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Comparison of Methods for Rotavirus Detection in Water and Results of a Survey of Jerusalem Wastewater

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Methods for the detection of viable rotaviruses and rotavirus antigen in water were developed and compared. The methods included laboratory-developed enzyme-linked immunosorbent assays (ELISAs) with chromogenic and luminescent substrates, commercial Rotazyme and Enzygnost ELISAs, and an indirect immunofluorescent assay. Of the methods tested, the immunofluorescent assay and the Enzygnost ELISA were the most sensitive for the simian rotavirus SA-11. All of the methods were positive for human rotavirus from clinical specimens. Seeded SA-11 rotavirus was concentrated from water by adsorption to and elution from Zeta Plus filters followed by organic flocculation. Interference with the assays by components of the wastewater concentrates was minimal for the ELISAs, although the undiluted organic flocs were cytotoxic for the immunofluorescent assay. A survey of Jerusalem wastewater was carried out over the course of 1 year, and samples were assayed for rotaviruses and enteroviruses. Although enteroviruses were found in almost all of the samples, all samples were negative for rotaviruses. The concentration of rotaviruses in the wastewater was thus below the detection limit of the methods used.

Rotaviruses are enteric pathogens which cause diarrhea primarily in young children and are major causes of infant hospitalization and mortality worldwide (4). Rotavirus-induced morbidity has been reported in Israel (1, 13, 19), but little is known about its distribution in the Israeli water environment. The virus is transmissible by water and has been found in water and sewage in various parts of the world (5, 6). Rotavirus concentrations have been reported to exceed those of enteroviruses in some wastewater samples (18).

The purpose of this work was the development and testing of methods for the detection of rotaviruses in water concentrates to determine which methods are the most sensitive and useful for water monitoring. An evaluation of the relative usefulness of rotavirus and enterovirus assays for routine virus monitoring was made in a survey of untreated wastewater and activated sludge effluent from the Jerusalem wastewater treatment plant in Ein Karem, Israel, carried out over 1 year. The results are presented below.

MATERIALS AND METHODS

Simian rotavirus growth and assay in cell cultures. Simian rotavirus SA-11 was obtained from C. P. Gerba, University of Arizona, Tucson, and grown in MA-104 (fetal rhesus monkey kidney) cells as described previously (7).

Titration of the virus by cytopathic effect (CPE) in the MA-104 line of fetal rhesus monkey kidney cells was done as follows. Virus was added to the cells in four replicate wells of a microtiter plate in the presence of 20 μg of Bacto-Trypsin (Difco Laboratories, Detroit, Mich.) in RPMI 1640 medium without serum, and the CPE was observed 4 to 7 days after infection. Stock virus titers were generally 0.5×10^5 to 2×10^5 50% tissue culture infective doses per ml.

Enterovirus assay. Enteroviruses were detected by plaque assay on BGM (African green monkey kidney) cells as described previously (8). In some cases, cytotoxicity was reduced by washing the monolayers with 2% calf serum in saline after virus adsorption as described previously (11). Results are reported as enterovirus PFU, although virus identification was not carried out.

Human fecal specimens. Rotavirus-positive human fecal specimens diluted 1:3 in phosphate-buffered saline (PBS) were obtained from the Clinical Virology Laboratory, Hadassah Hospital, Ein Karem, Jerusalem, Israel, and stored at −70°C. The samples were diluted 1:5 in RPMI medium and further processed by one of two methods. (i) Sample 464 was diluted 1:5 with medium and subjected to centrifugation at 100 × *g* for 5 min; the supernatant was then filtered through a 0.22-μm (nominal pore size) filter (FP 030/3; Schleicher and Schüll, Dassel, Federal Republic of Germany). (ii) Samples 594 and 790 were diluted 1:2 with medium, sonicated in a water bath sonicator (Astrason model 2E ultrasonic cleaner; Ultrasonics, Plainview, N.Y.) for 10 min, and centrifuged for 30 min at 12,000 × *g*. The negative specimen (Ca) was obtained from a child after oral poliomyelitis vaccine administration and contained 50 PFU of poliovirus per ml (data not shown). The sample was treated by method (ii) above.

Antiserum preparation. Simian rotavirus SA-11 was purified by centrifugation at 100,000 × *g* for 90 min at 4°C in an SW 28 rotor (Beckman Instruments, Inc., Palo Alto, Calif.). The pellet was suspended in 6 ml of PBS containing 0.5% Triton X-100. The virus was subjected to sonication in a water bath sonicator for 5 min and layered on a 20 to 40% sucrose gradient in PBS. Following centrifugation for 90 min at 100,000 × *g* at 4°C in an SW28 rotor, the gradient was fractionated and the virus band was identified by A_{260} and CPE. The fractions containing virus were pooled, and the virus was further concentrated by centrifugation at 100,000 × *g* for 90 min at 4°C. The pellet was suspended in PBS and stored at −70°C.

The purified virus was diluted 1:1 with Freund adjuvant and injected intramuscularly into the thighs of two rabbits. Three shots, at monthly intervals, each containing 1.5×10^8 to 7.5×10^8 viral infectious units, were followed by a fourth after a 2-month interval. At 2 months after the final injection,

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was blood was taken from the heart and separated, and the serum was stored at −70°C.

Antibody titers were determined by neutralization of 100 50% tissue culture infective doses of the virus after exposure to dilutions of the sera for 30 min at 37°C. The 50% neutralization point was reached at a dilution of 1:16,000.

Antibody purification and enzyme conjugation. Immunoglobulins were purified from the serum by the method of Joustra and Lundgren (12). Briefly, the serum was dialyzed against PBS and clarified by centrifugation for 60 min at 12,000 × *g*. The supernatant was passed through a column of QAE Sephadex, and the unbound material was precipitated with saturated ammonium sulfate. After centrifugation for 30 min at 12,000 × *g*, the antibodies were suspended in PBS and dialyzed against PBS containing 5×10^{-4} M thimerosal.

Conjugation of immunoglobulin G with horseradish peroxidase was done by the periodate method, as described previously (3). The conjugate was separated from unconjugated protein by chromatography on Sephadex G-200. Two peaks of enzyme activity were obtained, and the void-volume fractions were used in the immunoassays.

Virus immunoassays. (i) Indirect immunofluorescence (IF). Cells (MA-104, at a concentration of 1.5×10^6 per ml) were seeded onto microscope slides (12 holes per slide, 20 μl per hole) and grown for 24 h. The medium was removed, and the cells were washed twice with RPMI medium. The samples were diluted 1:10 in RPMI medium containing 20 μg of Bacto-Trypsin per ml, preincubated for 30 min at 37°C, and added to the cells at a volume of 20 μl per hole. After 20 h at 37°C in a 5% CO_2 atmosphere, the slides were washed with PBS, and the cells were fixed with acetone and stained as described previously (9). Infected cells were detected with anti-rotavirus antiserum at a dilution of 1:320 in PBS and fluorescein-conjugated anti-rabbit immunoglobulin G antibody (Miles-Yeda, Rehovot, Israel) diluted 1:20 in PBS as the second layer. Results are expressed as focus-forming units (FFU).

(ii) Laboratory ELISAs. Enzyme-linked immunoabsorbent assays (ELISAs) were developed with the antisera and purified immunoglobulin G described above. Anti-rotavirus antiserum was diluted 1:1,000 in 0.05 M carbonate buffer (pH 9.5), 150 μl was added to polystyrene test tubes (no. 4960; Lumac BV Systems AG, Schaesberg, The Netherlands) for 2 h at 37°C, and the tubes were incubated overnight at 4°C. The tubes were washed three times with Tris-saline buffer (TSB; 0.05 M Tris hydrochloride, 0.15 M NaCl [pH 7.6]) containing 0.1% bovine serum albumin (BSA). The test sample (150 μl) was adsorbed for 1 h at 37°C. Unadsorbed material was removed, and the tubes were washed three times with TSB containing 0.05% Tween 80. Antigen was detected with anti-rotavirus immunoglobulin G conjugated to horseradish peroxidase at a dilution of 1:100 (pyrogallol and luminol assays [see below]) or 1:4,000 (*o*-phenylenediamine [OPD] assay) in TSB containing 1.0% BSA. The conjugate (150 μl) was incubated for 1 h at 37°C and then washed three times with TSB containing 0.05% Tween 80.

The conjugate was detected by using one of three substrate systems. (i) For the pyrogallol system, 100 μl of 0.2% pyrogallol (Sigma Chemical Co., St. Louis, Mo.) in 0.18 M phosphate buffer (pH 6.5) and 50 μl of 0.74% H_2O_2 in 0.18 M phosphate buffer (pH 6.5) were added to each tube (20). Each tube was read in a luminometer (Biocounter 2000; Lumac) immediately after the addition of H_2O_2. (ii) For the luminol system, luminol (0.66 mg/ml; Sigma) and luciferin (0.032 mg/ml; Sigma) in TSB were mixed 1:1, and 100 μl was added to each tube. The reaction was read in the luminometer immediately after the addition of 50 μl of 0.74% H_2O_2 in 0.18 M phosphate buffer (pH 6.5). Control experiments showed that the sensitivity of the assay was the same as when the reaction conditions were as described by Whitehead et al. (22). (iii) For the OPD system, OPD (2.5 mg/ml; Sigma) in citrate-phosphate buffer containing 0.02% H_2O_2 was freshly prepared, and 150 μl was added to each tube (21). After the mixture was incubated for 15 min at 20°C, the reaction was stopped with 1 ml of 1 N HCl. The reaction was read at A_{492} in a Techtron spectrophotometer (Varian, Palo Alto, Calif.).

TABLE 1. Time course of SA-11 rotavirus detection by the IF assay[a]

Time (h)	Virus titer at following dilution[b]:	
	Undiluted	1:10
2	0	0
4	1.2×10^2	0
8	3.3×10^4	6.4×10^3
22	+++	1.1×10^5

[a] Cells were infected with simian rotavirus SA-11 and fixed at the indicated times after infection.

[b] The results are expressed as FFU per milliliter of the original inoculum. +++, Too numerous to count.

(iii) Commercial ELISAs. Rotavirus diagnostic kits were obtained and used as specified by the manufacturers. The two kits used were Rotazyme (Abbott Laboratories, North Chicago, Ill.) and Enzygnost Rotavirus (Ag) (Behring Institute, Marburg, Federal Republic of Germany).

Blocking test. The blocking test to demonstrate the specificity of the assays was carried out by incubating anti-rotavirus antiserum (diluted 1:20 in medium) with SA-11 rotavirus for 1 h at 37°C prior to the assay and measuring the level remaining. A decrease of 50% or more was considered a positive reaction (16).

Virus concentration methods. Virus was concentrated from water by filtration through Zeta Plus 60S cellulose–diatomaceous earth–charge-modified resin 142-mm-diameter filters (nominal pore size, 0.45 μm; AMF, Cuno Division, Meriden, Conn.) at pH 6.0 followed by elution with 75 ml of 3% beef extract (pH 9.0). In some experiments, the eluate was reconcentrated to a volume of 3 ml by organic flocculation, as described previously (7). Additional filters (the kind gift of AMF, Cuno Division) used were Zeta Plus 50S (nominal pore size, 0.75 μm), Zeta Plus 30S (nominal pore size, 1.0 μm), and 1MDS (surface-modified fiber glass-cellulose; nominal pore size, 0.2 μm) filters.

RESULTS

Development of laboratory rotavirus assays. To detect rotavirus in water, an IF assay and ELISAs were developed with immune reagents prepared against simian rotavirus SA-11. The IF assay required 22 h of infection for optimal virus titration (Table 1) and was dependent on the presence of trypsin. The ELISAs were developed by using three different substrates. Two of the assays involved the use of luminescent substrates (pyrogallol and luminol-luciferin), while the third involved the use of the chromogenic OPD.

The tests were developed with SA-11 rotavirus as a positive control. The specificity of the IF and luminol assays was shown by a blocking test with SA-11 antiserum (Table 2). The same procedure was also found to block the Enzygnost commercial assay, which was used for comparison (Table 2).

TABLE 2. Assay blocking by antiserum to SA-11 rotavirus[a]

Assay	Virus dilution	Virus titer[b]:		Negative control[c]
		Without antiserum	With antiserum	
IF	5×10^{-2}	$6.2 \times 10^5 \pm 4.7 \times 10^5$	0	0
Luminol	1×10^{-1}	7,000	1570 ± 570	860
	1×10^{-2}	3,200	1170 ± 290	860
Enzygnost	1×10^{-1}	0.322 ± 0.063	0.021 ± 0.002	0.000
	5×10^{-2}	0.206 ± 0.018	0.026 ± 0.006	0.000

[a] The rotavirus assays were performed in the presence or absence of rotavirus-blocking serum.
[b] Replicate samples are expressed as mean ± standard deviation. The units for the assays are as follows: IF, FFU per milliliter; Luminol, luminometer counts; Enzygnost, optical density.
[c] Medium without virus was used in negative-control determinations.

Comparison of assays for SA-11 rotavirus. The laboratory and commercial assays were compared with respect to sample volume, sensitivity, and time to perform (Table 3), with SA-11 rotavirus as a test sample. Of the two assays for detecting viable virus, the IF assay had the advantage of requiring less time but was less sensitive than CPE owing to the smaller sample volume applied. Among the ELISAs, the Enzygnost assay was the most sensitive but took slightly longer to complete than the laboratory assays. Of the laboratory ELISAs, the OPD and luminol systems were more sensitive than the pyrogallol substrate for SA-11 rotavirus.

Detection of human rotavirus by the assays. The detection of rotavirus in water requires methods which are sensitive for the human virus. Rotavirus-positive stool specimens were tested by the IF, OPD, luminol, and Enzygnost assays (Table 4). The three positive samples were positive in all of the ELISAs, while the negative sample was negative in all the ELISAs. The IF assay was positive in two cases, and the sample was toxic to the cells in the third. The relative amounts of virus in the positive human samples differed as detected by the IF and the ELISAs. Sample 790 contained more virus than sample 464 as detected by IF but less as detected by the Enzygnost assay, and they contained similar amounts as determined by the OPD and luminol assays. Variations in the infectivity of rotavirus isolates are not uncommon (23).

Detection of SA-11 rotavirus in water concentrates. The ability of the assays to detect rotavirus in water concentrates was tested by using water seeded with SA-11 rotavirus and then concentrated by membrane filtration (Table 5). The amount of virus in the input water varied in each experiment. In general, the ELISAs did not detect virus in the input samples but were positive for virus in the eluate, indicating that the virus was concentrated by the procedure. The IF was somewhat more sensitive than the ELISAs, detecting virus in most of the input samples. The IF was negative at an estimated 100 FFU/ml and positive at 800 FFU/ml. The cutoff of virus detection by the ELISAs was in the range of 3×10^3 FFU/ml, based on an estimate of the amount of virus seeded. The OPD assay appeared to be slightly less sensitive than the commercial assays.

In one case, the eluate was reconcentrated by organic flocculation and the floc was assayed by IF and the Rotazyme assay (Table 5), both of which gave positive results.

Lack of interference with virus detection by sewage concentrates. The possibility that components of sewage concentrates interfere with the sensitivity of the rotavirus immunoassays was tested as follows. Sewage eluates and flocs were prepared by membrane filtration and organic flocculation of wastewater. SA-11 rotavirus was seeded into the samples and in parallel into medium, and the samples and medium were diluted and assayed (Table 6). The IF was the most affected by the floc, which was cytotoxic until diluted. The more-diluted samples were all positive by IF, with the titers of the floc and eluate being slightly higher than those of the medium. The Enzygnost assay was more sensitive than the other ELISAs, and the floc or eluate had no effect on the sensitivity. The OPD assay was slightly less sensitive than the luminol assay but was essentially unaffected by the concentrates. The luminol assay gave a more mixed result, indicating possible minor interference by the eluate.

Comparison of filters for virus concentration from effluent. A comparison was made among several types of positively charged filters for the ability to recover seeded SA-11 rotavirus from effluent (Table 7). Parallel experiments were performed with unseeded effluent, and all of the fractions were negative for rotavirus.

Virus was detected in the input only by IF and the Enzygnost assay. The highest IF titer of virus was found in the 60S filter eluate, while no virus was recovered by the 1MDS filter. In most cases, the floc was cytotoxic.

The OPD and Enzygnost ELISAs indicated that the floc of the 30S filter contained the largest amount of viral antigen. The OPD assay detected similar amounts of virus in the Zeta Plus eluates, with less for the 1MDS filter. The luminol test was negative for the 50S and 1MDS eluates. Among those tested by the Enzygnost assay, the eluate of the 50S filter contained the most antigen. A mixed picture was obtained of the usefulness of the filters, although the 1MDS filter seemed the least useful for rotavirus recovery.

Additional attempts to recover rotavirus from unseeded effluents by using 60S and 30S filters were not successful. However, enterovirus recovery was higher for the 60S filters than the 30S filters (Table 8). The survey described below was carried out primarily with the 60S filters.

TABLE 3. Sensitivity of the assays for SA-11 rotavirus

Assay	Sample vol (μl)	Maximal positive dilution	Time to complete
Viable assays			
CPE	200	10^{-5}	5–7 days
IF	20	10^{-4}	22 h
Commercial ELISAs			
Rotazyme	200	10^{-2}	6 h
Enzygnost	150	10^{-3}	5 h
Laboratory ELISAs			
Pyrogallol	150	10^{-1}	3 h
Luminol	150	10^{-2}	3 h
OPD	150	10^{-2}	3 h

TABLE 4. Sensitivity of assays for human rotavirus in clinical samples

Assay	Sensitivity for sample:					
	464	594	790	Ca	Negative control[a]	Positive control[a]
IF[b]	+	T	+++	0	0	+++
OPD	0.30	0.40	0.35	ND[c]	0.09	0.35
Luminol	4,800	4,450	4,550	775	860	7,000
Enzygnost	0.845	0.779	0.617	0.012	0.000	0.413

[a] The positive control was a 10^{-1} dilution of SA-11 rotavirus stock, and the negative control was dilution medium.
[b] For the IF: T, cytotoxic; +, a few isolated positive cells; +++, too numerous to count. For the OPD, the unit is optical density. For the other units, see footnote *b*, Table 2.
[c] ND, Not determined.

Efficiency of enterovirus recovery from wastewater. Enterovirus recovery could be directly measured by comparing input and eluate titers in a number of samples concentrated. The average recovery was 34% (Table 9), while concentration factors ranged from 5- to 14-fold, depending on input volume. It may also be noted that in addition to concentrating the virus, filtration provided an eluate that was less cytotoxic than the wastewater effluent. The eluate of the primary wastewater effluent had an average recovery efficiency of 43 ± 38%, while the eluate of the untreated effluent averaged 24 ± 4%.

Recovery of virus in the filtrate. The possibility that the low recovery of enterovirus was due to interference with virus adsorption was tested as follows. Filtrates of two samples were refiltered through Zeta Plus filters, and the eluates of the second filtration were assayed for virus (Table 10). These second eluates contained enteroviruses, but fewer than contained in the original eluates. Thus, some virus is lost in the filtrate, but it does not appear to be the majority of the virus. Rotavirus was not detected in any of the samples.

Results of the virus survey. Samples of untreated and treated effluent were obtained from the Jerusalem sewage treatment plant in Ein Karem. The primary treatment was sedimentation, and the secondary treatment was with activated sludge followed by sedimentation. The samples were obtained over the course of 1 year, at approximately monthly intervals. The volumes of the samples ranged from 200 to 3,650 ml, with an average of 860 ml of wastewater, concentrated to 70 ml of eluate and 3 ml of floc. A total of 29 samples were assayed for enteroviruses, and, with one exception, viruses were detected on each date (Table 11). Titers varied, but in most cases viruses were detectable directly in the wastewater effluent. A maximum of 8.3×10^4 PFU/liter was detected in the untreated effluent. Virus reduction due to primary or secondary treatment was variable, and in some cases there was no decrease in virus titers.

The samples were also assayed for rotaviruses by the immunoassays, and the results are summarized in Table 12. In one case (the luminol assay), there was a borderline positive, but this was not confirmed by any other tests. The other samples were negative in all of the assays. Thus, the phenomenon of false-positives was not a major problem. Rotaviruses were therefore not present in large enough concentrations to be detectable by the methods used.

DISCUSSION

Human rotaviruses are a medically important group of pathogens which can be transmitted by water. They can be present in water in numbers exceeding those of viruses detected by standard tissue culture techniques (18). Since rotaviruses are known to be present in the feces of Israeli people, it was of interest to determine the relative abundance of these viruses in the local water.

The detection of rotaviruses requires the use of specific immune reagents, since they are difficult to detect by standard techniques. In this study, we developed and compared several rotavirus detection methods. IF was useful for the detection of simian and human rotaviruses, in agreement with the literature (5, 10, 14, 18). Of the ELISAs tested, the commercially available Enzygnost assay was the most sensitive. The Enzygnost assay has also been reported to be more sensitive than the Rotazyme assay for the detection of human rotaviruses in fecal specimens (15). Luminescent substrates did not increase the sensitivity of the ELISA, in comparison with the use of OPD. The use of OPD as a substrate required smaller amounts of conjugate than the use of luminescent substrates.

TABLE 5. Detection of SA-11 rotavirus in water concentrates[a]

Sample	Virus seeded	Fraction	Water vol (ml)	Titer[b] by following assay:					
				CPE	IF	OPD	Luminol	Enzygnost	Rotazyme
1	2.0×10^5	Input	2,000	+	−	−	ND[c]	−	−
		Eluate	70	+	+	−	ND	±	+
2	4.0×10^5	Input	500	+	+	−	ND	−	−
		Eluate	100	+	+	+	ND	+	+
3	2.5×10^6	Input	500	ND	+	−	−	−	−
		Eluate	70	ND	+	+	+	+	+
		Floc	3	ND	+	ND	ND	ND	+

[a] Virus was seeded into the indicated volume of deionized water and the sample was adsorbed to a Zeta Plus 60S filter. The adsorbed virus was eluted with the indicated quantity of beef extract and reconcentrated by organic flocculation (sample 3). Seeded virus titers were calculated from the stock titer.
[b] Symbols: +, positive; ±, greater than background but less than twice the negative; −, negative.
[c] ND, Not determined.

TABLE 6. Effect of sewage concentrates on assay sensitivities[a]

Assay	Dilution or titer	Sensitivity[b] of virus seeded into:		
		Medium	Eluate	Floc
IF	1:5	+ + +	T	T
	1:25	+ + +	+ + +	T
	1:50	+ +	+ +	T
	1:100	+ +	+	+
	1:200	+	+ +	+
	1:400	+	+	+
	Titer	3×10^5	5×10^5	6.3×10^5
OPD	1:5	+	+	+
	1:25	+	+	±
	1:50	±	±	±
	1:100	−	−	−
	1:200	−	−	−
	1:400	−	−	−
Luminol	1:5	+	+	+
	1:25	+	+	±
	1:50	+	−	+
	1:100	±	−	±
	1:200	−	−	−
	1:400	−	−	−
Enzygnost	1:5	+	+	+
	1:25	+	+	+
	1:50	+	+	+
	1:100	+	+	+
	1:200	±	±	±
	1:400	−	−	−

[a] Rotavirus was added to eluates, and flocs were obtained from concentrating wastewater by adsorption to and elution from Zeta Plus 60S filters, followed by organic flocculation. A parallel sample was maintained in medium. The samples were diluted with medium and assayed by the indicated tests.

[b] For the IF, +, + +, and + + + are relative virus levels, and the titers were calculated as FFU per milliliter; T is cytotoxic. For the ELISAs, −, negative; ±, greater than background but less than twice the negative; +, positive.

The ability to detect rotavirus antigen was improved by adsorption to and elution from Zeta Plus microporous filters followed by organic flocculation. Wastewater concentrates did not interfere with the ELISAs, although the organic floc was frequently toxic in the IF assay. All of the Zeta Plus filters concentrated rotavirus. The 30S filter provided organic flocs which apparently contained the highest amount of rotavirus antigen, and, generally, a higher volume of effluent could be filtered. The 60S filter had a slightly higher recovery of enteroviruses. Rose et al. (17) also reported that Zeta Plus filters with a smaller pore size (50S) are more efficient than 30S filters for the recovery of animal viruses from primary effluent.

TABLE 7. Comparison of filters for virus concentration from effluent[a]

Assay	Input	Fraction	Titer[b] of virus on filter type:			
			60S	50S	30S	1MDS
IF	2×10^4	Eluate	1.6×10^4	6.0×10^3	5.0×10^3	0
		Floc	T	0	T	T
OPD	−	Eluate	+	+	+	−
		Floc	±	+	+ +	±
Luminol	−	Eluate	−	±	±	−
		Floc	−	+	+	−
Enzygnost	±	Eluate	+	+ +	+	±
		Floc	+	+ +	+ + +	+

[a] Rotavirus SA-11 was seeded into untreated effluent, and 500 ml was filtered through the indicated filters. For the 1MDS filter, 400 ml was filtered.

[b] The symbols are as described in the footnote to Table 6. Relatively high ELISA values are indicated as + + or + + +.

TABLE 8. Enterovirus recovery by 60S and 30S filters[a]

Expt no.	Filter type	Fraction	Vol filtered (ml)	Concn factor (fold)	Virus concn (PFU/liter)
1	60S	Input	1,000	1	8,000
		Eluate	50	20	3,300
	30S	Input	950	1	8,000
		Eluate	50	19	1,520
2	60S	Input	500	1	T[b]
		Eluate	75	7	750
	30S	Input	1,500	1	T
		Eluate	70	21	560

[a] Untreated effluent was filtered through the designated filters in the amounts indicated. The input and eluate fractions were assayed for enterovirus by plaque assay, and the results are presented as PFU per liter of the unconcentrated wastewater. All of the fractions were tested for rotavirus by the IF, OPD, luminol, and Enzygnost assays and were negative.

[b] T, Cytotoxic.

The survey of Jerusalem wastewater was carried out over 1 year to determine the relative levels of rotaviruses and enteroviruses. Rotaviruses were not detected, even during the winter months when they might be expected to be found in increased numbers (10), while enteroviruses were found in almost all of the samples. Thus, Jerusalem wastewater was not found to be highly contaminated with rotaviruses, although other viruses were abundant.

The lack of detection of rotaviruses in the survey samples may be due to the insensitivity of the assays. An estimated detection limit of IF can be calculated by using the results of tests for SA-11 rotavirus. Generally, 7.6- to 15.2-ml equivalents of each wastewater sample was assayed, and, assuming complete virus recovery in the concentrates, a minimum of 66 to 132 FFU/liter could have been detected. Concentrations of rotaviruses in wastewater exceeding these levels have been reported (10, 18), although enterovirus levels were generally lower than those reported here (10). However, our estimate of the sensitivity of the assay is very rough, since human rotaviruses may differ from the simian virus in their detectability by IF. In addition, the organic

TABLE 9. Efficiency of enterovirus recovery from wastewater[a]

Expt no.	Type of wastewater	Vol filtered (ml)	Concn factor (fold)	Virus recovery (%)[b]
1	Primary	700	9	87
	Untreated	450	6	25
2	Primary	550	7	27
	Untreated	300	6	21
3	Primary	850	14	16
	Untreated	400	5	29

[a] Samples of primary and untreated wastewater were filtered through Zeta Plus 60S filters, and the input and eluate fractions were assayed for enterovirus by plaque assay. Recovery efficiency in the eluate was calculated relative to the amount of virus in the input.

[b] The mean percent virus recovery was 34 ± 26% (standard deviation).

TABLE 10. Recovery of virus from the filtrate[a]

Type of wastewater	Fraction	Virus titer (PFU/liter)	Virus recovery (%)
Untreated	Input	1.9×10^4	100
	Eluate	5.9×10^3	30
	Eluate of filtrate	1.4×10^3	7
Secondary	Input	1.2×10^4	100
	Eluate	3.3×10^3	28
	Eluate of filtrate	1.7×10^3	14

[a] Wastewater samples (525 ml of untreated and 650 ml of secondary effluent) were filtered through Zeta Plus 60S filters, and the input and eluate fractions were assayed for virus. The filtrates were refiltered through fresh Zeta Plus 60S filters, and the eluates of the second filtration were assayed (eluate of filtrate). The enterovirus titers are given in PFU/liter of the input wastewater, and the recovery efficiency was calculated from the input virus. The fractions were assayed for rotavirus by the IF, OPD, luminol, and Enzygnost assay and were all negative.

flocs, which presumably contained the most concentrated virus, were frequently toxic to the cells. It is also possible that the high levels of enteroviruses in the samples interfered with rotavirus infectivity, although the maximum levels of enteroviruses can be calculated to be 56 PFU per drop, which would not have overwhelmed the assay system. In the short time of the assay, single cells infected with rotavirus should have been detectable, even in the presence of this level of contaminating enteroviruses. More concentrated samples might, however, interfere with rotavirus detection.

The ELISAs were less sensitive than IF for SA-11 rotavirus, but a larger volume of each sample could be assayed (45 to 96 ml of wastewater equivalent), and the tests were not affected by wastewater contaminants. It can be calculated that the Enzygnost assay would detect a minimum of 3.3×10^4 to 6.6×10^4 FFU of SA-11 rotavirus per liter without correcting for loss in the concentration steps. Again, this is a broad estimate, since the ratio of antigenic mass to SA-11 or human rotavirus was not measured. It may be noted that the Enzygnost ELISA was more sensitive for the human virus than the other ELISAs were, in comparison with their sensitivity for the simian virus. This is likely to be due to the use of antisera against SA-11 rotavirus for both layers in the laboratory assays, while the Enzygnost kit uses anti-Nebraska calf diarrhea rotavirus antiserum for the second layer. In any case, these assays require high rotavirus concentrations for virus detection, and it is possible that more highly concentrated wastewater would have resulted in some positive samples.

TABLE 11. Enterovirus titers in wastewater survey samples[a]

Date (day/mo/yr)	Titers (PFU/liter) in wastewater type:	
	Untreated	Treated
1/2/85	8.0×10^3	—[b]
22/2/85	5.2×10^2	1.4×10^2
29/3/85	$<2.0 \times 10^2$	$<5.0 \times 10^1$
19/4/85	$<1.5 \times 10^2$	2.9×10^2
3/6/85	1.7×10^4	8.3×10^3
10/7/85	3.0×10^3	3.0×10^3
23/8/85	8.0×10^3	9.0×10^3
27/9/85	7.5×10^2	8.0×10^3
1/11/85	1.9×10^4	1.2×10^4
3/12/85	2.7×10^4	4.0×10^3[c]
27/12/85	8.3×10^4	4.2×10^4[c]
10/1/86	2.1×10^4	1.2×10^4[c]

[a] Samples of treated (secondary or primary) and untreated wastewater were concentrated by adsorption to and elution from Zeta Plus 60S filters followed by reconcentration by organic flocculation. The highest measured concentration of enterovirus (in PFU/liter) in each sample, after titration of the input, eluate and floc fractions, is indicated.

[b] —, No sample.

[c] Primary wastewater.

TABLE 12. Summary of rotavirus wastewater survey[a]

Assay	No. of days	No. of samples tested					
		Untreated	Treated	Total	Input	Eluate	Floc
IF	12	17	11	28	21	23	25
OPD	10	15	10	25	14	22	23
Luminol	11	15	11	26	19	22	24
Enzygnost	11	16	11	27	20	22	25
Rotazyme	4	3	4	7	6	7	0

[a] Wastewater samples and concentrates were assayed for rotavirus by the indicated methods on the dates indicated in Table 11, and the number of samples tested are listed. One sample was positive in one test (untreated wastewater eluate, December 1985, luminol assay), at a level of twice the background.

The problem of false-positive results, which has been reported when the Rotazyme assay was used for the assay of environmental samples (2, 10), was not apparent in the Jerusalem samples. The one positive result which was found was with the luminol assay, which in general was less consistent than the OPD test. Since the OPD test had a sensitivity similar to that of luminescent substrates, there does not appear to be an advantage to their use.

In summary, the replacement of standard tissue culture methods of virus detection by a rotavirus assay would not ensure the virological quality of Jerusalem wastewater. The ease of use and commercial availability of the Enzygnost assay might make it useful for supplemental monitoring of water for the presence of rotaviruses. The IF method is sensitive and detects viable virus but is not yet generally available. Thus, the testing of water sources with suspected rotavirus contamination or during rotavirus epidemics might be carried out with either the IF or the Enzygnost assay in combination with virus concentration. Future progress in increasing the sensitivity of detection methods might make the routine monitoring of water for rotaviruses a more feasible goal.

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