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Recovery of Viruses from Water by a Modified Flocculation Procedure for Second-Step Concentration

DANIEL R. DAHLING* AND BETTY A. WRIGHT

Virology Section, Biological Methods Branch, Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268

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A reduction in virus recovery efficiencies stemming from a change in the commercial processing of powdered beef extract was reversed by the addition of Celite analytical filter aid. Supplementing beef extract with this silicate is recommended as a modification to the organic flocculation procedure for second-step concentration in monitoring for waterborne viruses. Considerable differences in virus recovery were found among lots of beef extract and Celite preparations; this indicates that the performance of each lot of these substances should be checked before use.

The development of the secondary reconcentration procedure by Katzenelson et al. (8) in 1976 permitted elution of viruses from cartridge filters with large volumes of beef extract and subsequent reduction of the eluate volume to a workable level with minimal virus loss. Since this organic flocculation procedure is at present the method of choice (2, 9) for further concentration of viruses eluted from cartridge filters, it became important to modify the procedure when it was discovered (7) that recently marketed lots of powdered beef extract do not form the heavy precipitates necessary to obtain acceptable virus recovery.

The use of silicates, Cat-Floc, and aluminum sulfate were investigated as possible enhancement materials for virus concentration. Silicates (6, 10, 11, 14–17) previously used to remove viruses from water and wastewater have served as effective media from which viruses could be recovered. More recently, Cat-Floc and aluminum sulfate have been used (18, 19).

While it may be necessary at some point to establish the use of another eluent, modification of the present system seemed to be the prudent course of action in the interim.

MATERIALS AND METHODS

Cell culture and virus assay. The continuous African green monkey (*Cercopithecus aethiops*) kidney cell line designated BGM was used in this study at passage levels 110 through 170. The methods of propagation were previously described (1, 3, 4).

Plaque-purified strains of poliovirus 1 (Mahoney LP), echovirus 7 (Wallace), and coxsackievirus A9 (CME 456) were used for all experimental work. Virus assays were performed in screw-cap bottles by the plaque technique. A 1.0-ml sample was inoculated onto BGM monolayers and overlaid as described elsewhere (3).

Materials. The following silicates were tested in this study: two previously described (6) commercially available silicates, Micro-Cel T-70, a synthetic hydrous calcium silicate, and Celkate T-21, a synthetic hydrous magnesium silicate (both obtained as samples from Johns-Manville Products Corp., Toledo, Ohio); Celite analytical filter aid, a diatomaceous silica, lot numbers L665A, L665B, and L665C (Johns-Manville); Celite 503 E406-8, lot number 343350 (J. T. Baker Chemical Co., Phillipsburg, N.J.); Celite analytical filter aid C211, lot number 1087 (Fisher Scientific Co., Pittsburg, Pa.); kaolin powder 5-2242, an aluminum silicate, USP-FCC food grade, lot number 847338 (J. T. Baker Chemical Co.); Cat-Floc T, an organic polymer (19) (Calgon Corp., Pittsburgh, Pa.); and aluminum sulfate prepared as previously described (18) with the following modifications: 6 ml of a 10% $Al_2(SO_4)_3 \cdot 18H_2O$ solution was added per 100-ml sample, followed by adjustment of the final pH to 7.2.

Powdered beef extract was purchased from three sources: GIBCO Diagnostics, Madison, Wis., lot numbers 91190, 01920, and 98685; Oxoid Ltd., KC Biologicals, Lenexa, Kans., lot numbers 4911291 and 02815451; and Inolex Corp., Glenwood, Ill., lot number 6323A. Paste beef extract was purchased from Difco Laboratories (Detroit, Mich.), lot number 701628. Of the powdered beef extract lots, GIBCO lot number 91190 and Oxoid lot number 4911291 were older preparations that produced visible precipitates at pH 3.5. The paste beef extract also produced a visible precipitate at pH 3.5.

Test procedure. GIBCO beef extract lot number 98685 was used in the reference method and in all test procedures reported in Table 1 and Fig. 1 to 4; GIBCO beef extract lot number 01920 was used in the tests reported in Table 2 and in the reference method for tests reported in Table 3 in place of lot number 98685, the supply of which had been exhausted. Each beef extract tested in Table 2 was used in the reference method, and if further testing was to be done (as on the new lots of beef extract) they were subsequently used in the test method.

Tables 1 and 3 and Fig. 1 to 4 all consist of three replicate samples as listed, while Table 2, which also consisted of three replicate samples, is presented as a mean percent recovery owing to the large amount of data.

The basic test procedure was to inoculate a sample of 3% beef extract with test virus. After thorough mixing, 100-ml samples were dispensed into 250-ml beakers, each containing a stir bar. In each series, a set of three replicate 100-ml samples was tested by the original flocculation procedure as described by Katzenelson et al. (8). These served as the reference method (controls) against which all other results were compared. Other aliquots were treated in a similar manner, but with the addition of silicates, Cat-Floc, or aluminum sulfate. Silicates were used at a concentration of 0.1 g/100 ml of sample unless otherwise indicated.

The following series of tests were performed. (i) The

* Corresponding author.

TABLE 1. Effect of silicate concentration on recovery of poliovirus

Material tested	Total virus input (PFU/sample)	Replicate no.	Virus recovery (%)					
			Reference method[a]	With the following amt of material added (g/100 ml):				
				0.1	0.2	0.3	0.4	0.5
Filter aid (diatomaceous silicate)	94	1	60	109	98	89	106	107
		2	47	99	95	104	98	94
		3	49	100	91	102	127	93
		Mean	52	100	95	98	110	98
		CV[b]	13	9	4	8	14	8
T-21 (magnesium silicate)	94	1	60	95	98	89	95	81
		2	47	108	85	106	104	100
		3	49	112	82	108	94	79
		Mean	52	105	88	101	98	87
		CV	13	9	10	10	6	13
T-70 (calcium silicate)	94	1	60	63	54	56	80	52
		2	47	70	53	71	61	69
		3	49	71	80	78	51	89
		Mean	52	68	62	68	64	70
		CV	13	6	25	16	23	26
Kaolin (aluminum silicate)	94	1	60	95	101	91	69	84
		2	47	90	82	90	74	92
		3	49	89	79	85	86	96
		Mean	52	91	87	89	76	91
		CV	13	4	14	4	11	7

[a] Method as described in reference 7.
[b] CV, Percent relative standard deviation.

general test procedure was followed except that the concentration of test silicates was varied from 0.1 to 0.5 g/100-ml sample (Table 1); (ii) only the Celite filter aid was used in the general procedure to test various pH levels from 3.5 to 7.0 (Fig. 1); (iii) the mixing time allowed for virus adsorption was varied from 0 to 40 min (In the zero time sample, Celite filter aid was added to the beef extract [which was already mixing], allowed to thoroughly mix [about 5 s], and then poured into a centrifuge tube for further processing as directed) (Fig. 2); (iv) the contact time for the phosphate buffer elution was varied from 0 to 40 min (In the zero time sample, phosphate buffer was added to the Celite filter aid pellet, swirled to mix, and immediately recentrifuged to separate the buffer from the filter aid) (Fig. 3); (v) the effects of various amounts of a 10% solution of hydrated aluminum sulfate added to the 100-ml samples of beef extract in place of silicates was evaluated (Fig. 4); (vi) the general test procedure was followed and different lots of beef extract with several additives were evaluated, comparing them with GIBCO lot number 91190 and Oxoid lot number 4911291 powdered beef extract preparations that still formed heavy precipitates and Difco lot number 701628 (paste preparation) which also formed a heavy precipitate at pH 3.5 (these last three lots were not tested with any of the additives) (Table 2); (vii) the general procedure was followed and different brands of Celite filter aid were evaluated (Table 3).

Statistical analysis. An analysis of variance procedure was run for each set of data in Tables 1 to 3 and Fig. 1 to 4. In most cases, the Student-Newman-Keuls multiple comparisons procedure was used to obtain more detailed information about the differences among the methods (20).

Owing to the large amount of data, Table 2 is presented as means of three replicate samples with the relative standard deviations. Data in all other tables give the percent virus recovery for each replicate sample plus a mean and relative standard deviation.

RESULTS

Table 1 lists the results of tests on four different silicate preparations ranging in concentration from 0.1 to 0.5 g/100 ml of beef extract. Celite analytical filter aid ranked highest in overall virus recovery. The data were analyzed with Dunnett's test, a multiple comparison procedure which tests all treatments versus the reference method (5). The alpha level was set at 0.05. The filter aid and T-21 at all levels and kaolin at 0.1, 0.2, 0.3, and 0.5 g were found to be significantly better than the reference method. The filter aid was chosen for further study at this point because both the T-21 and the kaolin took longer to become completely dispersed in solution, and small unwetted clumps remained after mixing.

Additionally, a one-way analysis of variance run on the filter aid portion of Table 1, excluding the reference method, found no differences in virus recovery among the levels of filter aid.

Based on these results the next series of tests (Fig. 1 and 2) were conducted with only the Celite filter aid. Figure 1 gives the data for the effect of pH level on poliovirus recovery. Significant differences were found to exist among the reference method and the levels of pH ($P < 0.001$). The results of the Student-Newman-Keuls multiple comparisons procedure showed that pH levels of 3.5, 4.0, and 4.5 were significantly better than the reference method, with the highest recovery at pH 4.0. The five remaining pH levels had

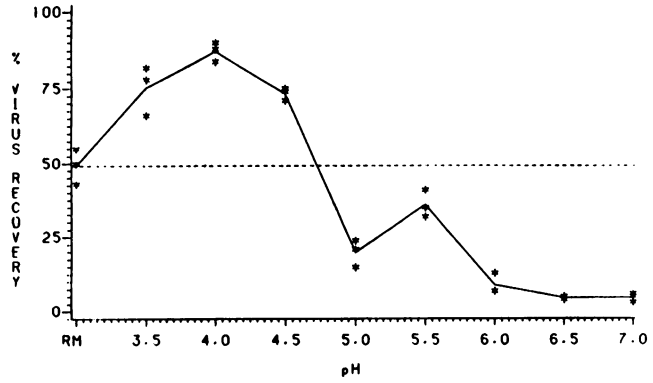

FIG. 1. Effect of pH on poliovirus adsorption. Samples were supplemented with 0.1% Celite filter aid. ——, Mean percent virus recovery; – – – –, mean percent virus recovery for reference method. RM, Reference method at a pH level of 3.5.

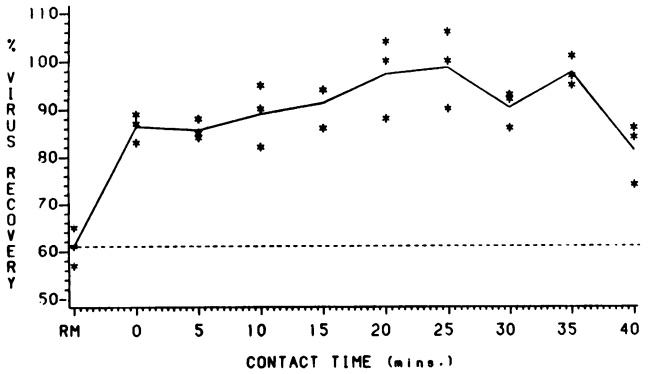

FIG. 3. Effect of contact time on poliovirus elution. Samples were supplemented with 0.1% Celite filter aid. ——, Mean percent virus recovery; – – – –, mean percent virus recovery for reference method. RM, Reference method with a 30-min contact time.

virus recoveries significantly lower than that of the reference method.

Figure 2 shows the effect of contact time on the adsorption of virus when the samples were treated with 0.1 g of Celite filter aid per 100 ml. Overall, a significant difference was found to exist among the contact times for the supplemented samples and the reference method ($P < 0.001$). The Celite-treated samples with contact times ranging from 5 to 40 min gave significantly higher recoveries than the reference method or the zero time sample. As few as 10 min were adequate to achieve maximum virus adsorption.

The elution of viruses adsorbed onto the Celite filter aid showed significant differences among the contact times for the samples with filter aid and the reference method (Fig. 3). The multiple comparisons procedure showed that all contact times (0 to 40 min) yielded significantly higher virus recoveries than the reference method.

The aluminum sulfate method described by Walter and Rudiger (19) was modified slightly to produce better flocculation. Figure 4 shows the results of a study conducted to determine the concentration of $Al_2(SO_4)_3 \cdot 18H_2O$ which would provide the maximum virus recovery from 3% beef extract samples. The multiple comparisons procedure showed that the addition of 3 to 6 ml of 10% aluminum sulfate solution provided significantly better recovery than the reference method. All other volumes tested were not as good.

Table 2 lists the results of a comparative study in which several silicates, Cat-Floc, and aluminum sulfate were tested in various lots of beef extract inoculated with either poliovirus 1, echovirus 7, or coxsackievirus A9. Of those lots of powdered beef extract that formed heavy precipitates (GIBCO lot number 91190 and Oxoid lot number 4911291), virus recoveries by the organic flocculation procedure (8) were as previously experienced with this method (2–4). The Difco (paste) beef extract (lot number 701628) consistently gave poor recoveries. The Inolex lot, which did not form a heavy precipitate, gave poor recoveries with echovirus and coxsackievirus. When this lot was supplemented with the various additives, the results still showed that the virus recoveries were generally below the levels of the other samples tested except for poliovirus. The beef extract materials produced by GIBCO showed substantially improved virus recoveries when Celite filter aid, T-21, or aluminum sulfate was applied to both lots. On the other hand, Oxoid lot number 02815451 showed almost no improvement (except with poliovirus and aluminum sulfate treatment) in virus recovery when results from the reference method were compared with those of the test procedures.

Analysis of the data in Table 2 by repeated measures analysis was difficult to interpret owing to the many factors

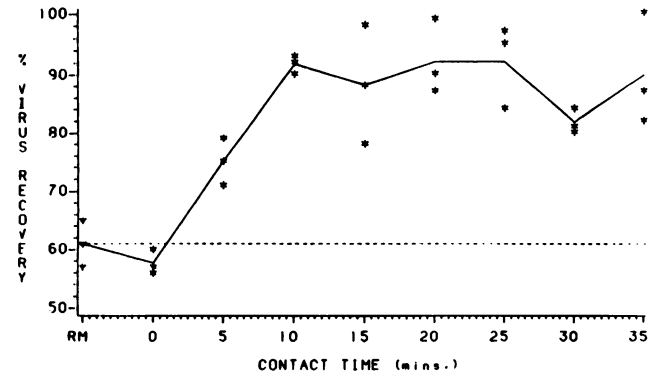

FIG. 2. Effect of contact time on poliovirus and adsorption. Samples were supplemented with 0.1% Celite filter aid. ——, Mean percent virus recovery; – – – –, mean percent virus recovery for reference method. RM, Reference method with a 35-min contact time.

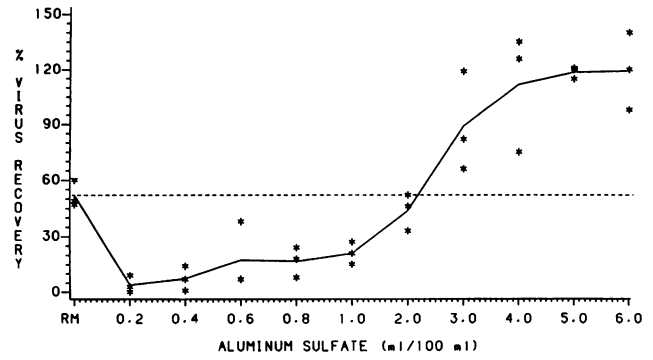

FIG. 4. Effect of aluminum sulfate concentration on poliovirus recovery. ——, Mean percent virus recovery; – – – –, mean percent virus recovery for reference method. RM, Reference method.

TABLE 2. Comparative virus recoveries from different lots of beef extract

Virus tested	Beef extract lot no.	Total virus input (PFU/sample)	Mean virus recovery (% [CV[a]])						
			Reference method[b]	Silicate tested				Cat-Floc method	Aluminum sulfate method
				Filter aid	T-21	T-70	Kaolin		
Poliovirus 1	GIBCO 91190	163	92 (9.5)						
	GIBCO 01920	135	71 (13)	96 (1.2)	93 (4.4)	56 (12)	62 (17)	29 (27)	93 (3)
	GIBCO 98685	134	53 (36)	91 (4.9)	94 (22)	37 (21)	51 (10)	11 (69)	92 (2.5)
	Difco 701628	41	12 (52)						
	Oxoid 4911291	112	143 (5)						
	Inolex 6323A	139	20 (30)	85 (11)	88 (11)	46 (6)	70 (13)	11 (24)	117 (4)
	Oxoid 02815451	140	84 (15)	88 (9)	78 (25)	73 (17)	100 (14)	59 (19)	116 (3)
Echovirus 7	GIBCO 91190	72	72 (11)						
	GIBCO 01920	59	80 (17)	104 (13)	82 (5)	24 (41)	90 (14)	43 (28)	72 (6)
	GIBCO 98685	62	47 (9)	72 (4)	70 (4)	5 (11)	62 (10)	43 (34)	84 (14)
	Difco 701628	286	8 (7)						
	Oxoid 4911291	256	114 (14)						
	Inolex 6323A	300	16 (10)	60 (13)	35 (15)	5 (25)	50 (18)	32 (44)	72 (7)
	Oxoid 02815451	383	71 (9)	67 (2.3)	63 (4)	14 (29)	72 (8)	50 (24)	75 (7)
Coxsackievirus A9	GIBCO 91190	31	87 (3)						
	GIBCO 01920	32	73 (14)	67 (7)	87 (9)	59 (18)	71 (28)	13 (77)	87 (23)
	GIBCO 98685	23	67 (37)	100 (17)	113 (10)	96 (32)	126 (12)	33 (67)	96 (24)
	Difco 701628	88	3 (58)						
	Oxoid 4911291	72	85 (23)						
	Inolex 6323A	121	11 (9)	59 (8)	59 (5)	38 (4)	46 (8)	6 (41)	13 (103)
	Oxoid 02815451	111	74 (19)	68 (13)	61 (26)	56 (15)	76 (18)	26 (22)	42 (19)

[a] CV, Percent relative standard deviation.
[b] Method as described in reference 7.

and interactions involved among viruses, lots of beef, and methods. All interactions were significant ($P < 0.001$), and the paired t test showed the filter aid to be significantly better than the reference method and T-70, but no better than the others.

A test of several different lots of Celite for their ability to recover the three viruses was conducted (Table 3). Using GIBCO beef extract lot number 01920, virus recoveries with the Johns-Manville and Fisher brand filter aids were about equal but better than with the Baker filter aid. The data were

TABLE 3. Virus recoveries with different brands of Celite filter aid

Virus tested	Total virus input (PFU/sample)	Replicate no.	Virus recovery (%)					
			Reference method[a]	Filter aid tested[b]				
				1	2	3	4	5
Poliovirus 1	326	1	70	95	90	91	96	76
		2	69	91	88	87	90	74
		3	69	90	89	88	91	73
		Mean	69	92	89	89	92	74
		CV[c]	0.8	2.9	1.1	2.3	3.5	2.1
Echovirus 7	118	1	76	90	91	88	86	75
		2	81	89	88	89	89	73
		3	78	91	89	87	87	71
		Mean	78	90	89	88	87	73
		CV	3.2	1.1	1.7	1.1	1.7	2.7
Coxsackievirus A9	190	1	46	73	76	72	69	61
		2	65	74	69	77	67	59
		3	57	74	73	72	71	60
		Mean	56	74	73	74	69	60
		CV	17	0.8	4.8	3.9	2.9	1.7

[a] Method as described in reference 7.
[b] Filter aids tested: numbers 1, 2, and 3, Celite by Johns-Manville, lots L665A, L665B, and L665C, respectively; number 4, Celite 211 by Fisher; and number 5, Celite 503 by J. T. Baker.
[c] CV, Percent relative standard deviation.

analyzed by a two-way analysis of variance. Owing to the presence of significant virus and lot of Celite interaction ($P = 0.0163$), the data were analyzed separately by virus. Filter aids 1 to 4 were found to be significantly better than the reference method for each virus. In addition, filter aid 5 gave significantly higher recoveries for poliovirus than the reference method.

DISCUSSION

The production process for the preparation of powdered beef extract has been altered (7), making the original organic flocculation procedure for concentrating viruses less effective. Therefore, the possibility of modifying the procedure (8) for recovering viruses from beef extract solutions was investigated.

Previous investigators (10, 11, 14–17) showed that silicates adsorbed viruses. Fass et al. (6) found he could not elute bacteriophage from the silicates, and the T-21 silicate was toxic to the phage. Our experiments with selected silicates showed that poliovirus 1, echovirus 7, and coxsackievirus A9 not only adsorbed to these materials but that most of the attached virus could be readily recovered. It was found that the act of adsorption was pH related (8) and that elution took place more rapidly than adsorption (Fig. 2).

Murray (12) has pointed out that virus recoveries from inorganic surfaces are affected by the extent of viral inactivation that occurs on the adsorbent surface. He showed that the longer the virus remained in contact with the surface before elution, the greater the inactivation.

From the comparative study in Table 2, it was determined that the new GIBCO beef extracts (lot numbers 98685 and 01920), which did not form heavy precipitates, could be brought up to virus recovery levels comparable to those of the old lots that formed heavy precipitates. The new Oxoid beef extract (lot number 02815451) had recoveries slightly lower than those of the previous lot which formed a heavy precipitate; however, recoveries with the Oxoid material still exceeded recoveries experienced with Inolex beef extracts.

Our data showed the Celite filter aid, T-21 silicate, and aluminum sulfate methods to be comparable. The Celite filter aid was chosen, however, because the T-21 silicate, while giving equal recoveries, was less wettable and took longer to totally disperse in solution. The aluminum sulfate, on the other hand, had one serious drawback. The precipitate, once it was recovered and placed into the citric acid-citrate buffer, only partially dissolved and remained in a kind of gelatinous state which did not allow the removal of bacterial and fungal contaminants by filtration (2) of the concentrate.

The Celite filter aid can be readily sterilized by autoclaving without altering the material, and it pellets readily on centrifugation, thus providing easy separation from the supernatant. Using this procedure we have encountered no virus reductions if antifoam is used and no toxicity problems with 3% beef extract as have been reported by Payment et al. (13). Based on our data the recommended procedure for use of the Celite filter-aid is outlined below, as it might be used for eluting viruses from cartridge filters with beef extract solutions. Collect beef extract eluate in a sterile beaker containing a sterile magnetic stir bar. Place the beaker on a magnetic stirrer and stir at a speed sufficient to develop a vortex. Add Celite (0.1 g/100 ml of eluate) to the beaker and mix for 1 min or until the Celite is dispersed evenly. Adjust the pH to 4.0 by slowly adding 1 M HCl. Stir the acidified suspension for 10 min. Pour the contents of the beaker into flat-bottomed centrifuge bottles and centrifuge at 2,500 × *g* for 15 min in a refrigerated centrifuge with a swinging bucket rotor. Discard the supernatants and resuspend the precipitates by dispensing evenly among the centrifuge bottles 0.15 M Na_2HPO_4 solution at 5 ml of Na_2HPO_4 for each 100 ml of initial eluate. Mix the suspension in the centrifuge bottles for 25 min either on a shaker at 160 rpm or with a stir bar on magnetic stirrers at a speed only sufficient to maintain an even suspension. Combine the suspensions in conical centrifuge tubes and centrifuge them at 2,500 × *g* for 15 min. Save the supernatant (discard the precipitate at this point) and check the pH. Adjust the pH to 7.0 to 7.5 if necessary with either 1 M HCl or 1 M NaOH. Assay the supernatant for viruses.

We showed that differences do exist, not only between lots of beef extract but also between brands of filter aid. Whether these differences in filter aids were a result of differing manufacturing processes or the result of the composition of the material itself could not be determined. However, it is important to test each new lot of beef extract and filter aid to determine now well they can be expected to perform.

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