximately 300 or 1,800 PFU of virus per ml. The virus pools were stored in 1-ml volumes at −70°C until used. The virus vials were assigned code numbers and dispatched on dry ice to the investigators.

Plaque assay. The details of the methods for quantifying enteroviruses were essentially those in use for several years (18). BGM cells grown in plastic flasks were used for plaque assay of poliovirus (5). The overlay medium consisted of Eagle basal medium, 2% fetal calf serum, 25 mM $MgCl_2$, 0.0017% neutral red, antibiotics, and 1% purified agar (Difco Laboratories, Detroit, Mich.). For purposes of assay, 0.1-ml samples of the concentrate were added to 12-cm^2 glass bottles, or 1- to 2-ml samples were added to 6-oz. (ca. 180-ml) flat bottles or 75-cm^2 plastic flasks. The viruses were adsorbed for 2 h at 36 ± 0.5°C. The inoculum was poured off, and the agar overlay was added. Plaques were marked and counted daily. Final counts were made on day 5 of incubation.

Virus adsorbents. Epoxy-fiber glass pleated cartridge filters of 25.4 cm length (Duo-Fine series; Filterite) and 0.45-μm nominal pore size were used.

Virus concentration procedures. (i) Filterite filters. Tapwater (100 liters) in a 100-gallon (ca. 379-liter) plastic container was dechlorinated with a final concentration of 0.05 mg of sodium thiosulfate per liter. The sample was acidified to pH 3.5 and conditioned with 0.0005 M $AlCl_3$. A 1-ml sample of virus from a coded vial was added, and the sample was mixed thoroughly by bubbling air. The virus-seeded water was forced through the Filterite filter with a water pump powered by a 0.75-horsepower electric motor at a rate of 10 liters/min. Duplicate experiments with low and high input levels were conducted on the same day. The filter was eluted with 1,600 ml of 3% beef extract-glycine (pH 9.0) or 0.05 M glycine (pH 10.5). The eluent remained in contact with the filter for approximately 1 min during passage. Eluates were immediately neutralized with 0.05 M glycine adjusted to pH 2.0.

(ii) Electropositive filters (Virozorb-1MDS). Positively charged, 25-cm-long, tubular filters (AMF-CUNO) made of cellulose, modified anion-exchange resin, and inorganic filter aids were used. Tapwater (100 liters) was collected in a 500-gallon Nalgene tank and dechlorinated with a 40% solution of sodium thiosulfate. The water was mixed by bubbling air, and its pH was noted. A 1-ml sample of virus was added, and the water was again mixed thoroughly. The sample was passed under positive pressure through the Virozorb filter. The filter was eluted with 1,600 ml of either 0.3 or 3% beef extract-glycine (pH 9.5), which remained in contact with the filter for 1 min. The pH of the eluate was immediately adjusted to 7.2 with 0.05 M glycine (pH 2.0). The primary eluate was reconcentrated by the organic flocculation method.

(iii) Electropositive and electronegative filter combination. Three filters were arranged in series. Dechlorinated tapwater (100 liters) at ambient pH was filtered through a Virozorb-1MDS filter, followed by a Zeta-Plus 60S filter (AMF-CUNO) with the pH of the water adjusted to 5.5. Then the pH was reduced to 3.5, and the sample was passed through a cellulose nitrate membrane (electronegative). All three filters had a nominal pore size of 0.45 μm and were eluted with 3% beef extract (pH 9.5). The eluate was further concentrated by polyethylene glycol hydroextraction.

Reconcentration procedures. All concentrates described below were divided into three samples and frozen at −70°C. One-third of the sample concentrate from each test run was assayed for virus in the individual laboratory, and one-third was run in the EPA EMSL laboratory. The remaining one-third was kept in reserve.

(i) Aluminum hydroxide precipitation plus hydroextraction. Neutralized glycine eluates (1,600 ml) were treated to contain 0.003 M $AlCl_3$. As this lowered the pH to between 4 and 5, the eluates were adjusted to pH 7.0 with 1 M sodium carbonate, and the fluids were stirred with a magnetic bar. The resultant floc was allowed to settle for 30 min. The supernatant fluid was removed by aspiration, and the remaining floc was pelleted by centrifugation at 1,000 × *g* for 3 min. Virus in the floc was eluted by mixing it with 3 volumes of 1 M glycine in fetal calf serum (pH 11.5). The mixture was centrifuged, and the supernatant was saved and neutralized by adding 1 M glycine (pH 2).

A dialysis tube was washed two or three times in sterile water, and one end of it was clamped. Neutralized eluate was introduced into the dialysis tube, and the open end was clamped. The filled dialysis tube was placed on a tray and surrounded by flakes of polyethylene glycol (molecular weight 20,000) at 4°C. The sample was hydroextracted until 10 ml of eluate remained in the tube. The outside of the tube was rinsed with distilled water, kept in cold phosphate-buffered saline at 4°C, and stirred with a magnetic bar for 1 h. The contents of the tube were poured into a sterile beaker; penicillin, streptomycin, and gentamicin were added. Details of the time taken for the reconcentration procedure, the volume of the final concentrate from different laboratories, and the cost of the materials are given in Table 1.

(ii) Organic flocculation. The primary eluate of beef extract-glycine (1,600 ml) was adjusted to pH 3.5 with 1 N HCl, and the sample was stirred slowly for 30 min to minimize foaming. This resulted in flocculation of the organic components, which could be collected by centrifugation at 2,500 × *g* for 15 min. The sediment is usually dissolved in 0.15 M Na_2HPO_4 (pH 9.0; 5 ml/100 ml of supernatant decanted). The concentrate was neutralized, and antibiotics were added.

(iii) Membrane disk filtration. The pH of the glycine primary eluate (1,600 ml) was adjusted to 3.5 with glycine (pH 2.0). The solution was filtered under positive pressure through 47-mm-diameter, 0.45-μm HA membrane filters (Millipore Corp., Bedford, Mass.). Virus was eluted with two 5-ml volumes of 0.05 M glycine (pH 10.5). The pH of the combined eluate was adjusted to 7.5 with glycine (pH 2.0). Fetal calf serum was added to the neutralized eluate to yield a serum concentration of 2%. Antibiotics were added to the eluate before storing it at −70°C. Difficulty was experienced in passing the 1,600 ml of primary eluate through a single 47-mm filter reconcentration step. A total of five 47-mm filter set-ups were used, resulting in a final concentrate of 50 ml.

(iv) Magnetic iron oxide adsorption-elution. The iron oxide used in these experiments was ferric ferrous oxide (black, Fisher Scientific Co., Pittsburgh, Pa.). The beef extract-glycine primary eluate (1,600 ml) was placed in a glass beaker and adjusted to pH 7.0. Two grams of iron oxide were added, and the sample was stirred intermittently with a glass rod for 30 min. A magnet was used to settle the iron oxide,

TABLE 1. Details of time and cost for the different methods investigated in the round robin testing for concentration of poliovirus from 100 liters of drinking water

Procedure	Time for reconcentration (h)[a]	Cost[b] ($)	Final vol of concentrate (ml)
Viradel with Filterite filters			
Aluminum hydroxide-hydroextraction	18	18	4–45
Organic flocculation	2	18	14–100
Membrane disk	2–3	18	50
Magnetic iron oxide	1.5	18	10
Viradel with Virozorb filters			
Organic flocculation	2	51	10–100
Electropositive followed by electronegative filters and hydroextraction	18	80	30

[a] Time taken for water conditioning, filtration, and elution of virus from filters is common in all methods, i.e., about 2 h; to this should be added the time required for reconcentration.

[b] Cost of materials: Filterite filter ($15), Virozorb-1MDS filter ($49), and HCl, $AlCl_3$, beef extract, glycine, membrane disk, dialysis membrane, polyethylene glycol, iron oxide, casein, etc.

and the supernatant was discarded. A 10-ml volume of 2% casein (pH 8.5) was added, and the mixture was stirred with a glass rod for 15 min. Again, the iron oxide was settled with a magnet. The casein eluate was poured into a bottle and treated with antibiotics.

RESULTS AND DISCUSSION

Measurement of virus input. A crucial aspect to determining the virus detection sensitivity or the percent virus recovery from the adsorbent systems under study required that the virus input be known with precision. In normal practice, for each experimental run a vial of the frozen virus pool would be thawed, and a sample would be removed for monitoring the virus assay. The plaque assay of the virus input and of the final concentrate would be conducted on the same day.

In the present investigation, except for Baylor and EPA EMSL, the participating laboratories did not titrate the virus stock. Thus, percent virus recovery for each method was based on the PFU of virus recovered in trial 2 (Table 2) at the EPA EMSL laboratory, since the samples of all the concentrates sent to this laboratory were reassayed at the time of this trial.

Table 2 presents the titers of the seed virus at Baylor and EPA EMSL. Average values of 350 and 860 PFU/ml, respectively, were found for the low dose, and average values of 1,837 and 4,689 PFU/ml, respectively, were found for the high dose of virus. Thus, the BGM cell assay system used at EPA was 2.5 times as sensitive as the system at Baylor.

Virus recovery based on laboratory and EPA assays. A comparison is presented in Table 3 between the absolute number of PFU of virus recovered in one-third volume of sample concentrates by the participating laboratories and the results obtained in a similar volume assayed in the EPA EMSL laboratory.

Aluminum hydroxide precipitation followed by hydroextraction. Results of analyses of sample concentrates by the aluminum hydroxide precipitation-hydroextraction procedure showed a wide recovery range: 0 to 67% for the low-input experiments and 0 to 18% for the high-input experiments (Tables 4 and 5). Two of the laboratories obtained only 9 to 11% recovery and four laboratories reported 17 to 34% recovery in low-input virus experiments. In the high-input experiments, results were even poorer, with 3 to 5% average recovery by two laboratories and 11 to 18% recovery by the others. The overall efficiency of the method, estimated from the results of 23 experiments, was 20% for the low-input and 10% for the high-input virus.

The procedure as outlined in the Viradel method and examined in the round robin testing has not been evaluated previously by numerous investigators, although portions of it have been examined. Wallis and Melnick (28) demonstrated that viruses could be concentrated on aluminum hydroxide flocs. Based on these results, they developed a procedure for concentrating viruses from water. Farrah et al. (6) seeded 1,900 liters of tapwater with both low and high inputs (5×10^6 to 8.5×10^6 PFU) of poliovirus and passed the sample through a fiber glass depth cartridge filter, followed by a pleated epoxy-fiber glass filter. The glycine eluates from these filters were reconcentrated by aluminum hydroxide flocculation. Viruses were eluted from the flocs, and no further reduction in the volume of the floc eluate by hydroextraction was attempted. An average virus recovery of 40 to 50% was reported. In a recent study, Farrah et al. (9) obtained virus adsorption on aluminum hydroxide flocs generated at ambient pH in tapwater, trapped them by filtering the sample through a membrane filter, and eluted the virus from the flocs. The virus was reconcentrated by adsorption to and elution from aluminum hydroxide flocs followed by hydroextraction. This procedure recovered virus from 1,000 liters of water in a final eluate of 20 to 80 ml, with an average efficiency of 70%.

Similarly, hydroextraction has been used to concentrate viruses directly from water (4) and wastewater (29) and as a second-stage concentration step in recovering viruses from estuarine water (8). The method is simple, but time consuming.

Organic flocculation. The average recovery of seeded virus in the low-input experiments was reported in the range of 8 to 20% by two laboratories, 49 to 63% by two laboratories, and 198% by one laboratory. For the high-input experiments, two laboratories reported virus recoveries of 6 to 12%, and four laboratories obtained recoveries in the range of 26 to 46% (Table 5).

Katzenelson et al. (15) reported that organic flocculation operated at a mean efficiency of 75% (range, 69 to 123%) for

TABLE 2. Titration of the virus stock used in virus recovery experiments from water

Testing laboratory	Trial	No. of vials tested	PFU detected in individual vials	
			Low dose	High dose
Baylor[a]	1	5	321, 345, 314, 369, 296	1,930, 1,870, 1,740, 1,770, 1,515
	2	1	378	1,950
	3	2	352, 448	2,040, 1,880
EPA EMSL[b]	1	6	680, 760, 860, 500, 680, 920	2,560, 2,360, 5,200, 4,400, 2,460, 2,360
	2	4	1,060, 1,070, 1,020, 1,050	7,700, 6,450, 6,750, 6,650

[a] Average values for low-dose and high-dose experiments were 350 and 1,837, respectively.

[b] Average values for low-dose and high-dose experiments were 860 and 4,689, respectively.

TABLE 3. Quantity of virus recovered in identical volumes of the sample concentrates from two methods in the EPA EMSL assay (A) compared with individual laboratory assay (B)

Participating laboratory	Aluminum hydroxide-hydroextraction procedure					Organic flocculation procedure				
	Sample no.	Vol[a] (ml)		PFU[b]		Sample no.	Vol[a] (ml)		PFU[b]	
		(a1)	(a2)	(A)	(B)		(a1)	(a2)	(A)	(B)
1	76 (L)[c]	10	27	0	0	130 (L)	40	90	1,652	404
	112 (L)	8	24	75	10	48 (L)	30	93	142	56
	347 (H)	8	27	0	0	301 (H)	40	90	924	126
	353 (H)	11	33	137	68	67 (H)	30	92	540	267
2	226 (L)	13	42	219	87	348 (L)	23	83	150	150
	390 (L)	6	15	0	0	166 (L)	33	106	217	156
	173 (H)	13	43	68	53	221 (H)	29	82	394	300
	241 (H)	14	45	141	87	77 (H)	28	80	904	633
3	124 (L)	7	20	105	25	62 (L)	31	92	18	8
	38 (L)	8	23	85	33	60 (L)	31	94	37	Toxic
	197 (H)	10	29	138	44	355 (H)	31	93	156	22
	291 (H)	13	38	406	102	373 (H)	30	91	98	Toxic
4	324 (L)	1	4	7	27	210 (L)	7	22[d]	27	0
	242 (L)	4	16	89	52	380 (L)	7	21	112	28
	196 (L)	3	9	53	51	7 (H)	4	14	112	126
	273 (H)	4	13	342	413	117 (H)	5	15	423	65
	9 (H)	3	10	241	317					
5	332 (L)	4	14	17	22	366 (L)	30	93	247	172
	396 (L)	6	20	34	30	224 (L)	32	94	89	83
	141 (H)	7	20	293	139	151 (H)	31	93	816	576
	277 (H)	5	18	179	152	139 (H)	30	95	844	1,377
6	84 (L)	9	26	80	66	98 (L)	26	80	214	186
	253 (H)	9	28	402	228	181 (H)	22	65	1,071	408

[a] (a1), Volume of the eluate concentrate tested; (a2), total volume of the eluate concentrate.

[b] PFU recovered from the volume tested.

[c] L, Sample containing low number of virus (1,050 PFU) as measured by EPA assay; H, sample containing high number of virus (6,887 PFU) as measured by EPA assay.

[d] Since the commercial beef extracts available failed to generate a large amount of floc, the sediment was suspended in the small volumes indicated.

poliovirus type 1. Morris and Waite (19) seeded 20 liters of tapwater with 450 PFU of poliovirus type 2, filtered it through cartridge-type epoxy-fiber glass filters (Balston) with 8-μm pores, and reconcentrated the primary eluates of beef extract by organic flocculation. The authors obtained an average 45% (range, 14 to 81%) virus recovery. However, recovery of coxsackieviruses B2, B4, and B5 and echovirus 1 was very poor (3 to 22%). Sobsey and Glass (22) seeded 1,000 liters of tapwater with 10^7 to 10^8 PFU of poliovirus and reported 34 to 35% virus recovery, whereas low input (199 to 392 PFU) resulted in 16 to 41% virus recovery (average, 23%).

Since publication of the procedure in 1976 (15), organic flocculation as a reconcentration step has been tested in several countries, and the reports of virus recovery in general have been satisfactory. The method is simple, requiring only a centrifuge, and the concentrates are small and nontoxic to cell cultures. During this round robin testing, a number of the participating laboratories found that the commercial beef extracts used yielded poor virus recoveries. Adjustment of the pH to 3.5 did not result in a visible floc, and, after centrifuging the sample, very little deposit was obtained. The poor recoveries by some of the laboratories may have been caused by inadequate floc formation, resulting in loss of virus in the discarded supernatant. It has since been learned that a change in the manufacturing process of beef extract has been introduced recently by some companies. Thus, the reconcentration procedure should be carried out with satisfactory beef extracts that have been pretested.

Comparison of trials. From Table 4 it can be seen that the average percent recovery results from low-input experiments tended to be greater than from the high-input experiments, with both the aluminum hydroxide-hydroextraction and the organic flocculation procedures. However, when the results of individual tests are examined, the differences are not always apparent.

Methods other than the Viradel procedure. The numbers of laboratories conducting these additional experiments and the results obtained are shown in Tables 4, 5, and 6.

(i) Virozorb-1MDS filters. Three laboratories provided results of experiments conducted with positively charged Virozorb-1MDS filters. Laboratory 4 recovered only 4 and 24% for two low-input experiments, whereas the high-input experiments gave only 1 and 10%. Laboratories 1 and 6 conducted one experiment with each dose of virus and reported recoveries of 26 and 15% and 42 and 38%, respectively, for low and high inputs.

One of the advantages attributed to positively charged filters was that the water did not require pH adjustment to acidity or the addition of salts to obtain virus adsorption. Sobsey and Jones (23) studied the effect of tapwater pH on poliovirus adsorption to 47-mm-diameter 50S Zeta-Plus filters and observed that virus adsorption was 98 to 99% in the pH range of 5.5 to 7.5. Recovery of virus was 63 to 69%.

TABLE 4. Summary of Viradel testing[a]

Reconcentration method	Laboratory no.	Low input			High input		
		Expt. 1	Expt. 2	Avg.	Expt. 1	Expt. 2	Avg.
Aluminum hydroxide-hydroextraction[b]	1	0	21	11	0	6	3
	2	67	0	34	3	6	5
	3	28	23	26	6	17	12
	4	3	34, 15	17	16	12	14
	5	6	11	9	12	9	11
	6	22	ND[c]	22	18	ND	18
Organic flocculation[d]	1	354	42	198	30	24	27
	2	51	66	59	16	37	26
	3	5	11	8	7	4	6
	4	8	32	20	6	18	12
	5	73	25	49	35	39	37
	6	63	ND	63	46	ND	46

[a] After the first large-volume adsorption-elution procedure, the results of two reconcentration methods were compared (percent recovery of virus based on EPA EMSL assay).

[b] The ranges for low-input and high-input experiments were 0 to 67% and 0 to 18%, respectively. The averages were 20 and 10%, respectively.

[c] ND, Not done.

[d] The ranges for low-input and high-input experiments were 5 to 354% and 4 to 46%, respectively. The averages were 66 and 26%, respectively.

However, at a pH of 8.5, only 26% of virus was adsorbed on the filters, and only 1% of the total input virus was recovered. The authors indicated that the ambient pH of tapwater at Chapel Hill, N.C., was 7.0 to 7.3; in this pH range, efficient adsorption of poliovirus to Zeta filters was achieved with no addition of multivalent cation salts. However, the ambient pH of tapwater recorded by laboratory 4 in Houston was in the range of 8.2 to 8.8 when these experiments were conducted. The poor virus recoveries of 1, 4, 10, and 24% in the four trials may have been due to poor adsorption of seeded virus to the filters in this pH range. It may be pertinent to point out that the median value of ambient pH of 19 samples of tapwater in Cincinnati during a survey (14) was 8.4 (range, 8.0 to 9.3). From these results it appears that a pH adjustment to near neutrality may be necessary to process water by Virozorb filters to obtain efficient adsorption of virus.

(ii) Combination of positively and negatively charged filters. In a sequential filtration of water involving a positively charged filter at ambient pH followed by a negatively charged filter at pH 3.5, conducted by laboratory 3, virus recovery results were extremely poor (0.4%). It is quite likely that the low recovery of virus may not be due to failure in the adsorption of virus to the filters, but to the poor elution of the virus. Elution of virus from electropositive filters has been found to be difficult in a number of laboratories. This approach is costly, cumbersome, and, in view of the poor recovery of virus, does not offer any promise for future use.

(iii) Membrane disk. Recovery of seeded poliovirus was 6% for the low-input experiments and 4% for the high-input experiments. Such a poor recovery may be attributed to the omission of an important step in the Viradel procedure, i.e., the addition of $AlCl_3$ to the primary eluate of glycine in the reconcentration procedure. The original procedure of Wallis and Melnick indicated the need for lowering the pH of the eluate and adding $AlCl_3$ to readsorb the virus on a smaller diameter cellulose membrane. Using a membrane disk to reconcentrate virus from primary eluates obtained from four different microporous filters, Jakubowski et al. (14) and Hill et al. (13) showed recoveries of 29 to 45% of poliovirus seeded into tapwater. In their experiments, $AlCl_3$ was added to the primary eluates besides lowering the pH to 3.5

Another important point that deserves mention is that 1,600 ml of the primary eluate from Filterite filters is too large for filtration through a single 47-mm-diameter Millipore filter during the reconcentration procedure. It requires at least five filtration setups to process the whole volume, and this results in increasing the volume of the final concentrate to about 50 ml. Attempts to isolate low numbers of viruses from field samples usually require assay of the complete eluate, resulting in the use of a large number of tissue culture flasks, which is not economical.

(iv) Iron oxide. Results from three low-input experiments gave 21, 45, and 64% recovery, or an average of 43% of the input virus. With regard to high doses of virus, results from two experiments yielded 24 and 26%. The overall recovery was 36%.

Adsorption of a variety of viruses suspended in water to magnetic iron oxide was demonstrated by Rao et al. (20). Bitton et al. (3) carried out a detailed study on the adsorption of poliovirus type 1 to magnetite (Fe_3O_4). Recently, this method was used to concentrate virus from tapwater. Virus recovery ranged from 60 to 80% (21). In the present investigation, iron oxide was used as a method for reconcentration. The method is simple, does not require any special apparatus, can be completed in 1.5 h, is inexpensive, and gives final eluates of 5 to 10 ml that are nontoxic to cell cultures.

Selection of poliovirus as a model enterovirus. The attenuated poliovirus type 1 was selected as the model enterovirus because it has been used as such in all environmental virology laboratories and has been the virus most frequently isolated from urban sewage. In addition, the methods developed for poliovirus have been able to detect the other enteroviruses.

Conclusions. An analysis of the evaluation of the present round robin testing undertaken by six laboratories elicits the following comments.

(i) Quality of test samples. Differences in water quality may be an important reason for the wide variation in virus recoveries obtained by different investigators. There is evidence in the literature that dissolved and colloidal sub-

TABLE 5. Average recovery of poliovirus type 1 seeded into 100 liters of tapwater by the Viradel method in round robin testing

Reconcentration method	Low-input experiments			High-input experiments		
	% Recovery (avg)	Range	No. of laboratories reporting	% Recovery (avg)	Range	No. of laboratories reporting
Aluminum hydroxide-hydroextraction	9	9–11	2	3	3–5	2
	11			5		
	17	17–34	4	11	11–18	4
	22			12		
	26			14		
	34			18		
Organic flocculation	8	8–20	2	6	6–12	2
	20			12		
	49	49–63	3	26	26–46	4
	59			27		
	63			37		
				46		
	198		1			

TABLE 6. Virus recovery with methods other than the Viradel procedure

Method[a]	Laboratory no.	pH of water	% Recovery of virus	
			Low input	High input
Virozorb	1	7.5–7.6	26	15
	6	Not indicated	42	38
	4	8.2–8.8	4, 24	1, 10
Sequential filtration through Virozorb, Zeta-Plus, cellulose nitrate	3	7.1–7.3	0, 0.6	1.2, 0.1
Membrane disk	4	8.2–8.8	9, 3	3, 5
Magnetic iron oxide	4	8.2–8.8	45, 64, 21	24, 26

[a] Virozorb and sequential filtration through Virozorb, Zeta-Plus, and cellulose nitrate methods are concentration methods, whereas membrane disk and magnetic iron oxide are reconcentration procedures.

stances in water may interfere with virus recovery, and the types and concentrations may vary (10, 11, 24). Unfortunately, chemical data were not generated in the present investigation, and therefore emphasis cannot be placed on any single characteristic of the water samples used. One way of circumventing this problem in future studies is to arrange for a supply of reference tapwater samples to be supplied to all participating laboratories. However, this approach involves transportation of large volumes of water, which is costly and cumbersome. On the other hand, a realistic approach requires the testing of the Viradel method in waters of different qualities (as undertaken in this study) because in the real world situation, virus isolation from drinking water must be conducted in different regions whenever disease due to water contamination is investigated.

(ii) Assay of sample concentrates. Examination of one-third of the sample concentrates in the individual laboratories served to indicate the variability in the susceptibility of the BGM cell assay to poliovirus. Future testing of methods should continue to arrange for assays of seed virus as well as eluate concentrates in one central laboratory. The testing of virus inputs by the participating laboratories together with their concentrates will yield further comparative data.

(iii) Volume of the eluate concentrate. A volume of about 100 ml for the final concentrate (organic flocculation) is too large to examine economically. When experiments are conducted with low numbers of viruses, the complete eluate must be assayed. Efforts should be made to develop a procedure to keep the volume of the final concentrate low.

(iv) Uniformity of materials. Comparison of results from different laboratories requires a high level of uniformity in certain crucial materials used in conducting the experiments. Filtration media and eluents are two such items. It is important that these materials be procured by one central laboratory in multiple numbers and supplied to the participants.

Based on the data generated in the present study, the American Society for Testing and Materials Committee on Viruses in the Aquatic Environment voted to recommend the Viradel-organic flocculation procedure as a provisional method for the recovery of human enteroviruses from drinking water. Details of the method will be published in the U.S. EPA *Manual of Methods for Virology*.

Further study of methods evaluation should include (i) positively charged filters, with special emphasis on adjustment of the sample pH to neutrality, and (ii) iron oxide reconcentration. The latter method deserves more study because of its economy in equipment and time when compared with other procedures.

LITERATURE CITED

1. **American Public Health Association.** 1976. Standard methods for the examination of water and wastewater, 14th ed. Section 913: detection of viruses in water and wastewater. American Public Health Association, Washington, D.C.
2. **Berg, G., H. L. Bodily, E. H. Lennette, J. L. Melnick, and T. G. Metcalf (ed.).** 1976. Viruses in water. American Public Health Association, Washington, D.C.
3. **Bitton, G., O. Pancorbo, and G. E. Gifford.** 1976. Factors affecting the adsorption of poliovirus to magnetite in water and wastewater. Water Res. **10**:973–980.
4. **Cliver, D. O.** 1967. Detection of enteric viruses by concentration with polyethylene glycol, p. 109–121. *In* G. Berg (ed.), Transmission of viruses by the water route. Interscience, New York.
5. **Dahling, D. R., G. Berg, and D. Berman.** 1974. BGM, a continuous cell line more sensitive than primary rhesus and African green kidney cells for the recovery of viruses from water. Health Lab. Sci. **11**:275–282.
6. **Farrah, S. R., C. P. Gerba, C. Wallis, and J. L. Melnick.** 1976. Concentration of viruses from large volumes of tap water using pleated membrane filters. Appl. Environ. Microbiol. **31**:221–226.
7. **Farrah, S. R., S. M. Goyal, C. P. Gerba, R. H. Conklin, C. Wallis, J. L. Melnick, and H. L. DuPont.** 1978. A simple method for concentration of enteroviruses and rotaviruses from cell culture harvests using membrane filters. Intervirology **9**:56–59.
8. **Farrah, S. R., S. M. Goyal, C. P. Gerba, C. Wallis, and J. L. Melnick.** 1977. Concentration of enteroviruses from estuarine water. Appl. Environ. Microbiol. **33**:1192–1196.
9. **Farrah, S. R., S. M. Goyal, C. P. Gerba, C. Wallis, and J. L. Melnick.** 1978. Concentration of poliovirus from tapwater onto membrane filters with aluminum chloride at ambient pH levels. Appl. Environ. Microbiol. **35**:624–626.
10. **Farrah, S. R., S. M. Goyal, C. P. Gerba, C. Wallis, and P. T. B. Shaffer.** 1976. Characteristics of humic acid and organic compounds concentrated from tapwater using the Aquella virus concentrator. Water Res. **10**:897–901.
11. **Farrah, S. R., C. Wallis, P. T. B. Shaffer, and J. L. Melnick.** 1976. Reconcentration of poliovirus from sewage. Appl. Microbiol. **32**:654–658.
12. **Henderson, M., C. Wallis, and J. L. Melnick.** 1976. Concentration and purification of enteroviruses by membrane chromatography. Appl. Environ. Microbiol. **32**:689–693.
13. **Hill, W. F., W. Jakubowski, E. W. Akin, and N. A. Clarke.** 1976. Detection of virus in water: sensitivity of the standard method for drinking water. Appl. Environ. Microbiol. **31**:254–261.
14. **Jakubowski, W., W. F. Hill, and N. A. Clarke.** 1975. Comparative study of four microporous filters for concentrating viruses from drinking water. Appl. Microbiol. **30**:58–65.
15. **Katzenelson, E., B. Fattal, and T. Hostovesky.** 1976. Organic flocculation: an efficient second-step concentration method for the detection of viruses in tap water. Appl. Environ. Microbiol. **32**:638–639.
16. **Melnick, J. L., and C. P. Gerba.** 1980. Viruses in water and soil. Publ. Health Rev. **9**:185–213.
17. **Melnick, J. L., C. P. Gerba, and C. Wallis.** 1978. Viruses in water. Bull. WHO **56**:499–508.
18. **Melnick, J. L., H. A. Wenner, and C. A. Phillips.** 1979. Enteroviruses, p. 471–534. *In* E. H. Lennette and N. J. Schmidt (ed.), Diagnostic procedures for viral, rickettsial and chlamydial infections, 5th ed. American Public Health Association, Washington, D.C.
19. **Morris, R., and W. M. Waite.** 1981. Evaluation of procedures for recovery of viruses from water. I. Concentration systems. Water Res. **14**:791–793.

20. **Rao, V. C., R. Sullivan, R. B. Read, and N. A. Clarke.** 1968. A simple method for concentrating and detecting viruses. J. Am. Water Works Assoc. **60**:1288–1294.

21. **Rao, V. C., S. V. Waghmare, and S. B. Lakhe.** 1981. Detection of viruses in drinking water using magnetic iron oxide. Appl. Environ. Microbiol. **42**:421–426.

22. **Sobsey, M. D., and J. S. Glass.** 1980. Poliovirus concentration from tap water with electropositive adsorbent filters. Appl. Environ. Microbiol. **40**:201–210.

23. **Sobsey, M. D., and B. L. Jones.** 1979. Concentration of poliovirus from tapwater using positively charged microporous filters. Appl. Environ. Microbiol. **37**:588–595.

24. **Sobsey, M. D., C. Wallis, M. Henderson, and J. L. Melnick.** 1973. Concentration of enteroviruses from large volumes of water. Appl. Microbiol. **26**:529–534.

25. **Wallis, C., M. Henderson, and J. L. Melnick.** 1972. Enterovirus concentration on cellulose membranes. Appl. Microbiol. **23**:476–480.

26. **Wallis, C., A. Homma, and J. L. Melnick.** 1972. Apparatus for concentrating viruses from large volumes. J. Am. Water Works Assoc. **64**:189–196.

27. **Wallis, C., and J. L. Melnick.** 1967. Virus aggregation as the cause of the nonneutralizable persistent fraction. J. Virol. **1**:478–488.

28. **Wallis, C., and J. L. Melnick.** 1967. Concentration of viruses on aluminum phosphate and aluminum hydroxide precipitates, p. 120–138. *In* G. Berg (ed.), Transmission of viruses by the water route. Interscience, New York.

29. **Wellings, F. M., A. L. Lewis, and C. W. Mountain.** 1976. Demonstration of solids-associated virus in wastewater and sludge. Appl. Environ. Microbiol. **31**:354–358.

30. **World Health Organization Scientific Group.** 1979. Viruses in water, wastewater and soil. W.H.O. Tech. Rept. Ser. No. 639. World Health Organization, Geneva.