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Round Robin Investigation of Methods for Recovering Human Enteric Viruses From Sludge

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To select a tentative standard method for detection of viruses in sludge the American Society for Testing and Materials D19:24:04:04 Subcommittee Task Group initiated round robin comparative testing of two procedures that, after initial screening of several methodologies, were found to meet the basic criteria considered essential by the task group. Eight task group member laboratories agreed to perform round robin testing of the two candidate methods, namely, The Environmental Protection Agency or low pH–$AlCl_3$ method and the Glass or sonication-extraction method. Five different types of sludge were tested. For each particular type of sludge, a single laboratory was designated to collect the sludge in a single sampling, make samples, and ship it to the participating laboratories. In most cases, participating laboratories completed all the tests within 48 h of sample arrival. To establish the reproducibility of the methods, each laboratory tested each sludge sample in triplicate for the two candidate virus methods. Each processed sludge sample was quantitatively assayed for viruses by the procedures of each individual round robin laboratory. To attain a more uniform standard of comparison, a sample of each processed sample from all laboratories was reassayed with one cell line and passage number by a single laboratory (Environmental Protection Agency Environmental Monitoring and Support Laboratory, Cincinnati, Ohio). When the data were statistically analyzed, the Environmental Protection Agency method was found to yield slightly higher virus recoveries for all sludge types, except the dewatered sludge. The precisions of both methods were not significantly different. On the basis of these and several other considerations both methods are recommended as tentative American Society for Testing and Materials standard methods.

As a result of primary and secondary sewage treatment, most of the human enteric viruses present in domestic sewage become an integral part of waste sludge. A large percentage of viruses may be inactivated during activated sludge treatment and subsequent sludge digestion, but some may survive these processes (2, 3, 5, 9). To determine the fate of viruses and the magnitude of possible public health implications associated with sludge disposal, it is important to have available simple and reliable methods that can quantitatively detect low levels of viruses in sludge. Although a number of methods have recently become available (7, 8, 11–13), a standard method has not been recognized to date.

In 1979, the American Society for Testing and Materials Task Group responsible for developing standard methods for virus recovery from solids in waters (D19:24:04:04/05) initiated the development of a standard method for virus concentration and recovery from wastewater sludges. During 1980, screening studies were performed on a number of candidate methodologies. Based on initial results, two candidate methods were selected for further testing. The two candidate methods were as follows: (i) the Environmental Protection Agency (EPA) method or low pH-aluminum chloride method developed by Donald Berman of the EPA Environmental Monitoring and Support Laboratory, Cincinnati, Ohio; (ii) the glass method, a sonication-extraction method developed by Steven Glass of the Department of Biology, New Mexico State University, Las Cruces.

In 1981, the task group initiated round robin comparative testing of these two methods. Eight task group member laboratories served as round robin test participants and provided activities including sludge sample collection and shipment, chemical characterization of sludge, and the actual round robin testing of the two candidate sludge methods.

MATERIALS AND METHODS

Sludge samples. Five sludge types were tested. Of the eight round robin test laboratories, five were asked to collect and distribute one representative sludge type each. The designated laboratory collected sludge in a single large sampling on a specified sampling date. The sludge was mixed thoroughly, and samples of 1-liter were placed in sterile plastic bottles. These were then chilled in an ice bath for 2 to 3 h, placed in shipping containers, iced, and air shipped by Priority Mail to each participating laboratory. The laboratory gathering a specific sludge type also performed a chemical analysis on that sludge to determine pH, alkalinity, and total and volatile suspended solids (1). The sludge treatment history was recorded. The types of sludges examined and their physicochemical characteristics are listed in Table 1.

EPA method for virus recovery from sludge. The EPA procedure was used for all wastewater sludges except pri-

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TABLE 1. Types of sludges examined and their physicochemical characteristics

Sludge type	pH	Alkalinity (mg/liter) as $CaCO_3$	Suspended solids (g/liter)		Source
			Total	Volatile	
1. Anaerobic, high rate digested (mesophilic 29 to 38°C)	7.4	5,200	31.4	14.6	Dayton, Ohio
2. Anaerobic standard rate digested (mesophilic, 29 to 38°C)	7.0	3,630	39.5	22.7	Cibolo, Tex.
3. Aerobic, digested (17°C)	5.7	174	24.5	16.5	Gainesville, Fla.
4. Primary, undigested	5.1	1,535	41.2	31.0	Las Cruces, N.M.
5. Anaerobic, digested, dewatered	6.9	900	116.0	76.0	Houston, Tex.

mary sludges. A 300-ml sample of well-mixed sludge was stirred on a magnetic stirrer and adjusted to pH 3.5 with 5 M HCl. It was then adjusted to a final $AlCl_3$ concentration of 0.0005 M by the addition of 0.05 M $AlCl_3 \cdot 6H_2O$. After additional stirring for 30 min, the pH of the sludge was readjusted to 3.5, if necessary, and it was then centrifuged at 2,500 × *g* for 15 min at 4°C. The supernatant was discarded, and the sedimented, conditioned sludge was suspended in 300 ml of 10% buffered beef extract solution (30 g of beef extract powder, 4.02 g of $Na_2HPO_4 \cdot 7H_2O$, and 0.36 g of citric acid dissolved in 300 ml of distilled water). The mixture was stirred on a magnetic stirrer for 30 min. To minimize foaming, the stirrer was operated only at the minimum speed sufficient to develop a vortex. The conditioned sludge-eluate mixture was centrifuged at 10,000 × *g* for 30 min at 4°C. The supernatant fluid was decanted and then filtered through a stack of 3.0-, 0.45-, and 0.25-μm-porosity Filterite filters (Duo-Fine series; Filterite Corp., Timonium, Md.). The filtrate was diluted with sterile distilled water at a ratio of 7 ml of water for every 3 ml of beef extract. The diluted filtrate was adjusted to pH 3.5 with 1 M HCl, stirred for 30 min, and then centrifuged at 2,500 × *g* for 15 min at 4°C. The supernatant was discarded, the precipitate was suspended in 0.15 M $Na_2HPO_4 \cdot 7H_2O$ with 5 ml of buffer for every 100 ml of diluted filtrate. The pH of the final mixture was adjusted to 7.0 to 7.5, and the final concentrate was then stored.

The EPA procedure was modified for recovering viruses from primary sludge and consisted of stirring 100 ml of sludge on a magnetic stirrer, adjustment of pH to 3.5, addition of 1 ml 0.05 M $AlCl_3 \cdot 6H_2O$, and mixing for 30 min. Subsequently, 75 ml of Freon (Du Pont Co., Wilmington, Del.; TF) was added and vigorously mixed for another 5 min. The mixture was then poured into a Buchner funnel that contained a 127-mm size AP25 filter (Milipore Corp., Bedford, Mass.) to which vacuum was applied. As soon as the solids appeared dry, a wash consisting of 100 ml of 0.0005 M $AlCl_3$ at pH 3.5 was poured into the funnel. This wash procedure was repeated four more times. The vacuum was then turned off, the filtering flask was replaced, and 100 ml of 10% buffered beef extract was poured on the solids. After a contact period of 10 min, the vacuum was turned on, and the beef extract filtrate (eluate) was collected in a filtering flask. This eluate was further filtered through a stack of 3.0-, 0.45-, and 0.25-μm-porosity Filterite filters. The filtrate was aliquoted into three equal portions, and these were stored at −70°C.

Glass method for virus recovery from sludge. Liquid sludge (800 ml) was poured in a blender jar, and 0.4 ml of antifoam B and 19.2 g of beef extract powder were added. For the dewatered sludge, 40 g of dry sludge was mixed in 800 ml of 3% beef extract solution containing 0.4 ml of antifoam B. The mixture was blended at low speed for 1 min and then at high speed for 2 min. The mixture was next transferred to a beaker, and its pH was raised to 9.0 with 2 N NaOH. After stirring for 25 min, the mixture was poured into centrifuge bottles placed in an ice bath, and a sonicator probe (Labline; model 9100 with 9106 probe or equivalent) was inserted 1 cm below the surface of the liquid. The mixture was sonicated at 100 W for 2 min. After centrifugation at 10,000 × *g* for 30 min, the sediment was discarded, and the supernatant was adjusted to pH 3.5 with 2 N HCl and then stirred for 30 min. After another centrifugation at 10,000 × *g* for 30 min, the supernatant was discarded, and the beef extract precipitate (floc) was suspended in 10 ml of 0.15 M Na_2HPO_4. The suspension was adjusted to pH 7.5 and then poured into a glass bottle and detoxified as follows.

A 100-mg sample of diphenylthiocarbazone (dithizone; Eastman Chemical Products, Inc., Kingsport, Tenn.; no. 3092 or equivalent) was dissolved in 1,000 ml of chloroform and stored in amber bottles at 4°C (shelf life, 1 month). On the day of use the stock dithizone solution was diluted 1:10 in chloroform, and 10 ml of this working dilution was added to the suspended floc. After blending at high speed for 1 min, the mixture was centrifuged at 10,000 × *g* for 30 min. The upper, aqueous layer was gently removed and placed in a sterile test tube containing 0.05 ml of 0.1% $CaCl_2$. This aqueous supernatant was gently aerated (approximately 1 bubble per s) with a sterile Pasteur pipette attached to a filtered air line for 10 min to remove residual chloroform. The final sample was treated with antibiotics and divided into three samples, and these were stored frozen at −70°C until assayed.

Sludge testing protocol. Most participating laboratories initiated and completed round robin tests within 48 h of sludge arrival, but two laboratories processed one or two sludges in 72 h. Each type of sludge was analyzed in triplicate by each virus recovery procedure. In most cases, duplicate sludge processing by each procedure was performed on the first test day, followed by a single replicate of each procedure the following day. Thus, each participating laboratory performed a total of six experiments on each sludge type. All laboratories took great effort to adhere to the stated procedures, including the examination of optimum sludge volume as mentioned in each respective methods protocol. However, on occasions the methods were modified slightly to compensate for problems in dealing with sludges. The problems encountered and modifications required for each sludge and virus recovery method were documented. After completion of the virus recovery procedures, each final sample was divided into three equal portions and frozen at −70°C for at least 24 hours before virus enumeration. One portion from each sample was assayed for viruses by the round robin test laboratory by using a cell line of their choice, and the second portion was saved as a backup in case of virus assay problems. The third frozen portion of each processed sample was held at −70°C until all sludge samples had been concentrated and assayed. These third portions were then shipped frozen in dry ice to the EPA

reference laboratory, where the samples from all the eight laboratories were quantitatively assayed on the same passage lot of a single cell line (buffalo green monkey kidney [BGM] cells).

Virus enumeration. Each round robin test laboratory performed a quantitative plaque assay for each replicate-processed sludge sample from each virus recovery procedure (10). All laboratories used BGM cells for virus assay, except laboratories 5 and 7, which used HeLa and human rhabdomyosarcoma (RD) cells, respectively. All virus assays were reported in terms of PFU per liter of sludge.

Statistical analysis. Virus data from each participating laboratory (including the EPA reference laboratory) were statistically analyzed (4, 14) by the U.S. Army Medical Bioengineering Research and Development Laboratory as follows.

(i) Data handling. Of a total of 240 data points anticipated (2 virus recovery methods × 8 laboratories × 5 sludges × 3 replicates), 19 and 26 were not reported in the round robin comparison data set and the reference laboratory quality assurance data set, respectively (Table 2). In addition, some data points were not used in the analysis due to use of estimated virus levels (detection limits of the methods) rather than the measured virus PFU values for the endpoints. The total percentage of non-analyzable data, including missing values, was 32% for the round robin laboratories comparison data and 19% for the reference laboratory analysis quality assurance data. Before analysis all virus counts were transformed to $\log_{10}$ values.

(ii) Precision estimation. The $\log_{10}$ precision of each method for each sludge type was estimated by using the standard deviation of the variation among replicates within laboratories. This estimate was obtained from an analysis of variance (ANOVA) on each sludge type and method combination by using a model containing only the laboratory effect. The method precisions were compared for each sludge type and overall with an F_{max} test (4) at the 99% level of confidence. The precisions of each method also were compared across sludge types by the F_{max} test to determine whether precision was sludge type dependent. Relative precisions also were calculated, expressing the precisions as percentages of the estimated population $\log_{10}$ population means for each sludge type and method combination. The estimated $\log_{10}$ population means were obtained from the comparison testing ANOVA described below and represent the predicted statistical estimates of the population mean for each method.

(iii) Comparison testing. Comparison testing was done via another ANOVA with a model containing the following factors: laboratory, method, and the interaction of laboratory and method for each sludge type. The interaction represents the dependence of method effects on the labora-

TABLE 2. Comparative recovery of viruses[a] from sludges

Sludge type	Method	Trial no.	Recovery of viruses (PFU/liter) from laboratory no.: 1		2		3		4		5		6		7		8	
			IA[b]	CA[c]	IA	CA	IA	CA	IA	CA	IA	CA	IA	CA	IA	CA	IA	CA
1. Anaerobic, high rate	EPA	1	<16[d]	83	<10	21	130	140	10	160	–[e]	–	<11	20	<25	50	295	100
		2	<23	124	<10	72	140	30	41	210	–	–	<12	10	50	20	735	90
		3	19	150	<10	40	100	80	30	190	–	–	<11	10	<25	50	688	170
	Glass	1	290	17	8	22	30	40	<4[d]	4	37	7	<5	14	50	635	29	22
		2	75	150	15	14	12	11	<4	4	14	14	34	43	<28	29	31	25
		3	85	103	4	36	8	4	<4	7	26	29	9	36	<28	101	48	7
2. Anaerobic, standard rate	EPA	1	1,039	488	170	280	–	500	3,437	1,400	900	–	74	200	854	1,350	1,710	1,000
		2	160	1,040	120	380	753	360	5,859	1,250	910	868	<21	90	656	1,110	2,030	970
		3	894	830	200	630	1,237	460	1,793	1,550	430	638	<11	10	354	980	397	270
	Glass	1	1,550	743	229	505	151	256	758	466	570	2,804	56	466	502	1,155	54	<4
		2	2,360	865	619	931	98	112	536	440	560	1,900	112	1,051	387	1,011	143	<4
		3	420	638	544	1,119	451	549	533	426	470	1,151	17	318	326	693	–	–
3. Aerobic	EPA	1	<36	–	<10	10	12	10	10	–	11	8	<8	10	<25	10	69	110
		2	<30	–	10	10	12	20	10	20	22	9	<7	10	<24	10	74	10
		3	<36	–	<10	10	<12	20	20	40	12	8	<13	10	<25	10	70	30
	Glass	1	<14	4	4	4	4	4	4	14	<2	7	<4	4	<4	4	4	7
		2	<18	–	8	4	<4	4	<4	7	<3	<3	<3	4	<7	4	25	4
		3	<27	–	4	7	<4	<4	<4	7	3	4	<3	4	<6	4	22	7
4. Primary	EPA	1	720	2,407	1,350	2,303	150	242	–	–	2,000	6,261	355	1,389	–	–	17,280	132
		2	681	2,838	1,620	9,303	1,484	727	–	–	2,100	4,342	<390	1,539	–	–	4,850	2,667
		3	783	2,857	3,210	5,274	6,630	6,242	–	–	1,400	3,150	375	1,218	–	–	7,600	2,545
	Glass	1	<338	533	938	2,034	544	643	–	–	2,400	2,700	344	1,227	1,384	2,871	1,190	1,054
		2	<400	960	566	1,300	536	578	–	–	2,300	2,606	264	1,350	469	375	1,203	1,213
		3	288	901	581	2,159	701	751	–	–	2,500	3,112	87	729	212	3,379	1,431	1,177
5. Anaerobic, dewatered	EPA	1	<611	278	250	80	450	200	1,022	900	75	243	<11	160	565	500	945	729
		2	<611	149	280	220	441	180	287	480	<62	373	<11	40	344	260	955	397
		3	<370	444	170	160	–	–	–	–	71	707	<12	20	551	160	949	94
	Glass	1	<407	1,503	896	296	57	137	215	108	75	593	<4	217	206	40	325	137
		2	<333	1,139	848	108	158	188	432	217	56	475	<5	116	840	340	786	101
		3	<370	2,591	413	318	–	–	–	–	69	409	<5	108	–	–	–	–

[a] All samples were assayed in BGM cells, except samples from laboratories 5 and 7, which were assayed in HeLa and RD cells, respectively.
[b] IA, Individual laboratory assay.
[c] CA, Comparative assay by EPA reference laboratory.
[d] Detection limits, based on sludge sample volume and eluate volume.
[e] –, Not done.

tory. The adequacy of this model was assessed with the coefficients of multiple determination (R^2), which quantifies the percent of variation in the data accounted for by the model. Estimates of the population $\log_{10}$ means and 95% confidence intervals on the mean method differences for each laboratory, and overall pooled means across laboratories were obtained from the model. Pooled precision estimates were also obtained from the comparison ANOVA model results, pooling over both methods within each sludge type. The pooled precisions were tested for equality across sludge types, and an overall estimate of precision was obtained that applies to both methods.

(iv) Graphical display. Graphical displays of estimated population $\log_{10}$ laboratory means for each method were obtained for each sludge type and for the pooled data across laboratories for each sludge type and virus recovery method. By plotting the Glass method means on the vertical axis and the EPA method means on the horizontal axis, the dispersion of points about the 45° line could be seen. Points falling below the 45° line indicate that EPA method sample means were higher than Glass method sample means, and the opposite is true for points falling above the 45° line.

(v) Quality assurance comparisons. Comparisons were made among the reference laboratory quality assurance results and the round robin laboratories' comparison data analysis results to check for consistency and reliability of the results obtained by these separate labs. Since the quality assurance data experienced a different pattern of missing data points than the comparison data set, some differences would be expected, although large differences should not occur solely due to random missing values.

RESULTS

Comparative recovery of viruses from sludges. The amounts of virus recovered from five different sludge types by eight different laboratories using two candidate methodologies are shown in Table 2. The data from individual laboratories are also compared with those generated by the reference laboratory. It appears that the EPA procedure gave relatively higher virus recoveries when compared with the Glass method. Thus, of a total of 102 replicate samples assayed by the EPA reference laboratory, 69 samples processed by the EPA method gave higher virus recoveries than those processed by the Glass method. It appears from Table 2 that the viral assays performed by the EPA reference laboratory were, in general, more sensitive than those performed by the round robin test laboratories.

Precision of the methods. The $\log_{10}$ precisions (analytical reproducibilities) for each method and sludge combination, together with the degrees of freedom on which each is based, are listed in Table 3. The F_{max} statistics (Table 4) indicate no detectable statistical difference in the precisions of the two methods for each sludge type and overall at the 99% level of confidence. The comparison of precisions across sludge types for each method also yields no statistical difference, indicating that the method precisions are not sludge type dependent. The relative precisions are also listed to indicate precision as a precentage of the estimated population log means (Table 5). The estimated precision of the EPA method is 0.26 $\log_{10}$ units, and the Glass method is 0.24 $\log_{10}$ units based on the round robin test data (Table 3).

The reference laboratory quality assurance data precisions for each sludge type and method were compared with the individual laboratory comparison data results by using an F_{max} statistical test (Table 6). No statistical difference between the quality assurance and comparison data precisions were detected at the 99% confidence level. The relative precisions for the quality assurance data are also listed in Table 5 for comparison with the round robin relative precisions. Relative precisions were highest for sludge 3, except for the EPA round robin results. The average relative precisions range from 10 to 16% of the means, with no significant differences between methods. Overall precision estimates, pooling methods for each sludge type, were obtained from the comparison testing ANOVA (Table 7). The F_{max} tests detected no statistical difference in precisions across sludge types for either method and no difference in precisions of the two methods for each sludge type. The weighted average overall pooled precisions estimate for the round robin comparison data is 0.22 and 0.29 $\log_{10}$ units for the quality assurance data. Thus, the analytical reproducibility of both methods is on the order of 0.25 $\log_{10}$ units, based on the laboratories in this study.

TABLE 3. $\log_{10}$ precisions of the methods

Sludge type	$\log_{10}$ precision (df)			
	Round robin comparison		Reference laboratory analysis quality assurance	
	EPA	Glass	EPA	Glass
1	0.23 (6)	0.28 (11)	0.22 (12)	0.42 (16)
2	0.27 (14)	0.27 (15)	0.29 (15)	0.20 (14)
3	0.13 (7)	0.26 (6)	0.33 (7)	0.17 (8)
4	0.39 (11)	0.22 (12)	0.41 (12)	0.22 (14)
5	0.21 (9)	0.18 (9)	0.30 (12)	0.24 (13)
Overall	0.26 (47)	0.24 (53)	0.31 (58)	0.26 (65)

TABLE 4. F_{max} statistics for round robin and quality assurance $\log_{10}$ precisions

Sludge type	Round robin comparison		Reference laboratory analysis quality assurance	
	F_{max}	Critical value[a] (df)	F_{max}	Critical value (df)
1	1.4	7.5 (8)	3.7	4.4 (14)
2	1.0	4.4 (14)	2.1	4.4 (14)
3	4.0	11.1 (6)	4.1	8.9 (7)
4	3.1	5.4 (11)	3.4	4.6 (13)
5	1.3	6.5 (9)	1.6	4.9 (12)
Overall	1.2	2.2 (50)	1.4	2.0 (61)
Pooled EPA[b]	8.4	11.1 (9)	3.6	10.6 (11)
Pooled Glass[b]	2.3	9.6 (10)	6.4	7.5 (13)

[a] Value that must be exceeded to reject equality of precisions at the 99% level and degrees of freedom associated with the critical value (the geometric mean of the degrees of freedom of the precisions being compared).
[b] Comparison of five precisions across sludge types for each method.

TABLE 5. $\log_{10}$ relative precisions of the methods

Sludge type	$\log_{10}$ relative precision[a]			
	Round robin comparison		Reference laboratory analysis quality assurance	
	EPA	Glass	EPA	Glass
1	11.8	17.0	11.9	30.5
2	10.0	11.1	10.7	7.1
3	10.7	38.4	28.0	22.9
4	12.1	7.8	12.5	7.2
5	8.5	7.3	12.9	9.9
Avg	10.6	16.3	15.2	15.5

[a] Percentages of estimated $\log_{10}$ population means.

TABLE 6. F_{max} of $\log_{10}$ precisions between reference laboratory analysis quality assurance and the round robin comparison data

Sludge type	EPA		Glass	
	F_{max}	Critical value (df)	F_{max}	Critical value (df)
1	1.1	7.5 (8)	2.3	4.6 (13)
2	1.1	4.4 (14)	1.9	4.4 (14)
3	6.3	8.9 (7)	2.6	8.9 (7)
4	1.2	5.4 (11)	1.0	4.6 (13)
5	2.1	5.9 (10)	1.7	5.4 (11)
Overall	1.4	2.1 (52)	1.2	2.0 (59)

An ANOVA was also performed on each sludge and method combination to test for trends in the replicates. No evidence of a linear or nonlinear trend was found at the 95% level of confidence for any combination of method and sludge type. Thus, there is no evidence of a "learning curve" in the replicates for either method.

Comparison testing. The results of the ANOVA for the comparison of methods appear in Table 8. Statistical evidence of differences are judged by P values. A P value less than 0.05 is considered evidence of statistical differences in the data and corresponds to the 95% level of confidence. Round robin test laboratory effects were detected for all sludge types in the comparison data and in three out of five sludge types of the reference laboratory quality assurance data. Variation among laboratories is an expected outcome in collaborative testing due to the variation in environmental and other factors across laboratories. This phenomenon does not invalidate the test for virus methods since both methods are tested in each laboratory. However, interaction effects of laboratory with method as observed in this study make interpretation of method effects difficult, as the interaction indicates dependence of method effect on laboratories. Interactions were detected in sludge types 1, 2, and 5 for both the round robin comparison data and the reference laboratory analysis quality assurance data, indicating that there is a dependence of method effect on laboratory for these cases. For sludge types 3 and 4, method effects were clearly indicated with no interactions detected.

In general the ANOVA results for both the round robin comparison data and the reference laboratory analysis quality assurance data are consistent. For sludge type 2 a method effect was detected in the comparison data, but not in the quality assurance data, and for sludge type 3 a laboratory effect that was detected in the comparison data was not detected in the quality assurance data. It is felt that this may be due to the different sample sizes and laboratories analyzed within each test due to different patterns of missing values.

The adequacy of the statistical model to account for the variability in the data ranges from 76 to 90% for the comparison data and from 53 to 72% for the quality assurance data.

It is not clear why the model fit was consistently better for the round robin comparison data analyses than for the reference laboratory quality assurance data analyses. Given the estimated analytical precision of approximately 0.25 $\log_{10}$ units, this degree of explanatory power in a statistical model is not disappointing. Thus, there is little evidence to reject the statistical model used to evaluate the methods.

Graphical displays. Figure 1 for round robin comparison data and Fig. 2 for the reference laboratory analysis quality assurance data show the joint distribution of the estimated population laboratory method means for each sludge type and the overall means for each sludge type. The 45° line represents zero sample difference in method means, where the Glass virus recovery method mean is plotted on the vertical axis and the EPA virus recovery method mean is plotted on the horizontal axis. The dispersion of laboratory means in the figures represents the variation in laboratories. The further a point is from the 45° line, the more likely a statistical difference in method means will be detected. The interaction of laboratory and method can be seen by the distribution of points above and below the 45° line, representing cases where some laboratories had higher recovery by the Glass method and some had higher recovery by the EPA method. The overall sludge means (Fig. 1 and 2) were averaged over all laboratories and represent the overall effect of the methods for each sludge type. The EPA method in general provides higher average virus recovery, except for sludge 5, where no differences could be statistically detected (Fig. 1 and 2).

TABLE 7. Estimated pooled $\log_{10}$ precisions from ANOVA

Sludge type	$\log_{10}$ precision (df)		F_{max}[a]
	Round robin comparison	Reference laboratory analysis quality assurance	
1	0.23 (10)	0.35 (26)	2.32
2	0.27 (29)	0.24 (27)	1.27
3	0.20 (13)	0.27 (13)	1.82
4	0.30 (21)	0.30 (24)	1.00
5	0.20 (18)	0.27 (25)	1.82
Overall	0.22 (91)	0.29 (115)	1.74
F_{max} (over sludge types)	2.31 (17)[a]	2.03 (22)[a]	

[a] None were significant at 99% level of confidence.

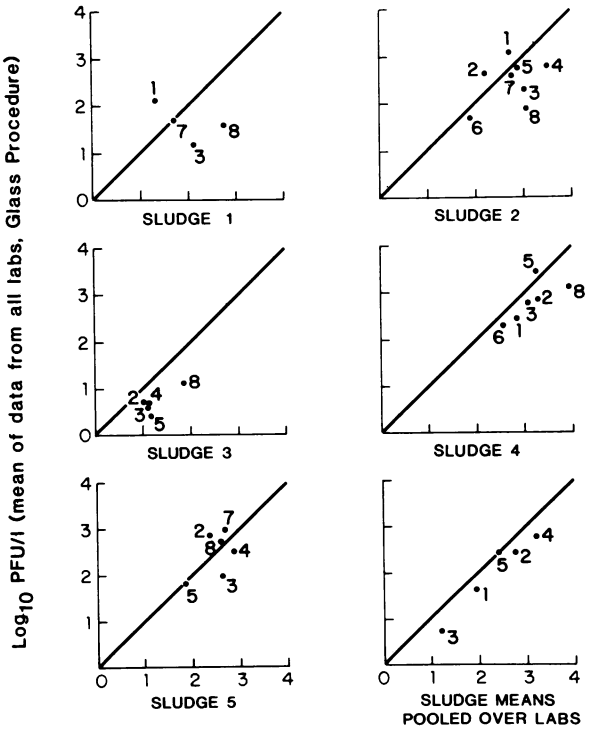

FIG. 1. Round robin comparison data for sludges.

TABLE 8. ANOVA P values[a] and R^2 values

Sludge type	Round robin comparison				Reference laboratory analysis quality assurance			
	P values				P values			
	Laboratory	Method	Laboratory × method	R^2 (%)	Laboratory	Method	Laboratory × method	R^2 (%)
1	0.0139	0.0239	0.0005	90	0.1004	0.0006	0.0006	72
2	0.0001	0.0026	0.0006	81	0.0001	0.1295	0.0005	77
3	0.0004	0.0001	0.5962	88	0.2186	0.0020	0.5504	67
4	0.0001	0.0042	0.2021	76	0.0110	0.0353	0.6756	53
5	0.0001	0.8528	0.0044	87	0.0007	0.3644	0.0385	70

[a] P values of less than 0.05 are considered statistically significant.

An alternative graphical display is to plot the mean method differences and 95% confidence intervals. When this was done for the round robin laboratory data (Table 9), the average method differences were found to be small in log magnitude (from 0.01 to 0.55 $\log_{10}$ units), and in the case of some sludge types the 95% confidence intervals encompassed the zero value. In general, the EPA method provided greater recovery for most sludge types, but the lower bound of the 95% confidence intervals on differences approaches zero in many of these cases.

To test whether the method effect was consistent across sludge types, the number of points above and below the 45° line were counted for both the comparison data set and quality assurance data sets (Fig. 1 and 2). A Fisher's exact test was performed at 95% confidence level to test for systematic difference in the dispersion of points above and below the 45° line across sludge types. No difference was detected for the comparison data (P = 0.41) or the quality assurance data set (P = 0.08). There is, therefore, no evidence from this test that the difference in the methods depends on sludge types. This test, however, is less sensitive than the comparison ANOVA, which detected a method effect for sludge types 3 and 4 with interaction, and interactions of method and laboratory in other sludge types.

As a final test, the dispersion of points above and below the 45° line was compared between the comparison data set (Fig. 1) and the quality assurance data set (Fig. 2) with Fisher's exact test again. No difference was detected at the 95% level of confidence between the two data sets with respect to dispersion above and below the 45° line, indicating consistency of the single laboratory analysis quality assurance and round robin comparison data sets. From these results, the following can be concluded: (i) the precisions or analytical reproducibilities of two methods were not statistically different; (ii) there was a dependence of method effect on laboratory for sludge types 1, 2, and 5 because, for these sludge, interactions were found between laboratory and method; (iii) the EPA method was more sensitive than the Glass method, except for dewatered sludge; (iv) the magnitude of the method effect was small (from 0.01 to 0.55 $\log_{10}$ units) and was statistically close to zero for many sludge types at the 95% confidence level; and (v) there was no statistically significant difference between the reference laboratory quality assurance data and the round robin comparison data for precision or comparison testing of the methods.

DISCUSSION

The results of this study indicated that the EPA method is slightly more sensitive than the Glass method for recovering viruses from all sludge types, except the dewatered sludge. For the latter sludge type, no statistically significant difference was found in virus recovery by either method. It also appears that the EPA method may be more readily adaptable to field monitoring of viruses in wastewater sludges because of its greater simplicity and the shorter time period required for its completion (with the possible exception of eluate filtration). However, bacterial and fungal contamination of the final sample is a problem associated with this method. Also, the final sample volume of the EPA method is too large to be assayed economically. Certain other advantages and disadvantages of both methods are listed in Table 10. On the basis of these observations, both procedures are recommended as tentative standard methods for the recovery of human enteroviruses from sludge. The EPA procedure for primary sludges was found to be too cumbersome and is recommended to be abandoned in favor of the EPA method for "all other sludges." Since environmental samples usually contain low numbers of viruses, it is important to assay the complete concentrate for viruses. To accomplish this economically, the final sample volume should to kept at a minimum. It is recommended, therefore, that efforts to

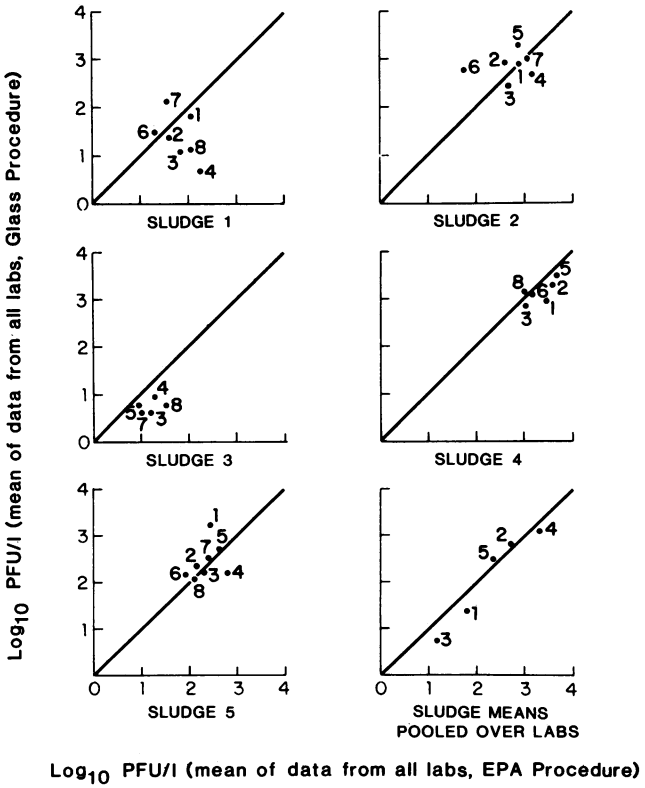

FIG. 2. Reference laboratory quality assurance data for sludges.

TABLE 9. Estimated round robin method population $\log_{10}$ means and difference by laboratory and sludge type

Sludge type	Laboratory	$\log_{10}$ mean		Difference	95% Confidence intervals	
		EPA	Glass			
1	1	1.28	2.09	−0.81	−1.33	−0.29
	2					
	3	2.09	1.16	0.93	0.56	1.30
	4					
	5					
	6					
	7	1.70	1.70	0.0	−0.64	0.64
	8	2.73	1.55	1.18	0.81	1.55
	Overall	1.95	1.62	0.33	0.09	0.57
2	1	2.72	3.06	−0.34	−0.79	0.11
	2	2.2	2.63	−0.43	−0.88	0.02
	3	3.0	2.28	0.72	0.27	1.17
	4	3.47	2.78	0.69	0.24	1.14
	5	2.85	2.73	0.12	−0.33	0.57
	6	1.87	1.68	0.19	−0.44	0.82
	7	2.76	2.60	0.16	−0.29	0.61
	8	3.05	1.94	1.11	0.61	1.61
	Overall	2.74	2.46	0.28	0.12	0.44
3	1					
	2	1.00	0.69	0.31	−0.16	0.78
	3	1.08	0.61	0.47	−0.02	0.96
	4	1.11	0.59	0.52	0.05	0.99
	5	1.13	0.43	0.71	0.37	1.05
	6					
	7					
	8	1.85	1.13	0.72	0.38	1.06
	Overall	1.24	0.69	0.55	0.37	0.73
4	1	2.86	2.46	0.40	−0.29	1.09
	2	3.28	2.83	0.45	−0.03	0.93
	3	3.06	2.77	0.29	−0.19	0.77
	4					
	5	3.26	3.38	−0.12	−0.60	0.36
	6	2.56	2.30	0.26	−0.28	0.80
	7					
	8	3.93	3.10	0.83	0.35	1.31
	Overall	3.16	2.81	0.35	0.14	0.56
5	1					
	2	2.36	2.83	−0.47	−0.78	−0.16
	3	2.65	1.98	0.67	0.28	1.06
	4	2.73	2.48	0.25	−0.14	0.64
	5	1.84	1.82	0.02	−0.29	0.33
	6					
	7	2.68	2.98	−0.30	−0.61	0.01
	8	2.62	2.70	−0.08	−0.47	0.31
	Overall	2.48	2.47	0.01	−0.13	0.15

refine both procedures should be continued so that the final sample volume is as low as possible without sacrificing sensitivity.

In this study, uniformity was maintained in regard to the source of the sludge and amount of time lapsed between sludge collection and processing. Also, part of the final sample from all replicates from all laboratories was assayed by a central laboratory. Differences existed in terms of source of beef extract and other chemicals, the type and age of cell cultures, and the method of plaque assay and cell culture procedures used by the individual laboratories. It is no surprise therefore that wide variations were observed in virus recoveries obtained by different investigators. Among other things, this may have been due to inadequate mixing of initial sludge sample; inadequate elution of viruses embedded in sludge; laboratory-to-laboratory variability in sludge processing, cell culture, and viral assay procedures; and the past experience of a laboratory with a particular procedure. In future studies some of these variables can be avoided by providing uniform chemicals, media, dyes, filters, and eluents to all participating laboratories. It should be pointed out that the personnel at all eight test laboratories are very experienced in environmental virology and that a greater variability in results would be expected with less experienced laboratory workers.

Differences between the plaque assay results from individual round robin laboratories and those from the reference laboratory may indicate the variability in susceptibility of BGM cells, different assay procedures, plaque confirmation efforts, and quantity of concentrate assayed. In future studies, one central laboratory should always reassay the final samples from all laboratories to generate comparative data such as this. Also, a single passage number of a certain cell type should be distributed to all laboratories for use in viral assays.

The data generated in this study have reinforced results from previous studies which indicated the inadequacy of anaerobic digestion for complete removal of infective virus from sludge (2, 5, 9). It is also evident from this study that aerobically digested sludge contains the least amount of infective virus. Although the virus isolates were not identified in the present study, it may be advantageous to do so in future studies to determine whether a particular type of sludge treatment selectively eliminates certain viruses (6).

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TABLE 10. Comparisons of EPA and Glass methods for virus recovery from sludge

Procedure	Advantages	Disadvantages
EPA	No special or unusual equipment is required	Final sample volume is too large (ca. 50 to 100 ml)
	Easier to process several samples simultaneously	Problem with bacterial and fungal contamination
		Filtration step is difficult and time consuming to perform, particularly with primary sludge
		Most investigators found the primary sludge method cumbersome
Glass	Final concentrate volume to be assayed for virus is small	Needs sophisticated equipment such as sonicator
	The method is relatively simpler with single samples	Resuspension of organic floc proves difficult sometimes
	No problem with bacterial or fungal contamination	Excessive foaming with aerobically digested sludge
		The anaerobic sludges do not pack well after sonication and centrifugation
		It is difficult to obtain the top aqueous layer after dithizone treatment

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